Preface

The work presented in this PhD thesis was mainly performed in the laboratory of Peter A. Andreasen at the Department of Molecular Biology and Genetics, University of Aarhus, Denmark. In addition, my stay in a number of other laboratories contributed with important data to the thesis. My stay in the laboratories of Serge Muyldermans, Vrije Universiteit, Brussels, Belgium, and of Paul J. Declerck, Katholieke Universiteit, Leuven, Belgium was important for the generation of the nanobody immune libraries, and the selection and production of anti-uPA nanobodies. Also the collaboration with other laboratories was important for the results of the thesis. Thus, the laboratory of Jacky Chi Ki Ngo, School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China solved the X-ray crystal structure of the nanobody Nb4 in complex with human uPA; The Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark, generated the anti-muPA monoclonal antibodies. Also, the contribution from the laboratory of Knud J. Jensen, Department of Chemistry, Faculty of Science, University Copenhagen, Denmark was important for the outcome of the peptide work.

This thesis will start with an introduction focussing on the general function and biology of the S1 serine peptidases. Different mechanisms for regulation of peptidase activity are highlighted such as allosteric regulation by naturally occurring ligands. Next, different means of molecular intervention with the activity of peptidases is introduced with focus on orthosteric and allosteric regulation. The result section is divided into two main parts. The first summarises the main results from six manuscripts, which is also included in the appendixes (I-VI), in which more details can be found. The second part includes a more detailed description of results, which has not been covered by the manuscripts, including materials and methods. The discussion is divided into three main sections directly relating to the main purpose of the PhD project.

The appendixes contain: Six full manuscripts (referred to by roman numerals I-VI in the main text) and experimental data from the results, which has not been included in a manuscript (referred to as 7-10 in the main text).
This thesis contains the following published peer reviewed articles and manuscripts in preparation.

I. **A Cyclic Peptidic Serine Protease Inhibitor: Increasing Affinity by Increasing Peptide Flexibility.**

II. **Selection of High-Affinity Peptidic Serine Protease Inhibitors with Increased Binding Entropy from a Back-Flip Library of Peptide-Protease Fusions.**

III. **Interconversion of Active and Inactive Conformations of Urokinase-type Plasminogen Activator.**

IV. **Allosteric Inactivation of a Trypsin-like Serine Protease by an Antibody Binding to the 37- and 70-loops**

V. **Unravelling an Allosteric Regulatory Mechanism in Trypsin-Like Serine Proteases by the use of a Camelid Derived Antibody Fragment**
    Tobias Kromann-Hansen, Eva Louise Lange, Lin Lin, Hans Peter Sørensen, Jan K. Jensen, Gholamreza H. Ghassabeh, Serge Muyldermans, Mingdong Huang, Paul J. DeClerck and Peter A. Andreasen (Manuscript in preparation)

VI. **A Camelid-Derived Antibody Fragment Targeting the Active Site of aTrypsin-like Serine Protease Balances Between Inhibitor and Substrate Behaviour**
    Tobias Kromann-Hansen, Emil Oldenburg, Kristen Wing Yu Yung, Gholamreza H. Ghassabeh, Serge Muyldermans, Paul J. DeClerck, Mingdong Huang, Peter A. Andreasen and Jacky Chi Ki Ngo (Manuscript in preparation)
Summary

Trypsin-like serine peptidases play an important role in many physiological and pathophysiological processes, the latter including cardiovascular diseases and cancer. Binding of natural ligands to functional sites on the peptidase surface balances the level of activity and substrate specificity of peptidases. Thus, any disturbance to the balance between ligand and peptidase is detrimental to the function of the peptidase in its biological context. Orthosteric regulation, in which the ligand bind at the active site of the peptidase, and allosteric regulation, in which the ligand bind elsewhere on the peptidase and allosterically modulate the function of the active site, represents two important activity-regulating mechanisms in trypsin-like serine peptidases.

Development of specific orthosteric agents as therapeutics is a challenge due to similar active site topology within the trypsin-like serine peptidase. The thesis describes how X-ray crystal structure analysis and biochemical analysis were used to demonstrate new concepts for orthosteric regulation of activity in the trypsin-like serine peptidase urokinase-type plasminogen activator (uPA), studying two types of orthosteric agents, namely cyclic peptides and Camelid derived antibody fragments, so-called nanobodies.

Allosteric regulation of activity in trypsin-like serine peptidases is in general poorly understood, as the propagation of the allosteric signal from the ligand binding site to the active site in some cases is subtle and based exclusively on a change in side chain and backbone dynamics along the allosteric trajectory. This thesis describes the characterisation of two allosteric monoclonal antibodies and the development and characterisation of an allosteric nanobody against murine uPA. Insights into their binding mechanisms, using X-ray crystallography to determine crystal structures of active and inactive conformations of muPA, combined with biochemical analysis, elucidated an allosteric regulatory mechanism, which is now believed to be highly conserved in the trypsin-like serine peptidases.

Targeting zymogen activation represents an attractive approach for molecular intervention with the function of trypsin-like serine peptidases. In the
thesis, I also describe the development of nanobodies that specifically target zymogen activation of uPA, by preventing its proteolytic cleavage by plasmin.
Dansk Resume (Danish Summary)


Ortosterisk regulering, hvor liganden binder til det aktive site af peptidase, og allosterisk regulering, hvor liganden gennem binding til et område uden for det aktive site påvirker aktiviteten af peptidaseen gennem et allosterisk signal, er to vigtige aktivitets-regulerende mekanismer i trypsin-lignende serin-peptidaser.

Fremstillingen af specifikke ortosteriske hæmmere er udfordrende idet det aktive site i forskellige serin-peptidaser er meget ens. Denne afhandling bekskriver hvordan man ved hjælp af røntgenkrystallografianalyse og biokemiske analyser kan udvikle nye koncepter til fremstilling af specifikke ortosteriske hæmmere rettet mod den trypsin-lignende serin-peptidase urokinase-type plasminogen aktivator (uPA), ved at studere to nye typer af hæmmere; mono-cykliske peptid-hæmmere og antistoffragmenter fremstilet i dyr fra kamel familien.


Hæmning af pro-enzym aktiveringen er en attraktiv strategi for molekylær intervention af aktiviteten af trypsin-lignende serin-peptidaser. Denne afhandling beskriver ligeledes hvordan man kan anvende kamelantistoffragmenter til at hæmme pro-enzym aktiveringen af uPA.
Acknowledgements

I would like to thank my supervisor Professor, dr. scient. Peter A. Andreasen for very skilful scientific supervision and his guidance. It has been a true pleasure working in your laboratory, and I cannot comprehend how much I have learned during my 5-years under your supervision. A special thanks to my former colleague Associate professor Hans Peter Sørensen. I am truly grateful for your guidance to the practical work in the laboratory, and for providing me with basic research skills. I wish to thank Paul Declerk and Maarten Hendrickx from the Katholieke Universiteit in Leuven and Serge Muylldermans and Gholamreza Ghassabeh from the Vrije Universiteit in Brussels for introducing me to the nanobody technique and for giving me a great time in Leuven and Brussels. I also wish to thanks our collaborators Mingdong Huang and Jacky Chi Ki Ngo, which I have had the pleasure to visit in Fuzhou (China) and in Hong Kong to discuss our joint projects.

A special thanks to associate professor Jan K. Jensen, and PhD Jesper Johansen for introducing me to the X-ray crystallography. Thanks for always being present and for having time to scientific discussions. I also wish to thank the two master students Eva Louise Lange and Emil Oldenburg for good working relationship regarding the nanobody projects. Anni Christensen and Daniel Dupont also deserve special thanks for always being ready to help with any kind of problem. Also thanks to all present and former colleagues for creating a great work-environment and for a good working relationship. Finally, I wish to send me deepest thanks to Janni Kromann-Tofting who has been my greatest support during the years.

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Abbreviations

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>huPA</td>
<td>Human urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>Serpin</td>
<td>Serine protease inhibitor</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase-type plasminogen receptor</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>RCL</td>
<td>Reactive centre loop</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>VH</td>
<td>Variable heavy domain</td>
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<tr>
<td>CH</td>
<td>Constant heavy domain</td>
</tr>
<tr>
<td>VL</td>
<td>Variable light chain</td>
</tr>
<tr>
<td>CL</td>
<td>Constant light chain</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementary determining regions</td>
</tr>
<tr>
<td>Fabs</td>
<td>Fragment antigen binding</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain fragment variable</td>
</tr>
<tr>
<td>HCAbs</td>
<td>Heavy chain antibodies</td>
</tr>
<tr>
<td>VHH</td>
<td>Variable heavy domain of the heavy chain antibodies</td>
</tr>
<tr>
<td>Nb</td>
<td>Nanobody</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>EGF:EGR</td>
<td>Epidermal growth factor:Epidermal growth factor receptor</td>
</tr>
<tr>
<td>muPA</td>
<td>Murine urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy DNA</td>
</tr>
<tr>
<td>HBS</td>
<td>Hebes buffered saline</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>RU</td>
<td>Response units</td>
</tr>
<tr>
<td>EGR-cmk</td>
<td>Glu-Gly-Arg-chloromethylketone</td>
</tr>
<tr>
<td>HA-tag</td>
<td>Human influenza hemagglutinin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>HGFA</td>
<td>Hepatocyte growth factor activator</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>HDX-MS</td>
<td>Hydrogen deuterium exchange mass spectrometry</td>
</tr>
<tr>
<td>PARs</td>
<td>Protease Activated Receptors</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropyl phosphonofluoridate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl</td>
</tr>
<tr>
<td>PPACK</td>
<td>D-Phe-Pro-Arg-CH2-Cl</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
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<tr>
<td>AIA</td>
<td>Antigen-induced arthritis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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Introduction

Searching the MEROPS database reveals that at present more than 300,000 peptidase sequences have been classified into 61 clans and 242 families (1). A distinction of five classes of proteolytic enzymes has been recognized based on their catalytic mechanism: aspartate, cysteine, metallo-, threonine and serine peptidases. Bioinformatics analysis of the human genome determined that the human degradome, which is composed of 561 protease genes and more than 156 protease inhibitor genes, represent more than 2 % of the total gene repertoire (2). This clearly indicates that proteolysis is an important function in biology. Consequently peptidase activity influences many important biological processes such as apoptosis, blood coagulation, extracellular tissue remodelling, and complement activation during an immune response.

Serine Peptidases

Serine peptidases (Clan PA) constitute over one-third of all peptidases, and they are distributed into 15 clans with 45 families. They utilize the amino acid serine as a nucleophile to catalyse the hydrolysis of peptide bonds. The structural context, in which this catalytic serine is situated, classifies the serine peptidases into their respective families. The serine peptidase family S1 contains the serine endopeptidase, in which the catalytic serine is part of a so-called charge relay system including a histidine residue and an aspartic acid residue (His, Asp, Ser). There are three main P1 specificity types within the S1 family; cleavage on the C-terminal side of positive residues (Arg or Lys), cleavage on the C-terminal side of hydrophobic residues (Phe, Trp, Tyr) or cleavage on the C-terminal side of an alanine residue. A representative member of the S1 serine peptidase is trypsin, which like most S1 peptidases have substrate selectivity for arginine or lysine side chains in the P1 position.

Structural features and substrate specificity of the S1 serine peptidases. The S1 serine peptidases share a characteristic structural architecture in their catalytic domain, where two six-stranded β-barrels come together, to host at their interface
the residues of the catalytic triad (figure 1a). Two residues of the catalytic triad, His57 and Asp102 (chymotrypsin numbering), are donated by the N-terminal β-barrel, whereas Ser195 is donated by the C-terminal β-barrel. The six strands of each β-barrel are connected by five surface-exposed loops. For the N-terminal β-barrel these are referred to as the 37-, the 50-, the 60-, the 70-, and the 90-loops and for the C-terminal β-barrel as the 140-, the 170-, the 180-, the 200-, and the 220-loops. The surface-exposed loops are important to the catalytic machinery of the S1 serine peptidases as they contribute to substrate specificity by forming the substrate-binding pockets and exosites for macromolecular substrates. Residues 189-192, 214-216 and 224-228 create the primary S1 specificity pocket (figure 1b). The S2 and S3 specificity pockets are created by residues from the 90-, and the 220-loops (residues 97, 99, 215 and 216), whereas the S1’, S2’ and S3’ sites are created by residues from the 37-, the 60- and the 140-loops (residue 35, 41, 62, 143 and 151). Residues in position 189, 216 and 226 primarily determine the substrate specificity of the S1 serine peptidases. In the S1 serine peptidases with substrate specificity like trypsin (trypsin-like) Asp189, Gly216 and Gly219 create a negatively charged S1 pockets into which the positively charged Arg or Lys side chain of the substrate is inserted. In S1 serine peptidase with substrate specificity like chymotrypsin (chymotrypsin-like) Ser189, Gly216 and Gly226 create a deep hydrophobic S1 pocket, which account for the specificity for cleaving after hydrophobic amino acids. S1 serine peptidases that prefer substrates with small aliphatic P1 residues such as elastase (elastase-like) has a shallow S1 pocket due to the presence of Val216 and Thr226. The interactions between the polypeptide substrate and the S2-S3 and S1’-S3’ specificity pockets of the S1 serine peptidases can be more or less specific. Trypsin is an example of a non-specific S1 serine peptidase, which accepts a range of different substrates but only cleaves after Lys and Arg residues. Contrary, urokinase-type plasminogen activator (uPA) is an example of a specific S1 serine peptidase accepting only polypeptide substrates with specific amino acids around the scissile bond. However, the specificity of the S1 serine peptidases does not only rely on interactions around the scissile bond, as interactions of a macromolecular substrate with exosites also contribute to the specificity. Besides the catalytic domain (B-chain) S1 serine peptidases also contains additional domains such as apple, CUB, EGF, fibronectin,
kringle and von Willebrand factor domains (A-chain). These domains are often linked to the catalytic domain by a highly conserved disulphide bond.

The catalytic mechanism of the S1 serine peptidases. S1 serine peptidases catalyses the hydrolysis of peptide bonds in polypeptide substrates through a mechanism involving the formation of two tetrahedral transition states and an acyl-enzyme intermediate. The hydrolysis is initiated by a nucleophilic attack by the hydroxyl O-atom of Ser195 on the carbonyl group of the scissile bond. His57 is acting as a general base in the reaction extracting a proton from Ser195. The nucleophilic attack forms the first tetrahedral transition state with a negative charged oxyanion, which is stabilised by the main chain NHs of the oxyanion hole (Gly193 and Ser195) (figure 1b). The acyl-enzyme intermediate is formed when the protonated His57 acts as a general acid donating a proton to the leaving group (C-terminal part of the substrate). The acyl-enzyme becomes deacylated through the reverse reaction pathway by formation of the second tetrahedral transition state, but with a water molecule acting as the nucleophile instead of Ser195. The result is the release of the N-terminal part of the substrate and the active site is restored (3, 4). The role of
Asp102 in the reaction is to stabilise the protonated His57 and to fix the configuration around the active site (5).

The S1 serine peptidases with trypsin-like P1 specificity. Most S1 serine peptidases display trypsin-like P1 specificity by cleaving substrates C-terminally to Arg or Lys side chains. The rest of this report will primarily focus on these S1 serine peptidases and they will be referred to as trypsin-like serine peptidases in order to distinguish them from the chymotrypsin-like S1 serine peptidases with hydrophobic P1 substrate specificity. It should be mentioned that the zymogen activation mechanism described below is a highly conserved mechanism, which applies to all S1 serine peptidases.

Trypsin-like serine peptidases are involved in important physiological processes including: digestion, blood coagulation, immunity, fibrinolysis and tissue remodelling (6). As examples: trypsins are involved in the breakdown of polypeptides in the digestive system (7); thrombin is involved the blood coagulation system by catalysing formation of fibrin to form an insoluble fibrin clot (8); the fibrin clot is later lysed by plasmin, which belong to the fibrinolytic plasminogen activation system (9); C1r is one out of nine trypsin-like serine peptidases of the complement activation system responsible for inducing a pro-inflammatory signal as a response to pathogen invasion (10); matriptases are membrane-bound trypsin-like serine peptidases involved in e.g. formation and differentiation of the epidermal barrier (11); the proteolytic activities of tissue kallikreins is involved in many processes including tissue remodelling of the extracellular matrix, apoptosis, innate immunity, and neurodegeneration, whereas plasma kallkrein is responsible for the regulation of blood pressure through the kinin system (12, 13). The above-mentioned trypsin-like serine peptidases are arranged into cascade-like systems in which the initial activity is amplified by continues feedback signal by one of the later peptidases in the cascade. A classical example is the blood coagulation systems, which includes more than ten trypsin-like serine peptidases (9).
**Regulation of activity in trypsin-like serine peptidases**

A prerequisite for normal function of any trypsin-like serine peptidase cascade is a tight control of the system to ensure that a specific function of a peptidase is only turned on when its activity is required by the system. Besides regulation at the transcriptional level, three main regulatory mechanisms control peptidase function including; zymogen activation, allosteric regulation by ligands or cofactors, and regulation of activity by trypsin-like serine peptidase inhibitors.

**Zymogen activation and the conformational equilibrium in trypsin-like serine peptidases.** The conversion of a low-activity single-chain zymogen to an active two-chain peptidase offers an attractive regulatory mechanism for explaining the arrangement of trypsin-like serine peptidases into their sequential catalytic activation cascades, in which a zymogen acts as a substrate in one step, and as an active peptidase in the subsequent step of the cascade. Conversion of the single-chain zymogen precursors into the mature two-chain peptidase occurs by proteolytic cleavage of the peptide bond between amino acids 15 and 16. The consequence of the cleavage is a rearrangement of several surface-exposed loops of the catalytic domain (figure 2). These loops, which are highly disordered in the zymogen state, are referred to as the activation domain and comprise: The activation loop (residues 16-21), the oxyanion stabilising loop (residue 184-194), the S1 entrance frame (residues 216-223) and the autolysis loop (residues 142-152). The activation loop folds back to insert the newly generated N-terminal amino acid 16 into a hydrophobic pocket to ion-pair with Asp194 (14). As Asp194 is adjacent to the catalytic Ser195 and Gly193 this interaction is critical for formation of the oxyanion hole. Conformational changes of the oxyanion stabilising loop and the S1 entrance frame organise the primary S1 specificity pocket. The autolysis loop also becomes more ordered and is centrally positioned, interconnecting the loops of the activation domain through several hydrogen bonds with the oxyanion stabilising loop and the activation loop (15).

A large variation to the change in activity is observed after zymogen activation in different trypsin-like serine peptidases. The activity of trypsin is enhanced $10^8$-times
after zymogen activation, whereas that of tissue-type plasminogen activator (tPA) is only enhanced 5-10 times due to a high level of activity in the zymogen state (16, 17). Similarly, coagulation factor VIIa remains in a zymogen-like state with low activity after cleavage of the residue 15-16 peptide bond, as residue 16 is not stably incorporated into the hydrophobic activation pocket (18). Thus, single-chain trypsin-like serine zymogens are able to express proteolytic activity and the cleaved two-chain peptidases are able to maintain a zymogen-like state.

Traditionally, allostery was defined as a binding of a ligand or cofactor at one site (allosteric site), which affected the function at another site e.g. an active site by inducing a distinct conformational transition (19). However, recent findings has highlighted the importance of protein dynamics in allostery and demonstrated that ligand binding alters enzyme function through redistribution of internal dynamics (conformational entropy) in the enzyme without inducing significant conformational changes (20-25). Several studies using NMR and thermodynamic analysis show that binding of cAMP to the catabolite activator protein (CAP) activates CAP for DNA binding by redistribution of the internal dynamics through subunits without changing the mean conformation of the enzyme (26-28). This dynamic view of allostery also applies to the trypsin-like serine peptidase. In general, it was believed that the surface-exposed loops of the activation domain is highly disordered in the zymogen state.
state, and that zymogen activation stabilises the loops in an active peptidase conformation (14). This “static” two-state view has been replaced by a more dynamic view, in which the surface-exposed loops remains highly dynamic even after zymogen activation. This implies the existence of a conformational equilibrium between less and maximally active states, and it has been supported by several studies using NMR or rapid kinetic measurements (29, 30). In particular, NMR studies with thrombin have demonstrated how several surface-exposed loops (including the loops of the activation domain) surrounding the active site remains highly disordered even after zymogen activation (31, 32). As each conformation is likely to be associated with a specific function or activity, the model referred to as conformational selection offers a conceptual framework for understanding how ligands and cofactors regulates the activity of trypsin-like serine peptidase. By differential binding to different conformations, ligands and cofactors are able to shift the conformational equilibrium by stabilising more or less active conformations of the peptidase. This ensures a specific function of a trypsin-like serine peptidase in its biological context.

**Allosteric regulation of activity in trypsin-like serine peptidases.** The cleaved two-chain trypsin-like serine peptidase coagulation factor VIIa (FVIIa) circulates in the blood as a zymogen-like enzyme with little activity. This is due to high flexibility of the surface-exposed loops of the activation domain, which leads to a lack of incorporation of the N-terminal Ile16 into the activation pocket (18, 33). FVIIa remains inactive until it binds to its cofactor tissue factor, which ensures a stable incorporation of Ile16 and ordering of the loops of the activation domain (33, 34). The crystal structures of free FVIIa and tissue factor bound FVIIa did not reveal any differences to the enzyme, and thus did not offer any explanation for the conformational change associated with the allosteric transition from the inactive to the active conformation (35, 36). However, alanine-scanning mutagenesis identified the amino acids important for the allosteric effect of tissue factor to reside in the 170-loop, which is adjacent to the 180- and 220-loops of the activation domain (37). Thrombin is another trypsin-like serine peptidase in which allostery is well documented (38-40). Thrombin is central to the blood coagulation systems and is a
dual-action peptidase with both pro- and anticoagulant activities. Thrombin cleaves several pro-coagulant substrates such as the protease-activated receptor 1 (PAR1) and fibrinogen and activates other coagulation factors such as FXI, FVIII, FV and FXIII to amplify its own formation. Thrombin also cleaves the anti-coagulant substrate protein C, which inactivates FV and FVIII to shut down the coagulation cascade (41). Two important allosteric sites in thrombin is the Na\(^+\)-binding site and the thrombomodulin binding exosite I (42). Although the Na\(^+\)-binding site is situated > 15Å away from the active site in a groove between the 180- and 220-loops, binding of Na\(^+\) enhances the catalytic activity of thrombin towards procoagulant substrates and zymogen coagulation factors (figure 3) (43-47). These observations led to the idea that thrombin circulates in a low activity state (E) and a high activity Na\(^+\)-bound state (E:Na\(^+\)). Later, ultra rapid kinetics identified a third state of thrombin (E*) incapable of Na\(^+\) binding. X-ray crystal structure analysis suggested that the E* conformation is inactive and does not bind Na\(^+\) due to a collapse of the 215-219 segment into the active site to occlude the S1 specificity pocket (figure 4) (48, 49). Besides the linkage between the Na\(^+\) binding site and the active site, an allosteric linkage has been shown between exosite I and the active site. Exosite I, which is an important PAR-1 and fibrinogen binding exosite, is located in the 37- and 70-loops > 20Å from the active site (figure 3). However, binding of the cofactor thrombomodulin changes the substrate specificity of thrombin away from the procoagulant substrates and enhances the catalytic activity of thrombin more than 1000-fold towards the anticoagulant substrate protein C (50). The allosteric effects of Na\(^+\) or thrombomodulin also seems to have more global effect as thrombomodulin binds more tightly to the E:Na\(^+\) high activity state of thrombin, indicating an allosteric linkages across the N- and C-terminal β-barrels (figure 3) (43, 51). In addition to the Na\(^+\)-binding site and exosite I, thrombin has another macromolecular binding site called exosite II located at the C-terminal helix (figure 3). Exosite II is important for the recognition of heparin and the coagulation factors V and VIII. Much effort has been directed towards understanding the allosteric nature of regulation of thrombin activity. Rapid kinetic measurements demonstrates that thrombin alone interconverts between active (E) and inactive (E*) conformations on a slow time scale (ms), and that this pre-existing equilibrium of conformations is
shifted upon binding of ligands to exosites or the active site (52). Although X-ray crystal structure analysis has identified distinct conformations of thrombin representing the E and E* states, techniques such as NMR and molecular dynamics suggests that the E and E* should be considered as ensembles of conformations which interconverts on a fast time scale (ns to ms) (31, 32).

Mechanisms of allosteric regulation in trypsin-like serine peptidases. The existence of active and inactive states as conformational ensembles is likely to apply to other trypsin-like serine peptidases besides FVIIa and thrombin. Conformational heterogeneity has also been observed in trypsin (29, 30), in complement factor D (53), in hepatocyte growth factor activator (54), in the kallikrein family especially kallikrein-related peptidase 2 (KLK2) (55), in coagulation factor Xa and activated protein C (56). Molecular and structural insight into how ligands and cofactors regulates the activity of trypsin-like serine peptidases has largely remained elusive as X-ray crystal structure analysis in many cases fails to reveal any structural changes.
between the ligand free and ligand bound peptidase. This was the case for the FVIIa:tissue factor complex (36) and for the thrombin:thrombomodulin complex (57). Only in a few cases have X-ray crystal structures documented significant conformational changes as observed in the crystal structure of the inactive thrombin mutant D102N in complex with the extracellular fragment of PAR1 (figure 4) (58). The crystal structure demonstrates how PAR1 upon binding to exosite I converts thrombin from inactive E* conformation to the active E conformation through rearrangement of a complex hydrogen bond network. The allosteric signal propagates from exosite I to the 140-loop, which re-establish a hydrogen bond network with the 180- and 220-loop to restore the active site and the specificity pockets of the peptidase.

In attempt to address the mechanisms behind the allosteric regulation several studies have used more dynamics techniques such as NMR, HDX-MS and molecular dynamics simulations. In the allosteric activation of FVIIa by tissue factor such studies have demonstrated how binding of tissue factor to the 170-loop tightens the
interconnectivity of the loops of the activation domain in FVIIa, and suggests that an allosteric signal, involving redistribution of internal dynamics, propagates from the tissue factor binding site to the active site of FVIIa through the 180- and 220-loops (33, 59, 60), in a mechanism much similar to that proposed for Na$^+$ activation of thrombin (31, 61). Exactly how thrombomodulin exerts its allosteric effect on thrombin to promote protein C activation has been under intense investigation for many years. Fluorescent spectroscopy, using active site (Ser195) fluorescent-labelled dansyl-thrombin, showed that binding of thrombomodulin to thrombin affects the active site (62, 63), and recently it has been demonstrated, using isothermal titration calorimetry and molecular dynamics, that thrombomodulin locally affects the dynamics of the 90- and 140-loops in thrombin, which improves access of substrates to the active site (64, 65). This mechanism is in reasonable agreement with the findings for the allosteric peptide hirugen, which is the C-terminal fragment of the potent natural inhibitor hirudin (66, 67). Binding of hirugen to exosite I increases the catalytic activity of thrombin (68), and reproduces the effects of thrombomodulin (52), by affecting the dynamics of the 140-, the 170-, and the 180-loops causing an more global stabilisation to the C-terminal β-barrel (31). Together these studies have demonstrated how ligands to allosteric sites regulates activity of the peptidase by activating allosteric networks, which lead to a redistribution of internal dynamics without (at least observed in the X-ray crystal structures) changing the mean conformation of the peptidase.

**Trypsin-like serine peptidase inhibitors.** The activity of trypsin-like serine peptidase is also regulated by another type of proteins interacting directly with the active site of the peptidase. Two dominating types of inhibitory mechanisms are utilised by such protein inhibitors and is termed the standard-mechanism and suicide-mechanism inhibitors.

Standard-mechanism inhibitors are divided into 19 families based on their structural fold. They form high-affinity interactions by inserting a so-called reactive loop in a substrate-like manner into the active site of the peptidase (69, 70). The reactive loop contains a single reactive peptide bond, which is hydrolysed extremely slowly by the peptidase. Thus standard-mechanism inhibitors act as poor substrates
with $k_{cat}$ and $K_M$ values a million times lower than normal values (71). This is a consequence of the tight-binding and high stability of the peptidase-inhibitor complex, which is reflected by a very slow dissociation rate constant. Another characteristic of the standard-mechanism inhibitors is that the cleaved inhibitor is able to associate with the peptidase to resynthesize the reactive peptide bond (72, 73). Many X-ray crystal structures of peptidase-inhibitor complexes have been solved. They all have an identical conformation of the inhibitor reactive loop and the reactive peptide bond is intact in all structures (figure 5) (74). It was recently suggested that the hydrolysis of the reactive peptide bond is retained at the acyl-enzyme intermediated and the reformation of the peptide bond is favoured due to a tight and favourable orientation of the P1’ leaving group in the active site of the peptidase, which prevents access of the hydrolytic water (73-75). The favourable orientation of the P1’ leaving group is highly dependent on the scaffold of the inhibitor that often contains a highly conserved disulphide bond pattern which connects the P and P’ side of the scissile bond thus contribution to the overall stability of the peptidase-inhibitor complex (70, 76).

**Figure 5. Standard-mechanism inhibitors.** A. Surface representation of trypsin (wheat) in complex with BPTI (red) (PDB:4yOy). B. Close-up view of the active site. BPTI inserts the P1 Lys15 into the S1 specificity pocket of trypsin forming an ion-pair with Asp189. The oxygen atom of the P1 carbonyl group is positioned in the oxyanion hole (NHs of Ser195 and Gly193). The side chain oxygen atom of Ser195 is in an optimal position (2.6Å) for a nucleophilic attack on the carbonyl group of the reactive peptide bond. The disulphide bond that connects the P and P’ side of the reactive peptide bond is shown in sticks.

Because the active site topology of trypsin-like serine peptidases is highly conserved the substrate-like binding of the standard-mechanism inhibitors often leads to
inhibitors that inhibit more than one peptidase. Bovine pancreatic trypsin inhibitor (BPTI) is the best characterised standard-mechanism inhibitor. It belongs to the Kunitz-type inhibitor family, and it potently inhibits almost all trypsin-like serine peptidase with specificity for an Arg residue in the P1 position.

The suicide inhibitors contain three different protein families of which macroglobulins and serpins constitutes the largest families. The third family is the p35, which contains the baculovirus p35 caspase inhibitor (70). Serpins are trypsin-like serine peptidase inhibitors that utilises the catalytic machinery of the peptidase to trap the peptidase-serpin complex in an irreversible covalently bond state (77). The serpin inserts a long protruding reactive-center loop (RCL) into the active site of the peptidase in a substrate-like manner (figure 6a). The Michaelis complex between the peptidase and the serpin is not only stabilised by interactions of the RCL with the active site of the peptidase, but also by exosite interactions (78-80). The catalytic Ser195 of the peptidase attacks the reactive peptide bond of the RCL to form the acyl-enzyme intermediate through the normal catalytic mechanism. However, the catalytic cycle is arrested at this step and the N-terminal part of the RCL, with the peptidase attached, inserts into the central β-sheet in the core of the serpin. This translocate the peptidase to the opposite side of the serpin and results in a collapse of the active site architecture of the peptidase, thus leaving the peptidase in a highly flexible and completely inactive state still covalently attached to the serpin (figure 6b) (81, 82). In an alternative mechanism the acyl-enzyme intermediate is deacylated to release the serpin in a cleaved inactive form. If this mechanism has physiological relevance for the inhibitory serpins is unknown, but it appears to play an important role for the non-inhibitory hormone-binding serpins (83). The macroglobulins also trap their target peptidase after cleavage of the macroglobulin by the peptidase in the highly susceptible bait-region. α-2-Macroglobulin binds and inhibits a large number of serine peptidases. After cleavage of the bait-region α-2-Macroglobulin undergoes a large conformational change that traps the peptidase in a central cavity inside the refolded tetrameric α-2-Macroglobulin. The trapped peptidase is still catalytic active, however the entrances to the cavities are two small for macromolecular substrates to enter (84, 85).
Considering the important physiological role of trypsin-like serine peptidase as positive or negative regulators of many important biological processes, it is easy to understand that a deficiency, a misdirected, or uncontrolled activity, of a peptidase, directly or indirectly, can contribute to several pathological conditions such as cancer, arthritis, neurodegenerative and cardiovascular diseases. Due to their extracellular abundance the trypsin-like serine peptidases represent attractive

Figure 6. The serpin reaction mechanism. A. The Michaelis peptidase-serpin complex between the trypsin-like serine peptidase uPA (wheat) and the serpin PAI-1 (red) (PDB: 3pb1). The reactive-center loop (RCL) of PAI-1 is coloured blue. The surface-exposed loops in uPA, which forms exosites for the interaction is coloured green. The catalytic triad of uPA and the P1 Arg residue in PAI-1 is shown in sticks. B. The translocated peptidase-serpin complex between trypsin (wheat) and α1-antitrypsin (PDB:1ezx). After cleavage of the scissile bond in the RCL of the serpin it becomes inserted into the central beta-sheet. This translocate the peptidase to the opposite site of the serpin, resulting in a highly disordered and covalent inhibited peptidase.

Strategies for molecular intervention with the activity of trypsin-like serine peptidases
targets for molecular intervention. Three types of agents dominates the development of inhibitory agents: Orthosteric agents that bind in the active site of the peptidase and block the access of substrates; allosteric agents that binds distantly from the active site, but modulates it through an allosteric mechanism; steric agents that prevents protein-protein interactions, such as peptidase-substrate or peptidase-receptor interactions, by sterically preventing the access of the substrates or receptors to exosites.

**Orthosteric agents.** The vast majority of orthosteric agents developed for inhibition of trypsin-like serine peptidases are synthetic small molecules inhibitors. Based on the idea of utilising the catalytic machinery of trypsin-like serine peptidases many compounds has been developed that covalently and irreversibly traps and inactivates the peptidase by reacting with the catalytic triad using a reactive warhead. These compounds, which show broad specificity, comprise the sulfonyl florides or the phosphonyl fluorides such as phenylmehhylsulfonyl floride (PMSF) and diisopropyl phosphonofluoridate (DFP) (86). Another large group of irreversible inhibitors is the peptidyl chloromethylketones. The reactive group of the chloromethylketone alkylates the active site histidine whereas the active site serine forms a tetrahedral adduct with the carbonyl group of the chloromethylketone. Specificity of the petidyl chloromethylketones can be achieved by altering the peptide sequence of the inhibitor. The P1 residue is almost exclusively an Arg residue whereas the P2 and P3 residues are altered to mimic the substrate specificity of individual peptidases. As an example D-Phe-Pro-Arg-CH₂Cl (PPACK) reacts almost 10,000-fold better with thrombin than with uPA, due to a deeper S2 pocket created by the side chains of Trp215, Leu99, His57, Tyr60a and Trp60d, which prefers hydrophobic residues especially Pro. Also the S3 pocket has hydrophobic character. On the other hand, uPA has a shallow S2 pocket where Leu97b, Ala98 and His99 create a hydrophobic barrier, and a positively charged S3 pocket created by the side chain of Arg217 (figure 1b). Consequently uPA is inhibited 120-fold better by Glu-Gly-Arg-CH₂Cl (EGR-cmk) than thrombin (86-88). But in general irreversible inhibitors are not specific towards one trypsin-like serine peptidase, and they are so reactive
that they will also inhibit other peptidases such as the cysteine peptidases. Thus, irreversible inhibitors are not in clinical use as therapeutics.

Small molecule orthosteric inhibitors, that act orthosterically, are due to their high oral bioavailability the most successful trypsin-like serine peptidase inhibitors to enter into clinical trials as therapeutics (89-91). Many of these inhibitors consist of an Arg or Lys mimicking moiety that interacts with Asp189 in the S1 specificity pocket. Exemplified by the benzamidine derivative \textit{para}-aminobenzamidine, which forms a salt-bridge between the positively charged amidino group and the negatively charged Asp189. Rational design from structural information or by high-throughput screens of large compound libraries has identified small molecule inhibitors against various trypsin-like serine peptidases including plasmin (92), the kallikrein-related peptidases (93), HGFA, hepsin, matriptase (94, 95), thrombin (96-98), factor Xa (99) and uPA (100-102). Although these inhibitors have different chemical groups attached to the Arg or Lys mimicking moiety, in order to gain specificity, they are often found to inhibit several related peptidase.

Peptidic inhibitors, of 10-20 amino acids, have been widely used as orthosteric agents to target the activity of trypsin-like serine peptidases. Compared to small molecule inhibitors peptidic inhibitors often form a larger interaction surface with the peptidase, which often result in higher specificity. The disadvantages of peptidic inhibitors are their modest target affinity, their low biodistribution when administered orally, their short circulation half-life and their susceptibility to other proteases (103, 104). However, a major advantage is that the inhibitors are selected from combinatorial phage-display peptide libraries that allow modification of the peptides in the form of introduction of one or multiple disulphide bonds or chemical cross-linking that increases their resistance to proteolysis (figure 7a and 7b) (105-108). Furthermore, affinity maturation by construction of focussed libraries or introduction of unnatural amino acids during peptide synthesis can improve the target affinity significantly (105, 109, 110). Such orthosteric cyclic and bicyclic peptides has been selected towards plasma kallikrein (105, 111, 112), FVIIa (113), FXa (114), FXIIa (115), and uPA (116-119). When structural data is available it demonstrates that the peptides inserts the P1 Arg residue into the S1 specificity pocket of the peptidase (figure 7c and 7d). Moreover,
the peptides often contain a Glu or Asp residue that interacts with the active site residue Ser195 and His57 to obstruct the charge relay system (116, 117, 120).

Naturally occurring trypsin-like serine peptidase inhibitors can be extracted from plants and has been used as a starting point for designing specific orthosteric peptide based inhibitor. Two such trypsin-like serine peptidase inhibitors are sunflower trypsin inhibitor-1 (SFTI-1) and the *Momordica cochinchinensis* (MCoTI), which is a member of the trypsin-inhibitor cysteine –knot family (121, 122). Despite that these inhibitors display broad peptidase selectivity inhibiting many different trypsin-like serine peptidases rational design based on structural information has
developed potent and selective inhibitor against matriptase (123-129), kallikrein related peptidase 4 (130, 131), and FXa. In other examples orthosteric inhibitors towards FVIIa, plasma kallikrein, FXIIa and matriptase was selected by using the Kunitz-domain of Alzheimer’s amyloid β-protein precursor (APPI) or the standard-mechanism inhibitor ecotin as scaffolds for generating a phage-display library from which inhibitors with modest affinity and specificity were retrieved (132-135).

Antibodies are considered as attractive inhibitors of trypsin-like serine peptidases as they offer exquisite specificity as compared to small molecule inhibitors and peptidic inhibitors. Moreover, they possess excellent pharmacokinetic properties and are widely used as therapeutic agents in the clinic (136-138). Although not strictly orthosteric, antibody fragments that targets the active site of matriptase, HGFA, uPA, plasma kallikrein and FXIIa has been selected by the phage-display technology (139-145). X-ray crystal structure analysis demonstrated how the anti-matriptase and anti-HGFA antibody fragments achieve specificity by using a concave cleft, formed by the CDRs from the heavy or light chain, to interact with convex shaped surface-exposed loops of the peptidase. At the same time inserting one or two of their protruding CDR loops into the active site of the peptidase to occlude the substrate binding pockets (figure 8). The anti-matriptase antibodies A11, S4 and E2 inserts one CDR loop (H3) into the active site of matriptase utilising the S1 specificity pocket by a sub-optimal insertion of a Arg residue to form a water-mediated hydrogen bond to Asp189. A11 and S4 prevents proteolysis by matriptase by extending an additional Arg residue into the S’ side of the peptidase resulting in a tight-turn of the CDR-H3 loop away from the S2 and S3 pockets (figure 8a) (139). The tight turn of the CDR-H3 loop restricts the access of Ser195 to the scissile bond and proteolysis is prevented. Contrary, the anti-matriptase antibody fragment E2 is hydrolysed by matriptase at low pH due to a different orientation of the CDR-H3 loop in the active site. The CDR-H3 of E2 inserts in a reverse (C- to N-terminal) orientation utilising both the S2 and S3 pockets placing the scissile bond exposed for nucleophilic attack by Ser195 (figure 8b) (140). The anti-plasma kallikrein antibody DX-2930 also interacts extensively with the active site of the peptidase by inserting two CDR loops (H1 and H3) (figure 8c). The CDR-H3 loop is inserted in a substrate-like N- to C-terminal orientation utilising the S1, S2 and S3 substrate binding pockets
An Arg residue is inserted into the S1 specificity pocket forming a salt-bridge to Asp189. DX-2930 prevents proteolytic cleavage by plasma kallikrein as the CDR-H3 loop after the P1 Arg residue turn away from the S’ sides so that the scissile bond is inaccessible to nucleophilic attack by Ser195. Furthermore, the CDR-H1 loop is interacting with residues in the S1’ pocket of the peptidase. The anti-HGFA Ab58 also utilises two CDR loops (H1 and H2) to occlude the S2 and S3 substrate binding pockets (figure 8d). However Ab58 do not interact with the S1 specificity pocket of HGFA (142, 146). No structural data is available for the anti-uPA and anti-FXIIa antibodies, thus their exact mechanism of inhibition remains elusive.

**Allosteric agents.** Dating back almost 40 years, allosteric activation of a normally inactive zymogen conformation was demonstrated by induction of activity in the zymogen trypsinogen by dipeptides mimicking the native N-terminus (Ile-Val) of trypsin (147). X-ray crystal structure analysis confirmed that the dipeptide inserts into the activation pocket of trypsinogen thereby causing an allosteric switch from the inactive zymogen conformation to the active peptidase conformation (148). Non-proteolytic induction of activity has also been demonstrated in the zymogen of uPA by using the dipeptides Ile-Ile or Ile-Val, which stimulated the activity of the single-chain zymogen 20- and 80-fold respectively (149). This concept of allosteric activation was later used to stimulate Met signalling by stabilising the peptidase conformation of the inactive zymogen hepatocyte growth factor (HGF) by a longer peptide sequences corresponding to the native N-terminus of two-chain HGF (VVNGIPTR) (150). The peptide only stimulated Met signalling to levels that are 25% of those for the wild-type two-chain HGF. This was later improved by generation of a so-called ZAPtide phage display library from which highly potent cyclic allosteric activation peptides was selected towards zymogen HGF and zymogen Protein C (151).
Allosteric cyclic peptides have also been selected from phage display libraries towards FVIIa. The allosteric peptide E-76 (from the E series of peptides) binds to the 37- and 70-loops, which is placed distantly from the active site in FVIIa. This site is homologous to exosite I in thrombin. E-76 inhibits factor X activation and amidolytic activity in a non-competitive manner (152). The mechanism of E-76 remained largely elusive due to stabilisation of the active conformation of FVIIa by including an active
site covalent inhibitor (D-FFRCMK) in the X-ray crystal structure analysis. The structure revealed that E-76 induces a conformational change to the 140-loop that causes a destabilisation of the active site due to breakage of a hydrogen bond between Gln143 and Lys192, which is adjacent to Gly193 of the oxyanion hole (figure 9). Although the 140-loop is stabilised in the altered conformation by crystal contacts, the findings demonstrated that the allosteric effect of E-76 upon binding to the 37- and 70-loops is associated with increased flexibility of the 140-loop. A-183 is another allosteric peptide (from the A series of peptides) against FVIIa (153, 154). A-183 binds in the vicinity of the active site to the 60-loop and the C-terminal helix, and inhibits FX activation with a mixed-type of inhibition, whereas the amidolytic activity towards chromogenic substrates was inhibited competitively (figure 9). A crystal structure between A-183 and a zymogen variant of FVII did not provide further information about the inhibitory mechanism of A-183 (155).

Hirugen, a synthetic peptide corresponding to the C-terminal fragment of the polypeptide thrombin inhibitor hirudin from the leech Hirudo medicinalus, binds to exosite I in thrombin. Hirugen competitively inhibits cleavage of fibrinogen by steric hindrance, but accelerated the amidolytic activity of thrombin (68, 156). X-ray crystal structure analysis comparing hirugen bound and PPACK bound thrombin demonstrated that binding of hirugen cause subtle changes to the Ala190-Gly197 segment in the active site region. This rearranges the side chain of Glu192 in order to
accommodate a substrate with an acidic residue at the P3 position, thus accounting for the accelerated amidolytic activity of thrombin towards such substrates (67). Later, the crystal structure of the low activity Na⁺-free state of thrombin (E) was determined and confirmed the important role of the orientation of Glu192 in substrate recognition (157). Recently, a NMR study suggested that the effect of hirugen is more global, as it allosterically reduces the dynamics of the surface-exposed loops in the C-terminal β-barrel of the peptidase catalytic domain through a allosteric network of residues connecting the C-terminal β-barrel to the hirugen binding site in the N-terminal β-barrel. Together, this results in a thrombin conformation, which is compatible with substrate binding (31).

Antibodies have also been used as allosteric modulators of activity in trypsin-like serine peptidases such as hepsin (158-160), HGFA (146, 161), thrombin (162), FVIIa (163, 164), FIX (165), kallikrein related peptidase 3 (KLK3) (166), and uPA (167). Most of the antibodies act as allosteric inhibitors, but the FVIIa antibodies F36 and F37, the FIX antibody 224AE3, and the KLK3 antibody 8G8F5 enhances the catalytic activity of their target peptidase. Although the exact allosteric mechanisms of the antibodies remains elusive, the crystal structure of the complex between 8G8F5 and KLK3 suggested that 8G8F5 stabilises the so-called kallikrein-loop (90-loop) in an active conformation. The kallikrein-loop is the major activity-regulating loop in kallikreins (55, 166). Opposite to the activity enhancing antibodies, the allosteric inhibitory antibodies destabilise the active conformation of the peptidase by pulling a convex surface-exposed loop of the peptidase into the concave shaped V_{H}/V_{L} binding interface (figure 10). The anti-hepsin hH35 antibody forms a deep binding pocket into which the 170-loop of hepsin is buried. The position of the V_{L} chain of hH35 displaces the adjacent 180- and 220-loops of hepsin which results in a disordering of the S1 specificity pocket (figure 10b). At the end of the 180-loop, Asp194 rotates to make a hydrogen bond to His40 instead of a salt-bridge to the N-terminus Ile16. Thus hH35 destabilises the entire activation domain of hepsin (159). By a very similar mechanism Fab-112 was found to convert the active conformation of uPA into an inactive zymogen-like conformation (figure 10c). Fab-112 pulls the 140-loop >12Å away from the core of the peptidase domain resulting in an inactive conformation of uPA. The inactive conformation display zymogen characteristics as
the N-terminus Ile16 is displaced from the activation pocket, and the oxyanion hole is not properly formed due to a 3.2Å displacement of the 180-loop. Moreover, the S1 pocket is highly disordered and occluded by Arg217 as a result of a >9Å movement of the 215-217 segment. Similar to the inactive hepsin conformation induced by hH35, Asp194 in the Fab-112:uPA complex has rotated to form a hydrogen bond to Tyr40 (167). This interaction between residue 194 and 40 is also a zymogen characteristics (16). The anti-HGFA antibody Fab40 utilises another allosteric mechanism than hH35 and Fab-112. It binds to the 90-loop of HGFA, and pulls it into an inactive conformation in which the S2 substrate binding pocket on the backside of the 90-loop has collapsed (figure 10d) (161). Thus, Fab40 is a competitive inhibitor opposite to hH35 and Fab112, which inhibited in a non-competitive manner (168).

Aptamers consists of 20-50 nucleotides (DNA or RNA), which fold up in structures that allow them to bind various targets. Aptamers are selected by an in vitro process called SELEX (169, 170). Several advantages, such as high affinity, high specificity and low immunogenicity, make them suitable as therapeutic agents. An RNA aptamer, APC-167, binding to activated protein C were found to inhibit hydrolysis of small chromogenic substrates in a non-competitive manner (171). Although the exact binding site of APC-167 is unknown, it seems to inhibit protein C activity by an allosteric mechanism. Aptamers that binds to exosite I and exosite II in thrombin has also been reported. HD-1, a DNA aptamer, binds to exosite I in thrombin and hinders the binding between thrombin and its exosite I binding substrates such as fibrinogen and PAR-1 (172, 173), whereas the DNA aptamers HD-22 binds to exosite II and inhibits FV and FVIII activation (174, 175). At first HD-1, and HD-22 was reported not to alter the active site of thrombin, but later other reports demonstrated that HD-1 and HD-22 might have allosteric effect as rates of hydrolysis towards various chromogenic substrates was affected upon aptamer binding (176, 177). HD-1 and HD-22 were also used to construct a bivalent thrombin aptamer, which displayed a higher potency (178).
Small molecule inhibitors are not commonly used as allosteric regulators of activity in trypsin-like serine peptidases, but recently a small molecule glycosaminoglycan mimetic plasmin inhibitor was described to allosterically inhibit plasmin activity (179). The binding site of the various allosteric inhibitors introduced is summarised in figure 11.
A wide range of agents has been developed that are not strictly orthosteric or allosteric. These agents typically block the function of the trypsin-like serine peptidase by preventing their interaction with macromolecular substrates. This is the case for a panel of antibodies towards uPA (180, 181). The antibodies bind to exosites and sterically hinder the interaction of uPA with its physiological substrate plasminogen, but not with small chromogenic substrates, demonstrating that the inhibitory mechanism is not allosteric. In a similar manner the anti-uPA antibodies mAb-112 and mAb-PUK were demonstrated to inhibit zymogen activation of single-chain uPA by sterically interfering with the interaction between zymogen uPA and plasmin (168, 181). Interestingly, mAb-112 was also found to inhibit the amidolytic activity of uPA towards small chromogenic substrates, as described previously. This dual-action effect of mAb-112 might be an important factor for its demonstrated ability to inhibit uPA dependent processes in vivo (182).
Aptamers that inhibits peptidase function sterically has been selected towards a wide range of trypsin-like serine peptidase from the coagulation system including FVII (183), FIX (184), FX (185), and thrombin as mentioned previously. These aptamers prolongs plasma-clotting times by sterically hindering the access of substrates to their respective exosites. In other examples aptamers has been used for molecular intervention of interactions of the A-chain in trypsin-like serine peptidases with cell surface receptors. One such aptamer, upanap-12, was found to inhibit the interaction between uPA and its cell surface receptor uPAR (186). In good agreement with this observation the binding site of upanap-12 was mapped to the uPAR interacting growth factor domain of uPA (187). Another uPA binding RNA aptamer, upanap-126, demonstrated that aptamers are able to affect protein-protein interactions distantly from their binding sites. Upanap-126 binds to the C-terminal helix in the catalytic domain of zymogen uPA (187). Nevertheless, upanap-126 covers more than 80% of the length of uPA, including the growth factor domain, to sterically interfere with the binding of zymogen uPA to uPAR and also activation of zymogen uPA by plasmin. Moreover, upanap-126 were also found to inhibit the binding of vitronectin to the zymogen uPA:uPAR complex, and to inhibit uPA depended processes in \textit{in vivo} (188).

A special type of inhibitors utilises both exosite and active site binding for inhibiting their target peptidase. Hirudin, the natural thrombin inhibitor from the medical leech, binds to exosite I through its C-terminal domain, whereas the N-terminal domain extents into the active site of thrombin to form a tight and almost irreversible complex even after cleavage at the scissile bond (66). Based on this naturally occurring bivalent inhibitor, several recombinant hirudin derivatives such as Bivalirudin and Lepirudin has been designed for use in treatment of heparin-induced thrombocytopenia (189). Recently a more potent thrombin inhibitor, variegin, was isolated from the tropical bond tick (190). Similar to hirudin, X-ray crystal structure analysis demonstrated that variegin binds at thrombin exosite I but extends the N-terminal domain into the active site of thrombin. Opposite to hirudin, the cleaved C-terminal domain of variegin called MH22 retained inhibitory activity by a mechanism involving disruption of the charge rely system of the catalytic triad (191). Bivalent peptide constructs that interact with an exosite and the active site
simultaneously have also been selected towards FVIIa. Based on the scaffold of the allosteric peptide A-183, which binds in the vicinity of the active site, a phage display peptide library was constructed to select for a C-terminal extension that would reach into the active site of the peptidase. This resulted in selection of a peptide, A-183X, that opposite to the A-183 completely inhibits FVIIa catalysed activation of factor X by extending the peptide into the active site where an Arg residue is inserted into the S1 specificity pocket (113).

**Naturally occurring heavy chain-only antibodies**

Naturally occurring heavy chain antibodies was first described in relation to a pathological disorder known as heavy chain disease (192). The heavy chain antibodies were found in the serum of a patient, but they were non-functional since the variable light chain (V_L) and part of the variable heavy chain (V_H) domain were missing. In 1993 functional heavy chain antibodies were found in the serum of species from the family of *Camelidae* (camels, dromedaries, llamas, and alpacas). Camelids have in addition to the classic antibody isotypes a unique class of heavy chain antibodies (HCAbs) that contributes to the immune response in the animals (193). Three distinct subclasses of IgGs can be isolated from camelid serum. IgG1s are the conventional antibodies (150 kDa), whereas IgG2 and IgG3 are the HCAbs (100 kDa) (193). When comparing the heavy chain between the IgG subclasses, the heavy chain of IgG2 and IgG3 is 10 and 12 kDa smaller than the heavy chain in the IgG1 subclass. It was demonstrated that the smaller size of the heavy chain in IgG2 and IgG3 is due to a point mutation in the splice signal at the 3’ splice site of the constant domain 1 (CH1) exon (194, 195). Hence, the exons coding for the V_H is spliced directly to the exon coding for the hinge region. This results in a loss of the CH1 domain in the HCAbs. The consequence for the lack of the CH1 domain is that the V_H domain does not pair with a light chain binding partner to from the normal V_H-V_L interface (196) (figure 12).
Hallmark features of the heavy chain antibodies. Without the light chain binding partner the HCAbs recognises their antigen by the aid of only one single domain – the variable heavy chain domain (V<sub>H</sub>). When produced recombinant the V<sub>H</sub> domain remains soluble, thus it represents the smallest antigen-binding fragment derived from functional immunoglobulins. Due to the small size (15 kDa) of the V<sub>H</sub> domain it has been referred to as a nanobody (Nb). When sequenced it became clear that the V<sub>H</sub> domain deviates from the conventional V<sub>H</sub> domain by substitution of five amino acids that are conserved in all V<sub>H</sub> domains of vertebrates (197). The substitution of Leu11, Val37, Gly44, Leu45 and Trp47 into Ser11, Phe/Tyr37, Glu44, Arg/Cys45, and Gly47 resulted in increased solubility of the V<sub>H</sub>’s, as these residues in conventional V<sub>H</sub> domains forms a hydrophobic surface patch that interacts with the light chain. These observations were later confirmed by the crystal structure of a nanobody in complex with lysozyme (198), which revealed three distinctive features of nanobodies (figure 13).
First, the CDR-H1, CDR-H2, and CDR-H3 can adopt a larger number of possible loop conformations that deviates significantly from those defined for conventional antibodies (199, 200). This is a direct result of the higher diversity in the CDRs of nanobodies, but also because nanobodies on average have a longer CDR-H3 than that found in conventional antibodies (197, 201). Secondly, besides the conserved disulphide bond between Cys22 and Cys92, the CDR-H3 of the nanobodies often forms an additional interloop disulphide bond with a Cys in the CDR-H1 or the CDR-H2. The extra disulphide bond restrains the otherwise flexible CDR-H3 loop and also dictates the CDR-H3 to fold over and cover the conserved hydrophobic amino acids Phe37 and Trp103 that otherwise would be solvent exposed (198). Thirdly, the CDR-H3 of the nanobody often forms long protruding loops, which bind into cavities or clefts on the antigen. This allows nanobodies to recognise epitopes that are difficult to target with conventional antibodies such as the catalytic site of enzymes (198,
These features enlarge the antigen-interaction surface of the nanobodies, but it also offers a large diversity to the antigen-binding repertoire, which is important in the light of the missing light chain binding partner. Together, these hallmark features of nanobodies results in antigen specificity, affinity, and kinetic binding properties, which is comparable to those found for conventional antibodies.

**Nanobody applications.** The construction of nanobody libraries from immunized camels, llamas or alpacas are easily obtained by standard cloning procedures, and antigen specific nanobodies can be identified by phage display (204). In research nanobodies have proven useful as affinity capture agents (205) or as crystallisation chaperones stabilising flexible proteins (206-209). Nanobodies also find applications within diagnostics, and especially as tracers in non-invasive in vivo imaging.

Nanobodies possess most of the requirements for an ideal tracer for positron emission tomography (PET) and/or single photon emission computed tomography (SPECT) imaging (210). They are stable in vivo, easily labelled, have low immunogenicity, and high target specificity and affinity. Furthermore their small size assures optimal tissue penetration and fast blood clearance, contributing to a high contrast (211). Currently, an anti-human epidermal growth factor receptor 2 nanobody for PET imaging of breast cancer is in clinical trials (210, 212-215). Limitations of nanobodies as imaging agents is their fast renal excretion leading to high kidney and bladder signals, and their inability to cross the blood-brain barrier. Nanobodies also find application as potential therapeutics, due to their low immunogenicity (216). However, modification of the nanobodies is required to increase their serum half-life. One strategy is the prolonging of half-life by PEGylation (217), whereas another, and more widely used strategy is to couple the nanobody to another nanobody that binds to serum albumin. This has successfully been demonstrated for nanobodies targeting tumour necrosis factor α (TNFα) for treatment of arthritis (218). Nanobodies have been used against envenoming by Aahl and Aahll toxins from scorpion (219) and against infections (220). In pathologies and cancer, nanobodies have found application as antagonist inhibiting ligand-receptor interactions. A nanobody binding to von Willebrand factor blocks thrombosis initiation (221), and nanobodies that prevent interaction of epidermal
growth factor and hepatocyte growth factor with their cell surface receptors, has been shown to delay the outgrowth of solid tumours in murine xenograft models (222-224). The structural basis for the inhibitory mechanism of the anti-EGFR nanobodies was later demonstrated by X-ray crystal structure analysis (225). Nanobodies has also recently been used for modulation of G-protein-coupled-receptors (GPCRs) function as demonstrated by selection of inhibitory nanobodies towards the chemokine receptors CXCR4 and CXCR7 (226, 227).

Three nanobodies against active trypsin-like serine peptidases has been described. Two nanobodies, A10 and B7, were found to bind to the active site cleft of FXIIa, whereas the nanobody called nAb-C8 were reported to target the active site of uPA (228, 229). However, their exact inhibitory mechanism remains elusive as crystal structures of the nanobodies in complex with their target peptidase has not been reported. Allosteric regulation of trypsin-like serine peptidases by a nanobody has not been demonstrated, but allosteric nanobodies towards other enzymes such as dihydrofolate reductase (DHFR), the Verona integrin-encoded metallo-β-lactamase (VIM) and furin has been described (230-233).

Finally, due to the small size of nanobodies they are easily manipulated into multispecific or multivalent constructs (234, 235). Recently multispecific antibody constructs has proved useful as combination-therapy in next-generation therapeutics for cancer (236-238), and multiformat nanobody constructs is expected to contribute within this field.

The Plasminogen Activation System

An extracellular trypsin-like peptidase system involved in tissue remodelling is the plasminogen activation system (figure 14). The cascade is initiated when zymogen uPA is activated by other peptidases. In vitro, peptidases including cathepsin B, cathepsin L, human kallikrein type 2, and matrix metalloproteinase-2 (239) have been reported to active uPA (240). However physiological activator of uPA has been identified as matriptase and hepsin (241-243), plasmin (9) and glandular kallikrein (244). Generated active uPA sequentially activates plasminogen and the active form of plasminogen, plasmin, is a non-specific peptidase that
degrades various extracellular matrix proteins such as fibronectin, vitronectin and fibrin. Plasmin also activates other zymogens such as the matrixmetallopeptidases, which further leads to degradation of the extracellular matrix. uPA also has a counterpart, tissue-type plasminogen activation (tPA), that activates plasminogen during fibrinolysis in the vasculature. The plasminogen activation system also includes the serpin plasminogen activator inhibitor-1 (PAI-1). However, other serpins such as plasminogen activator inhibitor-2, proteinase nexin-1, and protein C inhibitor has also been reported as physiological relevant inhibitors of uPA and tPA. The serpin α2-antiplasmin is also part of the system and inhibits plasmin in blood and tissues (245). The activation of zymogen uPA and plasminogen is tightly coupled to the cell surface, as both the zymogen form and active form of uPA binds to the urokinase-type plasminogen activator receptor (uPAR) with low nanomolar affinity (246). The binding of uPA to uPAR at the cell surface accelerates the activation of plasminogen due to cell surface co-accumulation of uPA and plasminogen (247, 248). Components of the plasminogen activation system are found pericellularly, at focal adhesion sites at the leading or tailing edge of cells, interacting with vitronectin, integrins and endocytosis receptors. This organisation of the plasminogen activation system agrees well with the involvement of the system in cell adhesion, migration and invasion (9, 245, 249). Furthermore binding of uPA to uPAR initiates intercellular signalling important for cell proliferation and migration (250).

**Urokinase-type plasminogen activator.** The trypsin-like serine peptidase uPA consists of two disulphide-linked polypeptide chains, a C-terminal B chain and a N-terminal A chain. The B chain contains the trypsin-like serine peptidase domain, whereas the A chain contains the uPAR binding growth factor domain, a kringle domain and an interdomain linker region. uPA is a highly specific peptidase as plasminogen has been reported to be only physiological substrate, and plasminogen is cleaved by uPA by hydrolysis of a single peptide bond in the sequence Cys-Pro-Gly-Arg560-Val561-Val-Gly-Gly-Cys. Although the molecular mechanism for uPA catalysed plasminogen activation remains elusive, studies with tPA suggests that the 140-loop forms an important exosite for plasminogen during activation (251).
uPA in Cancer. As a central peptidase in the plasminogen activation system it is well established that uPA plays a pivotal role in remodelling of the extracellular matrix, and high levels of uPA has been associated with pathological conditions in which the balance between extracellular protein degradation, cell proliferation, cell adhesion, cell migration and cell invasion is obstructed (9). One such condition is cancer, in which the invasive capacity of malignant tumours can be considered as uncontrolled tissue remodelling (249). uPA has been demonstrated to be important for tumour progression utilising both plasmin-dependent and plasmin-independent processes (9, 245). Considering cancer cell migration and invasion, the general accepted view is that uPA, produced by either cancer or stromal cells, catalyses the conversion of plasminogen into plasmin, which directly or indirectly by activation of pro-matrix metallopeptidases, degrades the extracellular matrix and the basement membrane, thus clearing the path for the migrating and invasive cancer cells (252). However, uPA also stimulates cancer cell proliferation, migration and invasion through activation of an uPAR-dependent intercellular signalling cascade, and by regulating the interaction of uPAR with vitronectin, integrins and endocytosis receptors (245). Furthermore, the delicate balance between uPA and its specific inhibitor PAI-1 seem to be decisive for regulating cell adhesion, and thereby migration (245). The experimental evidence supporting the role of uPA in tumour progression involves
several *in vitro* assays with cancer cell lines indicating that uPA catalysed plasminogen activation is required to gain maximum invasiveness, but also *in vivo* models showing that anti-uPA antibodies reduces the level of metastasis to the lung in chick embryos implanted with human tumour cells, and that inhibition of the uPA-uPAR interaction decreases the level metastasis (9). The exact contribution of uPA to the specific steps in the metastatic process remains elusive, however a recent study, using a mouse xenograft model, indicated that activation of pro-uPA, and the following generation of plasmin by active uPA, is a critical step for the initial escape of PC-3 prostate carcinoma cells from the primary tumour (182).

In agreement with the decisive role of uPA for tumour progression it was demonstrated that patients with breast tumours containing a high level of uPA activity had a shorter disease-free interval than patients with low activity of uPA in the tumours (253). It was also demonstrated that high levels of uPA antigen were correlated with shortened survival of breast cancer patients (254, 255). Also high tumour levels of PAI-1 predicts a poor prognosis in breast cancer patients, and both levels of uPA and PAI-1 can be used, as independent prognostic markers, to distinguish node-negative patients that will benefit from adjuvant chemotherapy from those that will not benefit (256-259). These findings have high clinical relevance and in 2007 the American Society of Clinical Oncology, and later in 2008 the German Breast Cancer Society recommended the use of uPA and PAI-1 as prognostic markers in breast tumours and node-negative breast cancer for evaluating prognosis and planning adjuvant treatment (260, 261). Moreover, the American Society of Clinical Oncology also concluded that uPA has potential as a valuable therapeutic target in breast cancer. Besides breast cancer high levels of uPA has also been suggested as a prognostic marker in other types of cancers such as lung, bladder, and stomach (9).

**uPA in Arthritis.** Beside its role in cancer progression, it has also been shown that uPA plays a role in inflammatory diseases such as rheumatoid arthritis. The synovial membrane is a thin layer of cells between the joint capsule and the joint cavity. It consists primarily of macrophages and fibroblast and it secretes the synovial fluid, which fills the joint cavity. In inflamed joints the synovial membrane is characterised by tissue hyperplasia, with increased numbers of activated immune cells such as
phagocytes and lymphocytes fibroblasts (262). These cells are believed to secrete elevated levels of uPA into the joint cavity upon activation by cytokines in the inflamed joints. This results in a burst of plasmin generation that either directly or by activation of matrix metallopeptidases degrades the cartilage in the joints (263). Especially the invasion of macrophages into the synovial membrane is a key driver of development of rheumatoid arthritis and seems to be dependent on the proteolytic activity of uPA (264, 265). However, contrasting results about the role of uPA in development of rheumatoid arthritis has been reported. Using a monarticular model of antigen-induced arthritis (AIA) using methylated BSA as antigen it was shown that plasminogen deficient mice (Plg⁻/⁻) developed more severe arthritis than wild-type mice (266), whereas a systemic model of collagen inducing arthritis (CIA) demonstrated that Plg⁻/⁻ mice were completely resistant to arthritis (267). A similar pattern was observed for uPA-deficient mice, with uPA deficiency leading to more severe arthritis development in the AIA model (266), but less diseases in the CIA model (268). As direct blockade of plasmin is risky due to likely complications of thrombosis or bleeding it has been proposed to consider uPA as a therapeutic target in rheumatoid arthritis. However, the observed conflicting roles of uPA in disease progression has challenged this view, and it has been suggested that potent and highly selective inhibitors of uPA could help resolve the role of uPA in disease progression and whether inhibition of uPA activity would have any therapeutic value (269).
Project Aims

The overall aim of this project is to develop new principles for pharmacological intervention with pathophysiological functions of trypsin-like serine peptidases. As described in the introduction - when uncontrolled, the activity of peptidases can lead to severe pathological conditions. There is therefore extensive interest in generating a framework for development of specific inhibitors to be used for molecular intervention with the activity of trypsin-like serine peptidases. In this project I will focus on three different types of inhibitors – orthosteric agents, allosteric agents, and inhibitors of zymogen activation. Furthermore, the availability of such inhibitory has allowed me to study molecular mechanisms of regulation of activity in trypsin-like serine peptidases.

Orthosteric agents

An improved understanding of the molecular mechanisms for targeting the active site of trypsin-like serine peptidases could form a basis for new approaches to regulate their activity. Two different types of inhibitors were used to target the active site region of the trypsin-like serine peptidase uPA. First, we used a cyclic peptide, originally selected against murine uPA, to study how modification of peptide-peptidase interactions can improve the affinity of the peptide (I). Next, we use the same peptide scaffold to construct a focussed peptide-peptidase fusion back-flip library from which we select a high affinity peptide with optimal binding entropy (II). The second type of orthosteric inhibitor investigated was Camelid derived antibody fragments (nanobodies). Our purpose was to select nanobodies that selectively targets the active site of uPA in order to improve the understanding of nanobodies as potent active site enzyme inhibitors as suggested by studies with nanobodies against lysozyme (202). This was investigated by selecting nanobodies against active human uPA and active murine uPA (VI).

Allosteric agents

Allosteric regulation of activity in trypsin-like serine peptidases is an important control mechanism utilised in nature. Allostery implies that the peptidases exist in
equilibrium between active and inactive conformations, and that the equilibrium can be shifted upon binding of natural ligands or cofactors that stabilises a distinct conformation, which is associated with a specific activity or function. The allostERIC nature of trypsin-like serine peptidase can also be exploited by selecting inhibitors such as monoclonal antibodies that binds and stabilises active and inactive conformations of their target peptidase. We aimed at understanding allostery in trypsin-like serine peptidases by selecting and characterising allostERIC monoclonal antibodies and nanobodies towards murine uPA (III, IV, V). Such types of allostERIC agents would allow a deeper understanding of the molecular mechanism of allostery in trypsin-like serine peptidases, which in general is poorly understood.

**Inhibition of zymogen activation by nanobodies**

The strategy of targeting zymogen activation has proven successful using monoclonal antibodies. One such antibody mAb-112 was shown to inhibit uPA depended processes *in vivo*. One major challenged for targeting the active mature peptidase is that the inhibitory agent has to associate very fast with the target peptidase in order to avoid even small amounts of peptidase activity. The idea is that targeting zymogen activation is a more efficient strategy to avoid activity. We aimed at selecting nanobodies that selectively binds the zymogen form of uPA thus preventing its conversion into a mature active peptidase.
Results

The results contain two sections. The first section covers a brief summary of the main results from the six manuscripts included in the appendixes in which more detailed descriptions of the results obtained can be found. The second section is a more detailed description of results, which has not been included in the six manuscripts. These results are divided into two parts. The first part covers the selection and characterisation of an anti-muPA nanobody called Nb22. The second part covers the selection and characterisation of nanobodies inhibiting zymogen activation of human uPA. It should be noted that the results not included in the manuscripts is still on-going work.

Summary of main results from the manuscripts

I. A cyclic Peptidic Serine Protease Inhibitor: Increasing Affinity by Increasing Peptide Flexibility

In this report we describe in molecular details how affinity of a cyclic peptidic inhibitor, mupain-1 (CPAYSRYLDC), targeting murine uPA (muPA) can be improved by substitution of the P1 Arg residue in the peptide with non-natural amino acids. Furthermore we found, unexpectedly that the affinity of the peptide can be improved by substituting Asp9 in the peptide with an Ala residue thereby removing a polar interaction. We conclude that D9A substitution increases the flexibility of the peptide, which allows it to adapt to more favourable exosite interaction in the enzyme.

My contribution to this work was generation of alanine mutants of muPA, and determination of inhibitory constants ($K_i$) of the peptides for different enzymes.
Mupain-1, mupain-1-12 and mupain-1-16 and X-ray crystal structure determination. The cyclic peptide mupain-1 (CPAYSRYLDC) was originally selected from a phage-display library and was found to inhibit muPA activity competitively with a \( K_i \) value of 0.5 \( \mu \)M (119). Two variants of mupain-1, with non-natural amino acids L-4-guanidino-phenylalanine (mupain-1-12) or L-3-(N-amidino-4-piperidyl)alanine (mupain-1-16) as P1 residues instead of the original Arg6, was later found to have 2- to 10-fold improved affinities towards muPA (109). We have not been able to crystallise muPA in complex with mupain-1, mupain-1-12 or mupain-1-16.

In the present work, we determined the X-ray crystal structures of mupain-1, mupain-1-12 and mupain-1-16 in complex with a “murinised” version of human uPA called huPA-H99Y. The structures revealed that all three peptides bind in a similar manner to huPA-H99Y. They insert the P1 Arg6 residue into the S1 specificity pocket forming a polar interaction with Asp189. The interaction surface also includes a salt-bridge between Arg35 in the 37-loop of huPA-H99Y and Asp9 of the peptides (figure 15a). The peptides were found to be inhibitors and not substrates as the distance between the nucleophilic oxygen atom of Ser195 and the putative scissile bond in the peptides are too large (3.9\( \AA \)). Moreover, the oxygen atom of the carbonyl group of Arg6 in the peptide was not properly aligned in the oxyanion hole (figure 15b).

![Figure 15. X-ray crystal structure analysis of huPA-H99Y in complex with peptidic inhibitors. A. Overall structure of the complexes between huPA-H99Y (wheat) and mupain-1 (PDB: 4x1q, red), mupain-1-16 (PDB: 4x1n, green), and mupain-1-16-IG (PDB: 4zbn, yellow). Red dashed lines indicate the salt-bridge between Arg35 of huPA-H99Y and Asp9 of mupain-1 and mupain-1-16. The position of mupain-1-16-IG is shown to illustrate that the IG mutation remove the salt-bridge. B. A zoom on the oxyanion hole of huPA-H99Y.](image-url)
Site-directed mutagenesis, ITC, and SPR. Site-directed mutagenesis of the peptides and huPA-H99Y largely confirmed the observations from the X-ray crystal structures. However, substitution of Asp9 in the peptides with an Ala residue (D9A) led to a 3- to 10-fold increase in affinity of the peptides, which was counterintuitive due to the Asp9-Arg35 salt-bridge (table 1 in appendix I). Isothermal titration calorimetry (ITC) demonstrated that the D9A mutation in mupain-1-12 and mupain-1-16 was associated with a binding entropy penalty, indicating that the Ala9 peptides are more flexible in solution than their original Asp9 variants (table 3 in appendix I). Next, surface plasmon resonance (SPR) analysis revealed that the increased affinity of the Ala9 peptides was due to a 3- to 4-fold increase in the association rate ($k_{on}$) and a 2- to 3-fold decrease in dissociation rate ($k_{off}$) (table 2 in appendix I). This suggests that the D9A substitution in mupain-1, mupain-1-12 and mupain-1-16 increases the flexibility of the peptides, which allows them to adapt to more conformations of huPA-H99Y thereby increasing the overall binding enthalpy and affinity.

Site-directed mutagenesis in muPA. Using site-directed mutagenesis we identified Lys41, Tyr99 and Lys143 in muPA as a part of the binding site for mupain-1, mupain-1-12 and mupain-1-16. Contrary to huPA-H99Y there was no effect of the D9A substitution to the affinities of the peptides to wild-type muPA. Interestingly, we observed an increase in affinity of the Ala9 peptides towards the K41A muPA mutant (table 4 appendix I). Based on this we concluded that Lys41 sterically hinders mupain-1, mupain-1-12 and mupain-1-16 from assuming the binding conformation with the lowest energy, and that the D9A mutation allows the peptide to adapt to the K41A mutation by forming alternative surface interaction with muPA.

Importance of the P1 residue in mupain-1, mupain-1-12 and mupain-1-16. We analysed the difference in interactions of the three P1 residues in mupain-1, mupain-1-12 and mupain-1-16 with the S1 specificity pocket by introducing two point mutations in the pocket. The S190A and V213T mutations resulted in different inhibitory profiles depending on the P1 residue (table 5 in appendix I). As an example: the affinity of mupain-1 towards the V213T mutant increases 2.5-fold as
compared to wild-type muPA, whereas it increases 5-fold for mupain-1-12 and mupain-1-16. This indicates that the different P1 residues utilises different interaction in the S1 pocket, which accounts for the increased affinities of the mupain-1-12 and mupain-1-16 peptides as compared to the original mupain-1. The increased affinities also rely on amino acids outside of the S1 pocket as the K143A mutation resulted in a 9-fold decrease in affinity for mupain-1, whereas the decrease for mupain-1-16 was only 2.9-fold. This suggests that Lys143 influences the effect of the P1 substitution and vice versa.

II. Selection of High-Affinity Peptidic Serine Protease Inhibitors with Increased Binding Entropy from a Back-Flip Library of Peptide-Protease Fusions.


In this report we describe a selection strategy for affinity maturation of peptidic inhibitors. We constructed a small fusion peptide-protease library consisting of mupain-1 (CPAYSRYLDC), with randomised residues in selected positions, fused to the catalytic domain of murine uPA (muPA) via a linker. We screened the library for activity modulating peptides and identified a high affinity peptide, which after substituting the P1 residue with non-natural amino acids, have a 250-fold increased affinity for muPA as compared to the original mupain-1 peptide. We also explain the rationale behind the enhanced affinity.

My contribution to this work was generation of alanine mutants of muPA, and determination of inhibitory constants ($K_i$) of the peptides for different enzymes.

Selection of a high affinity peptide from a protease-peptide back-flip library. The back-flip library was designed by recombinant fusing of the catalytic domain of muPA to mupain-1 (CPAYSRYLDC) via a linker containing two TEV protease sites. We validated the construct by measuring the $K_M$ for a chromogenic substrate before and after cleavage with the TEV protease. The TEV protease reduced the $K_M$ from 21.1 mM to 5.1 mM, which is similar to the $K_M$ for non-fused muPA, hence validating that
the linker was sufficiently long to allow mupain-1 to interact with the active site of muPA (figure 1 in appendix II). We next randomised mupain-1 in two positions generating a library of 400 fusion constructs (muPA-CPAYSRYXXC). From the library we isolated a peptide mupain-1-IG (CPAYSRYIGC), which increased the $K_M$ of the protease-peptide fusion protein approximately 2-fold to 38.5 mM. Substitution of the P1 Arg6 residue to L-4-guanidino-phenylalanine (mupain-1-12-IG) or L-3-(N-amidino-4-piperidyl)alanine (mupain-1-16-IG) decreased the $K_i$ by 55- and 275-fold towards muPA and 1500- and 1700-fold towards huPA-H99Y, as compared to the original mupain-1 peptide (table 1 in appendix II).

**The rationale behind the observed increase in affinity of the IG peptides.** X-ray crystal structure analysis of mupain-1-IG and mupain-1-16-IG in complex with the huPA-H99Y did not explain the improved affinities of the peptides following the L8I and D9G substitutions, as mupain-1-IG and mupain-1-16-IG binds similarly to mupain-1 and mupain-1-16. In fact, the improved affinities were counterintuitive as the Asp9-Arg35 salt-bridge between the peptides and huPA-H99Y was removed by the IG substitution.

Site-directed mutagenesis in muPA confirmed the interactions suggested by the crystal structure of the peptides in complex with huPA-H99Y. One interesting observation was that the K41A mutation in muPA led to a much stronger reduction in $K_i$ upon the IG substitution as compared to any other substitutions, hence suggesting that the affinity-enhancing effect of the IG substitution is related to interaction between the peptides and muPA in this area (figure 16). ITC experiments demonstrated that the increase in affinity of the IG peptides was enthalpy driven and associated with an entropy penalty (table 3 in appendix II). Thus, in agreement with our previous findings we concluded that the IG substitution renders the peptides more flexible in solution, which allows the IG peptides to adapt to different conformations of muPA resulting in a more stable bound state of the peptide. This hypothesis is in reasonable agreement with the observation that the IG substitution also increases the affinity of the peptides towards other trypsin-like serine peptidase such as plasma kallikrein.
III. Interconversion of active and inactive conformations of urokinase-type plasminogen activator


In this report we describe how the catalytic domain of muPA exists in a conformational equilibrium between partially and maximally active states. The allosteric monoclonal antibody mU3, and the orthosteric peptidic inhibitor mupain-1-16 were found shift the equilibrium towards the active conformations, whereas destabilising mutations in the catalytic domain of muPA shifts the equilibrium towards the less active conformations. We further demonstrated that the major difference between the active and less active conformations is the level of solvent-exposure of the N-terminus Ile16.

My contribution to this work was generation of alanine mutants of muPA, determination of $K_M$ and $K_i$ values for various mutants and muPA variants, SPR analysis, determination of second order rate constants between muPA and PAI-1, and the carbamylation assays.

Identification of a disordered muPA conformation. The truncated muPA variant called muPA$_{16-243}$, which only contains the catalytic domain, exists in a slightly distorted state, as it has lower affinity than the full-length enzyme for a chromogenic
substrate pyro-Glu-Gly-Arg-pNa (S-2444) and several active site binding inhibitors such as amiloride, p-aminobenzamidine and mupain-1-16 (table 2 in appendix III). Furthermore, using a technique called carbamylation, we show that the N-terminus Ile16 is more frequently solvent-exposed in muPA\textsuperscript{16-243} than in full-length muPA. The equilibrium can be shifted even further towards the low activity conformations, as two destabilising mutations F40Y and E137A affected the affinity for S-2444 and active site binding inhibitors more severely than for muPA\textsuperscript{16-243}.

**The effect of the monoclonal antibody mU3 on the level of disorder in muPA.** The monoclonal antibody mU3 was originally developed in the Finsen Laboratory in Copenhagen and was demonstrated to inhibit uPA-catalysed plasminogen activation, but not cleavage of small chromogenic substrates (270). Using alanine-scanning mutagenesis we mapped the binding epitope of mU3 to the 37- and 70-loops of muPA (figure 17a). Although the epitope of mU3 is located > 20Å from the active site we demonstrated that the binding of mU3 to the 37- and 70-loop, directly alters the function of the active site. mU3 shifts the equilibrium of conformations in muPA\textsuperscript{16-243} towards the active states as the affinity for S-2444 and the active site binding inhibitors increases to values similar to full-length muPA. Furthermore, binding of mU3 to the 37- and 70-loop decreases the level of solvent-exposure of the N-terminal Ile16 as measured by carbamylation. Moreover, mU3 were also found to restore the active conformation in the F40Y and E137A mutants (table 3 in appendix III). ITC experiments further validated our findings, as the entropy penalty associated with binding of the peptidic inhibitor mupain-1-16 to muPA\textsuperscript{16-243} was reduced in the presence of mU3, demonstrating that mU3 shifts the equilibrium of conformations towards the active states by stabilising one of these active conformations upon binding to the 37- and 70-loop (table 4 in appendix III).
IV. Allosteric inactivation of a trypsin-like serine protease by an antibody binding to the 37- and 70-loops


In this report we describe the inhibitory mechanism of an allosteric monoclonal antibody, mU1, than binds to the 37- and 70-loops situated >20Å from the active site in muPA. We show that binding of mU1 destabilises the architecture of the active site, as the N-terminal Ile16 is less efficiently stabilising the oxyanion hole. Also the S1 specificity pocket was found to be dysfunctional in the presence of mU1. Furthermore, we demonstrates that the A-chain in muPA stabilises the active conformation of the catalytic domain (B-chain), as deletion of the A-chain results in a disordered conformation of the catalytic domain, which on many parameters resembles the one stabilised by mU1.
My contribution to this work was generation of alanine mutants of muPA, determination of $K_M$ and $K_i$ values for various mutants and muPA variants, SPR analysis, and the carbamylation assays.

**The effect of mU1 on muPA.** The monoclonal antibody mU1 was developed in the Finsen Laboratory in Copenhagen, and was found to inhibit uPA-catalysed plasminogen activation, plasmin-mediated activation of single-chain muPA, and also uPA-dependent processes *in vivo* (270). In the present work, we demonstrated that mU1 did not prevent plasmin-mediated cleavage of the Lys15-Ile16 peptide bond in muPA, but once cleaved mU1 is able to maintain the generated two-chain muPA in an inactive conformation. We determined the mU1 epitope, by alanine scanning mutagenesis, to the 37- and 70-loops > 20Å from the active site of muPA (figure 17b). Nevertheless, binding of mU1 to the 37- and 70-loops alters the function of the active site through an allosteric mechanism, as mU1 was found to inhibit muPA hydrolysis of a small chromogenic substrate (S-2444) in a competitive manner (table 1 in appendix IV). We further demonstrated that the mU1-stabilised conformation was characterised by a more frequently solvent-exposed N-terminal Ile16 as measured by carbamylation (figure 7 in appendix IV). Moreover, the S1 specificity pocket and the oxyanion hole is inaccessible in the mU1 stabilised conformation as the S1 binding fluorescent probe $p$-aminobenzamidine is competitively displaced and as binding of diisopropyl fluorophosphates (DFP) in the oxyanion hole of muPA was inhibited by mU1.

**The inhibitory mechanism of mU1 and the intra-domain linker.** We proposed an inhibitory mechanism of mU1 based on three findings. Firstly, mU1 inhibit a truncated variant of muPA (muPA$^{16-243}$), containing only the catalytic domain 7-fold more potently than full-length muPA. Secondly, two mutations in the intra-domain linker, F(-2)A and K4G, in full-length muPA destabilise the active conformation of muPA resulting in reduced affinity for S-2444. Thirdly, mU1 inhibits the full-length muPA mutant F(-2)A-K4G with the same inhibitory constant $K_{D}^{APP}$ as for the muPA$^{16-243}$ variant (table 2 in appendix IV). Based on this we hypothesise that the residues in the catalytic domain contacting Phe(-2) and Lys4 might be responsible for the
allosteric communication between the mU1 epitope in the 37- and 70-loop and the active site of muPA, as quenching of these interaction by our mutations in the intra-domain linker in full-length muPA gives rise to a conformational change to the catalytic domain, which increases the affinity to mU1.

V. Unravelling an Allosteric Regulatory Mechanism in Trypsin-Like Serine Proteases by the use of a Camelid Derived Antibody Fragment

Tobias Kromann-Hansen, Eva Louise Lange, Lin Lin, Hans Peter Sørensen, Jan K. Jensen, Gholamreza H. Ghassabeh, Serge Muyldermans, Mingdong Huang, Paul J. Declerck and Peter A. Andreasen (manuscript in preparation)

In this report we describe several X-ray crystal structures of the catalytic domain of muPA. We crystallised a ligand free inactive conformation (muPA inactive), a muPA conformation stabilised by an inhibitory Camelid derived antibody fragment called Nb7 (muPA Nb7), and two fully active conformations with two different active site binding inhibitors (muPANb7:EGR-cmk and muPANb7:p-aminobenzamidine). The crystal structures allowed us to follow large and subtle conformational changes as muPA converts from a substrate-bound active conformation to an inactive ligand free conformation. Furthermore, the analysis suggests that binding of Nb7 to its epitope in the 37- and 70-loop of muPA activates a dynamic allosteric pathway that propagates through the 140-loop to the active site region of muPA.

My contribution to this works was development of the nanobody Nb7, X-ray crystal structure determination and analysis, site-directed mutagenesis of muPA, determination of $K_M$ and $K_i$ values for various mutants and muPA variants, the carbamylation experiments, and the limited proteolysis experiments.

**Crystallisation of an inactive muPA conformation.** Initial attempts to crystallise muPA in complex with the covalent inhibitor Glu-Gly-Arg-chloromethylketone (EGR-cmk) did not result in crystal suitable for diffraction experiments. However, ligand free muPA, which is fully active in solution crystallised readily. The structure revealed that ligand free muPA crystallises in a completely inactive conformation...
(\text{muPA}_{\text{inactive}}) \text{ in which the active site architecture is collapsed due to a } 180^\circ \text{ turn of the beta-strand connecting the 170-loop and the 180-loop (figure 18). This results in a } >20 \text{ Å translocation of the oxyanion stabilising loop (180-loop) to the opposite side of the enzyme as compared to its observed position in an active conformation of human uPA. The consequence of the translocation is a complete disorganisation of the S1 specificity pocket and the oxyanion hole due to the lack of the salt-bridge between Asp194 and Ile16. Moreover, the 70-loop and the autolysis loop (140-loop) were found to be highly flexible in the inactive conformation. The inactive conformation could convert to a fully active one (\text{muPA}_{\text{active}}), as muPA from dissolved crystals formed a covalent complex with the natural uPA inhibitor PAI-1. Thus we concluded that the formation of the crystals selects for the low-populated inactive conformation that reverts to an active one when entering the solution state again, demonstrating that the conformational equilibrium in solution is strongly shifted towards the active conformation.}

**Identification of an inhibitory Camelid derived antibody fragment.** We generated a nanobody library by immunising an alpaca with recombinant muPA. From the library we retrieved a nanobody, Nb7, which was found to inhibit muPA activity towards plasminogen and a small chromogenic substrate. \textit{P-}aminobenzamidine displacement and carbamylation experiments revealed that Nb7 stabilises a conformation of muPA in which the N-terminal Ile16 is more frequently solvent-exposed, and in which the S1 specificity pocket is dysfunctional. By alanine-scanning mutagenesis we mapped the binding epitope of Nb7 to the 37- and 70-loops in muPA. As the binding site is >20Å from the active site we concluded that Nb7 is an allosteric inhibitor.
X-ray crystal structures of Nb7 in complex with muPA in the absence or presence of active site ligands. The X-ray crystal structure between muPA and Nb7 confirmed the Nb7-binding epitope and revealed that Nb7 inserts a long protruding CDR-H3 into a hydrophobic cleft formed by the 37- and 70-loops displacing the 70-loop by 14 Å as compared to its observed position in structures of active human uPA. In the complex muPA assumes a conformation (muPA_{mu7}) in many respects similar to the active form of human uPA and presumably the unknown active form of muPA, as we did not observe any of the conformational changes expected by our biochemical

Figure 18. X-ray crystal structure of an inactive muPA conformation. The catalytic domain of muPA from residue 16 to 243 containing a C122A mutation was crystallised by hanging drop vapour diffusion in 1.8M Li_{2}SO_{4} as precipitant. A. The inactive conformation of ligand free muPA (muPA_{human}, wheat). Red spheres highlights residues that changes position following the β-strand swap (highlighted in red) as compared to the ligand free conformation of human uPA (blue, PDB:4dva). The N-terminal β-barrel is largely preserved, whereas the C-terminal β-barrel is completely disordered. B. After dissolving the crystals we reacted 2µg of muPA with 2-fold molar excess of PAI-1 and analysed formation of the covalent complex by non-reduced SDS-PAGE analysis.
assays. This is likely to be a result of crystal packing effects. To assess if the muPA_{Nb7} conformation was able to bind active site ligands, we soaked the crystal complexes with EGR-cmk or p-aminobenzamidine and re-determined the crystal structures. The re-determined structures revealed that the muPA_{Nb7} conformation was able to bind EGR-cmk (muPA_{Nb7}:EGR-cmk) and p-aminobenzamidine (muPA_{Nb7}:p-aminobenzamidine).

Although the overall conformation of muPA in the three crystal structures is identical, we compared the individual b-factors to monitor changes in dynamics upon binding of the active site ligands (figure 19). Four regions where highlighted by the analysis: the N-terminal activation loop (residue 16-23), the autolysis loop (residue 142-153), the oxyanion stabilising loop (184-194), and the S1 entrance frame (residue 216-223). These regions, which were originally defined as the activation domain, is ordered in muPA_{Nb7}:EGR-cmk and muPA_{Nb7}:p-aminobenzamidine but less ordered in muPA_{Nb7}. Thus binding of e.g. p-aminobenzamidine in the S1 pocket affects the dynamics of distantly positioned loops such as the activation loop and the autolysis loop (140-loop). The analysis further highlighted a cluster of amino acids including Ile16, Lys143, Lys192, Cys191 and Cys220, which interconnects the loops of the activation domain and changes dynamics when going from the muPA_{Nb7}:EGR-cmk or muPA_{Nb7}:p-aminobenzamidine to the muPA_{Nb7} conformation.

The Nb7-stabilised muPA conformation is highly flexible. The muPA_{Nb7} conformation was characterised by a highly flexible 140-loop, whereas it was less flexible in the muPA_{Nb7}:EGR-cmk and muPA_{Nb7}:p-aminobenzamidine conformations especially at the N- and C-terminal stems. This structural observation was validated by limited proteolysis experiments using endoproteinase Glu-C (V8), which cleaves C-terminally to Glu residues. The result shows that a single cleavage site in the 140-loop between Glu146-Ser147 was more frequently exposed in the presence of Nb7. In good agreement with the structural data, the effect of Nb7 on the flexibility of the 140-loop was reverted in the presence of EGR-cmk.
VI. A Camelid-Derived Antibody Fragment Targeting the Active Site of a Trypsin-like Serine Protease Balances Between Inhibitor and Substrate Behaviour

**Tobias Kromann-Hansen**, Emil Oldenburg, Kristen Wing Yu Yung, Gholamreza H. Ghassabeh, Serge Muyldermans, Paul J. DeClerck, Mingdong Huang, Peter A. Andreasen and Jacky Chi Ki Ngo (manuscript in preparation)

In this report we describe an orthosteric Camelid derived antibody fragment, Nb4, that inhibit the activity of human uPA by inserting a long protruding CDR-H3 loop into the active site of the enzyme in a substrate-like manner. We demonstrate
that the inhibitory properties of Nb4 depend on several intra-loop hydrogen bonds of the CDR-H3, which stabilises a tight interaction between Nb4 and uPA. The inhibitory mechanism of Nb4 thereby resembles those of natural occurring standard mechanism inhibitors.

My contribution to this work was development of the nanobody Nb4, the SPR analysis, p-aminobenzamidine displacement assays, X-ray crystal structure determination and analysis of the free form of Nb4, site-directed mutagenesis of Nb4 and the proteolysis experiments.

**Selection of an inhibitory Camelid derived antibody fragment.** From an anti-uPA nanobody-library we identified a nanobody called Nb4, which binds with high affinity to the active form of uPA as determined by SPR analysis. The sequence of Nb4 revealed an arginine-rich motif in the CDR-H3, and we demonstrated, using the p-aminobenzamidine displacement assay, that either Arg100f or Arg100g of the CDR-H3 inserts into the S1 specificity pocket as mutation of these arginines to alanines abrogated the effect of Nb4.

**X-ray crystal structure analysis.** We determined the X-ray crystal structures of Nb4 in its free form (Nb4<sub>free</sub>) and Nb4 in complex with the catalytic domain of uPA (uPANb4). The data demonstrated that the binding of Nb4 to uPA is not associated with large conformational changes to either Nb4 or uPA. Nb4 binds in a substrate-like manner to uPA by inserting the CDR-H3 loop into the active site of uPA utilising the S3 to S3’ substrate binding pockets in the active site region (figure 20a). Nb4 form a specificity determining exosite interaction between the CDR-H1 of Nb4 and the 37-loop in uPA. From the crystal structure of uPANb4 it was evident that Arg100f of Nb4 inserts into the S1 specificity pocket of uPA (figure 20b). The putative scissile bond of Nb4 between Arg100f-Arg100g is perfectly aligned in the active site for catalysis. The carbonyl group of Arg100f is 2.9Å from the Ser195 Oy atom, and the oxygen atom of the carbonyl group of Arg100f is stabilised in the oxyanion hole. In the CDR-H3 loop of Nb4 we observed a tight hydrogen-bond network that connects the P and P’ side of the putative scissile bond especially one hydrogen bond from the P1’ Arg100g to Asp95 seems to be important for the stability of the CDR-H3.
Inhibitor or substrate behaviour of Nb4. Incubation of Nb4 with uPA revealed that the putative scissile bond (Arg100g-Arg100f) in Nb4 is hydrolysed slowly by uPA (figure 20c). To determine the difference between inhibitor or substrate behaviour of Nb4 we performed cleavage experiments with the Nb4 mutants R100gA, D95A and Y110iA. The results demonstrated that under the given conditions the cleavage reaches a steady-state equilibrium as approximately 50% intact wild-type Nb4 could be detected after 96 hours of incubation with uPA. The R100gA, D95A and Y110iA mutants destabilises the hydrogen-bond network in the CDR-H3. Hence, the equilibrium for these mutants was shifted strongly towards substrate behaviour.
Thus, we concluded that the intra-loop hydrogen-bond network stabilises the CDR-H3 in the active site of uPA, and that Nb4 after cleavage at the scissile bond by uPA remains tightly bound due to the high stability of the intra-loop hydrogen-bond network, which contributes to a very slow dissociation rate of the cleaved inhibitor-enzyme complex, thus balancing the inhibitor/substrate behaviour of Nb4.

Results not included in the manuscripts

Development and characterisation of a nanobody targeting the active site of muPA

Development of an anti-muPA nanobody. A nanobody library was generated by immunisation of an alpaca (Vicugna pacos) with recombinant active two-chain muPA. Screening the library with two-chain muPA as bait, we recovered a nanobody (Nb22) that inhibits the activity of muPA towards its macromolecular substrate plasminogen with a \( IC_{50} \) value of 1.6 ± 0.2 nM (figure 21). Moreover, Nb22 also inhibited muPA hydrolysis of a small chromogenic peptide substrate pyro-Glu-Gly-Arg-pNa (CS-61(44)) with an \( IC_{50} \) value of 0.49 ± 0.10 nM (figure 21). Thus, Nb22 is a potent inhibitor of muPA activity.

Next, we immobilized Nb22 on a CM5 surface sensor chip and measured binding affinities of Nb22 for different muPA variants using surface plasmon resonance (SPR). The results show that Nb22 binds with high affinity to active two-chain muPA \( (K_d = 0.17 \pm 0.05 \text{ nM}) \) (table 1). We also determined the affinity of Nb22 towards the zymogen form of muPA using a muPA zymogen variant (single-chain muPA\(^{(2-243)}\)) containing and C122A mutation, and the first 10 amino acids of the intra-domain
linker before the intact peptide bond between Lys15 and Ile16 (chymotrypsin numbering). The analysis demonstrated that Nb22 also binds to single-chain muPA\(^{[2-243]}\), although with an approximately 150-fold lower affinity \(K_D = 27 \pm 15 \text{ nM}\) than for the two-chain active form. Moreover, Nb22 displays a >5000-fold decrease in affinity towards active two-chain muPA when the active site was blocked with the covalent inhibitor Glu-Gly-Arg-chloromethylketone (EGR-cmk). EGR-cmk occupies the S1, S2 and S3 specificity pocket of the active site in muPA. The reduction in affinity of Nb22 towards muPA:EGR-cmk could either be due to binding competition between Nb22 and EGR-cmk, or because binding of EGR-cmk to the active site of muPA causes a binding-deteriorating conformational change to the Nb22 binding epitope.

Table 1. SPR analysis of binding of Nb22 to two-chain muPA, single-chain muPA\(^{[2-243]}\) and active site inhibited two-chain muPA:EGR-cmk. Association rates \(k_{on}\), dissociation rates \(k_{off}\), and the equilibrium binding constants \(K_D\) were determined by fitting the SPR data to a 1:1 binding model. The data are reported as the mean ± standard deviation for the reported number (N) of determinations*Significantly different form the value for two-chain muPA \((p < 0.01)\). All p-values were calculated by Student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th>(k_{on} (\text{M/s}) \times 10^5)</th>
<th>(k_{off} (\text{s}) \times 10^{-5})</th>
<th>(K_D \text{ (nM)})</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-chain muPA</td>
<td>5.60 ± 2.02</td>
<td>8.03 ± 1.91</td>
<td>0.17 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>Single-chain muPA(^{[2-243]})</td>
<td>0.036 ± 0.017*</td>
<td>8.40 ± 0.71</td>
<td>27 ± 15*</td>
<td>2</td>
</tr>
<tr>
<td>Two-chain muPA:EGR-cmk</td>
<td>0.0013 ± 0.0004*</td>
<td>18 ± 3*</td>
<td>1500 ± 280*</td>
<td>2</td>
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Inhibition of plasmin-catalysed single-chain muPA activation. Based on the observation that Nb22 binds to single-chain muPA\(^{[2-243]}\), we analysed the effect of Nb22 on plasmin-catalysed cleavage of the Lys15-Ile16 peptide bond using the single-chain muPA\(^{[2-243]}\) variant (figure 22). The \(k_{cat}/K_M\) value for single-chain muPA\(^{[2-243]}\) is approximately 260-fold lower than that of the two-chain form, which is the expected value for zymogen uPA (271, 272). The activation of single-chain muPA\(^{[2-243]}\), as measured by activated muPA\(^{[16-243]}\) hydrolysis of CS-61(44), was inhibited by Nb22. Unexpectedly, the inhibitory activity of Nb22 seems not to be related to its inhibition of CS-61(44) hydrolysis, as the Lys15-Ile16 peptide bond in single-chain muPA\(^{[2-243]}\) was found to be intact as evaluated by SDS-PAGE analysis, which demonstrated that single-chain muPA\(^{[2-243]}\) is not converted to muPA\(^{[16-243]}\) in the presence of Nb22. As a positive control we used the anti-muPA monoclonal antibody
mU1 that was previously described to inhibit CS-61(44) hydrolysis, but not plasmin-catalysed cleavage of the Lys15-Ile16 peptide bond (272).

Epitope mapping. To determine the epitope of Nb22, we analysed the Nb22 concentration dependency of inhibition of CS-61(44) hydrolysis using alanine substitution mutants in two-chain muPA. We generated a panel of alanine mutants primarily focussing on introducing mutations in the surface-exposed loops surrounding the active site region. As evaluated by this method we identified Phe141, Lys143, Glu146, Tyr149 and Lys192 to be important for the inhibitory
activity of Nb22, as mutation of these residues to Ala (or Gln for Lys192) affected the IC$_{50}$ value for inhibition of CS-61(44) by Nb22 more than 5-fold (Appendix 7).

**X-ray crystal structure analysis.** The crystal structure of the catalytic domain of muPA in complex with Nb22 was determined to a resolution of 2.3Å ($R_{free}=0.337$, $R_{work}=0.281$) with one complex in the asymmetric unit (for X-ray statistics see appendix 8). The crystal structure revealed that Nb22 (Chain B) covers a surface area of 864 Å$^2$ on muPA (Chain A) utilising residues from framework region 2, CDR-H1, CDR-H2, and CDR-H3 for binding to muPA (figure 23a) (a full list of polar contacts is provided in appendix 9, and numbering of Nb22 is provided in Appendix 10). Phe37/B and Phe47/B from the framework region 2 create together with Trp101/B and Trp103/B from the CDR-H3 a hydrophobic pocket into which Tyr149/A from the 140-loop of muPA becomes buried (figure 23b). The 140-loop of muPA also engages in exosite interactions with the CDR-H1 and CDR-H2 of Nb22, as Ile51/B, Ser56/B and Asn58/B from the CDR-H2 hydrogen bonds with Ser147/A. There are also several water-mediated hydrogen bonds including Thr57/B to Ser145/A, Ser56/B to Glu146, from Trp52a/B to Glu146/A and from the CDR-H1 Ser31/B to Glu146/A (figure 23c). The N- and C-terminal stems of the CDR-H3 loop also contributes to the interaction surface with muPA. Asp95/B and Ala97/B at the N-terminal stem contacts Lys192/A of the 180-loop, whereas Ser100c/B at the C-terminal stem hydrogen bonds with Lys143/A and Tyr151/A of the 140-loop (figure 23b). The most extensive interaction surface between Nb22 and muPA involves the 12 amino acid long CDR-H3 of Nb22, which inserts into the active site of muPA. At the apex of the loop, Nb22 inserts the P1 Arg100/B into the S1 specificity pocket forming hydrogen bonds to Asp189/A and Ser190/A in the bottom of the pocket (figure 23d). However, Arg100/B also forms hydrogen bonds to Trp215, and a water molecule in the bottom of the S1 pocket facilitates possible hydrogen bonds from Arg100/B to Tyr172/A, Arg217/A, Gly219/A, Lys224/A, or Pro225/A (figure 23e). The oxygen atom of the carbonyl group of Arg100/B is stabilised by the NHs of Ser195/A and Asp194/A. At the P2 position Ser99/B inserts into the S3 pocket forming hydrogen bond to Gly216/A and Gly219/A. The P1’ residue Asp100a/B turns towards the S1’ site to interact with His57/A and Ser195/A of the catalytic triad, but it is also within
hydrogen bonding distance to Tyr99/A and Lys192/A. The S2, S2’ and S3’ pockets of muPA is not utilised in a substrate-like manner by the CDR-H3 of Nb22 (figure 23f).
Conformational changes in muPA associated with binding of Nb22. We have recently solved X-ray crystal structures of an allosteric inhibitory nanobody, Nb7, in complex with muPA in the presence or absence of the active site binding ligands EGR-cmk or p-aminobenzamidine. Comparing the Nb22-stabilised conformation of muPA (muPA_{Nb22}) with that of muPA in complex with Nb7 (muPA_{Nb7}), reveals that the 70-loop undergoes a >14Å conformational change (figure 24). In muPA_{Nb7}, the CDR-H3 of Nb7 inserts into a hydrophobic pocket comprised by Phe30, Pro37e, Pro38 and Phe40 from the 37-loop and Tyr67, Leu68, Tyr76, Pro78, Phe141 and Leu155 from the 70- and 140-loops. In muPA_{Nb22}, the 14Å movement of the 70-loop occludes the hydrophobic pocket, and adopts a conformation similar to that observed in the EGR-cmk stabilised active conformation of human uPA (PDB: 1lmw). We also observed subtle conformational changes of the 140-loop and the Asp194-Cys191 segment of the 180-loop. These conformational changes occur in muPA in order to accommodate the insertion of the CDR-H3 of Nb22 in the active site and in order to form the exosite interaction between the 140-loop of muPA and Nb22.

Para-aminobenzamidine displacement. Structural comparison between the muPA:Nb22 complex and the muPA:Nb7:EGR-cmk or muPA:Nb7:p-aminobenzamidine complexes revealed that EGR-cmk and p-aminobenzamidine occupies the S1 specificity pocket utilised by Nb22, thus explaining the reduced affinity of Nb22 for the muPA:EGR-cmk complex. To further evaluate the structural observation that Arg100 in the CDR-H3 of Nb22 inserts into the S1 specificity pocket,
we performed a fluorescent assay using p-aminobenzamidine. The reversible binding of p-aminobenzamidine in the S1 specificity pocket of muPA results in a high intensity fluorescent signal with $\lambda_{\text{max}}$ at 362nm. The low intensity signal of p-aminobenzamidine in solution has $\lambda_{\text{max}}$ at 375nm. Binding of Nb22 to muPA was found to displace p-aminobenzamidine from the S1 specificity pocket as the intensity of the signal decreases and a red shift has occurred as observed for free p-aminobenzamidine in solution. Mutating Arg100 in Nb22 to an alanine affected the ability of Nb22 to displace p-aminobenzamidine. This finding validates the structural observations.

![Fluorescent spectroscopy](image)

**Figure 25. Displacement of p-aminobenzamidine from the S1 pocket of muPA by Nb22.** Fluorescent spectroscopy of p-aminobenzamidine (60 µM) before (black dashed curve) and after (black curve) incubation with muPA (0.23 µM). Adding Nb22 (800 nM) decreases the fluorescent signal. A control nanobody (800 nM) showed no effect. The data shown is representative for three individual experiments.

**Evaluation of the specificity of Nb22.** To evaluate the specificity of Nb22 we tested the Nb22 concentration dependency of inhibition of hydrolysis of small chromogenic substrates by the uPA related human trypsin-like serine peptidases tissue-type plasminogen activator (tPA), matriptase, plasmin and thrombin together with uPA from different species (human, dog, rat and rabbit). Nb22 did not inhibit any of the tested peptidases measurably at a high 5 µM concentration (data not shown).
Development and characterisation of nanobodies that targets zymogen activation of human uPA

Development of highly specific nanobodies towards zymogen uPA. A nanobody library was generated by immunisation of an alpaca (Vicugna pacos) with recombinant single-chain human uPA. Screening the library with single-chain uPA as bait, we recovered three nanobodies (Nb39, Nb48 and Nb49), which was found to bind to single-chain uPA with nano- to pico-molar affinities as determined by surface plasmon resonance (table 2).

Table 2. SPR analysis of binding of Nb39, Nb48 and Nb49 to single-chain uPA. Association rates ($k_{on}$), dissociation rates ($k_{off}$), and the equilibrium binding constants ($K_D$) were determined by fitting the SPR data to a 1:1 binding model. The data are reported as the mean ± standard deviation for the reported number (N) of determination.

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<th>$k_{on}$ (1/Ms)x10^5</th>
<th>$k_{off}$ (1/s)x10^-4</th>
<th>$K_D$ (pM)</th>
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<td>Nb39</td>
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<td>1.75 ± 0.34</td>
<td>155 ± 29</td>
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<tr>
<td>Nb49</td>
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<td>2.13 ± 0.64</td>
<td>141 ± 38</td>
<td>3</td>
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</tbody>
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Epitope mapping. To determine the epitope of Nb39, Nb48 and Nb49 we used site-directed mutagenesis of single-chain uPA expressed recombinantly in HEK293 6E cells. We primarily mutated amino acids in surface-exposed loops as evaluated by a homology model of single-chain uPA based on chymotrypsinogen (there is no structure of single-chain uPA available). In order to estimate the binding of the single-chain uPA mutants relative to the wild-type, we used ELISA employing directly conditioned medium from the transfected HEK293 6E cells. In the ELISA, the single-chain uPA variants from the conditioned media were captured on polyclonal anti-uPA antibodies, and the relative binding of the nanobodies to the single-chain uPA variants were evaluated using a control in which the capture level of the various variants were estimated with a monoclonal anti-uPA antibody. For Nb39 a 10-fold reduction in binding was found for the mutants I17A and Q154A, whereas a slight 1.2-fold reduction was found for the F21A and Y149A mutants (figure 26a and 26b). For Nb48 a more than 20-fold reduction was observed for the I17A mutant, and
between 2- to 5-fold for F21A, Y149A and Q154A. For Nb49 we observed a 23-fold and 4-fold reduced binding to the Y149A and Q154A mutants. Thus the three nanobodies has overlapping epitopes. For all uPA mutants their activity towards the small chromogenic substrate CS-61(44) were tested to verify their single-chain
format. Furthermore, using the ELISA setup we identified Nb39 and Nb48 to bind preferentially to single-chain uPA, whereas Nb49 also show binding activity towards active two-chain uPA (figure 26b).

Mechanism of Inhibition of plasmin catalysed single-chain uPA activation. The activation of single-chain uPA, as measured by activated two-chain uPA hydrolysis of CS-61(44), was inhibited by all three nanobodies (figure 27). However, as evaluated by a subsequent SDS-PAGE analysis, Nb39 and Nb48 completely hinder plasmin-
catalysed cleavage of the Lys15-Ile16 peptide bond after 1 hour of incubation, whereas Nb49 delays the proteolytic cleavage of single-chain uPA by plasmin. However, the inhibitory effect of Nb49, as compared to e.g. Nb39, seems to be depended on its ability to inhibit the catalytic activity of the generated two-chain uPA as we found that Nb49 inhibits two-chain uPA hydrolysis of CS-61(44) with an $IC_{50}$-value of $206 \pm 11$ nM (figure 28a).

**Carbamylation experiments.** The presently described effect of Nb49 is reminiscent of a previously reported monoclonal antibody, mAb-112, which was found to bind to single-chain uPA with 350-fold higher affinity than two-chain uPA. mAb-112 also delays plasmin-catalysed cleavage of single-chain uPA, and inhibits hydrolysis of a small chromogenic substrate in a non-competitive manner (168). The crystal structure of mAb-112 in complex with the active catalytic domain of uPA demonstrated that mAb-112 rezymogenises uPA by a mechanism, which involves disruption of the ion-pair between the N-terminal Ile16 and Asp194 (167). To investigate if Nb49 utilise a similar mechanism we measured the rate of inactivation of two-chain uPA during modification of the exposed $\alpha$-amino group of Ile16 by chemical modification. The rate of inactivation by carbamylation with potassium cyanate is proportional to the fraction of the time the N-terminus Ile16 is solvent-exposed versus the time in which it is forming and ion-pair to Asp194. The analysis revealed that similar to mAb-112, Nb49 increases the rate of carbamylation (figure 28b). Hence, the Nb49-stabilised conformation of two-chain uPA is characterised by a more frequently solvent-exposed N-terminus Ile16.
Figure 28. A. Inhibition of uPA hydrolysis of the chromogenic substrate CS-61(44) by Nb39 and Nb49. 1 nM two-chain uPA was incubated with various concentrations of Nb39 and Nb49 (20-0 μM). CS-61(44) were added to 47 μM. The rates of hydrolysis were normalised to rates in the absence of nanobody. B. Carbamylation rates measurements reveals enhanced exposure of the N-terminal Ile18 upon binding of Nb49 and mAb-112. The figure shows rate of activity loss following carbamylation of two-chain muPA (1 μM) in the presence or absence of Nb39 (1 μM), Nb49 (1 μM) or mAb-112 (1 μM). The relative activity at each time point is calculated as the fractional activity compared to time point 0. For both A. and B, means ± standard deviations of three individual experiments are indicated.
Materials and Methods

The materials and methods used in the different manuscripts and projects are described in details in the appendixes. The following is a brief description of the materials and methods for the work not included in the manuscripts. When the method used has already been described in the appendixes it will be referred to accordingly.

Proteins. Single-chain uPA was a gift from Abbot Laboratories. Two-chain uPA was purchased from ProSpec (Rehovot, Israel). Site-directed mutants of human uPA was produced in HEK293 6E cells as described in (VI). Full-length murine uPA (muPA), muPA mutants, and single-chain muPA\(^{2-243}\) was expressed recombinantly in HEK293E cells and purified from the conditioned medium as described in (III, IV, and V). The catalytic domain of muPA for crystallisation was produced as inclusion bodies in \(E.coli\), refolded and purified as described in (V). The peptidases matriptase were purchased from R&D systems (Wiesbaden-Nordenstadt, Germany). Human plasminogen was purified from out-dated human plasma by lysine Sepharose (GE Healthcare) affinity chromatography, eluted with 6-aminohexanoic acid and dialysed (273). Tissue-type plasminogen activator was purchased from Boehringer Ingelheim (Ingelberg am Rhein, Germany). Plasmin, Thrombin, Rat uPA, Dog uPA and Rabbit uPA was purchased from Molecular Innovation (USA) and aprotinin were from Sigma-Aldrich (Germany). The activity of muPA was quenched with Glu-Gly-Arg-chloromethylkentone (EGR-cm) by incubating muPA (25 µM) with EGR-cmk in phosphate-buffered saline (PBS) for 60min at 25°C. Excess EGR-cmk were removed by extensive dialysis against PBS.

Antibodies. mAb-112 were purified from hybridoma-conditioned medium using G-Sepharose affinity chromatography as described in (168). The antibodies: rabbit anti-uPA polyclonal antibodies and anti-uPA clone 6 was previously described in (180) and (274). mU1 was produced as described in (270).
**Generation if anti-uPA nanobodies.** Two alpacas (*Vicugna Pacos*) was immunised with recombinant single-chain human uPA or two-chain muPA and construction of the nanobody phage library was conducted as described previously (275). Selection and production of anti-single-chain uPA and anti-muPA nanobodies was performed as described in appendix V and VI, but with single-chain uPA and two-chain muPA as bait.

**Surface Plasmon Resonance.** The equilibrium dissociation constant $K_D$, the association rate $k_{on}$, and the dissociation rate $k_{off}$, for binding of Nb22 to two-chain muPA, single-chain muPA$^{[2-243]}$ or the muPA:EGR-cmk or for binding of Nb39, Nb48 and Nb49 to single-chain uPA were determined by surface plasmon resonance on a BiaCore T200 (Ge Healthcare). In all experiments the uPA variants were diluted in running buffer containing 10 mM HEPES, 135 mM NaCl and 0.1 % bovine serum albumin, pH 7.4 (HBS+ 0.1% BSA). The nanobodies (0.5 µg/ml in 10 mM sodium acetate pH5) were immobilised on a CM5 sensor chip by amine coupling to approximately 100 response units. For Nb22 active full-length muPA (10-0 nM), single-chain muPA$^{[2-243]}$ (100-0 nM) or muPA:EGR-cmk (500-0 nM) were injected for 240s with a flow rate of 30 µL/min and the dissociation was monitored for 3600s before regenerating the surface with 10 mM Glycine, 0.5 M NaCl, pH 2.5. For Nb39, Nb48 and Nb49 single-chain uPA were injected for 600s with a flow rate of 30 µL/min, and the dissociation were monitored for 7200s. The kinetic parameters were determined at 25°C, and the experimental curves were fitted to a 1:1 binding model using the BiaCore evaluation software.

**Enzymatic assays.** All enzymatic assays were conducted in a buffer containing 10 mM HEPES, 135 mM NaCl and 0.1 % bovine serum albumin, pH 7.4. In all measurements the initial reaction velocities was monitored at an absorbance of 405 nm in a microplate reader (Multiscan Go, Thermo Scientific) The velocity of two-chain muPA and two-chain huPA catalysed hydrolysis of the chromogenic substrate pyro-Glu-Gly-Arg-pNa (CS-61(44)) (Aniara Diagnostica LLC, USA) was measured at 37°C. When nanobody inhibition of the catalytic activity of two-chain muPA and muPA alanine mutants and two-chain huPA were measured the enzyme (1 nM) was
pre-incubated with various concentration of nanobody, as indicated for each experiment, in HBS+0.1% BSA at 37°C before adding the chromogenic substrate. For muPA the substrate concentration used was 750 μM whereas it was 47 μM for huPA. For determination of IC₅₀-values, the reaction velocity in the presence of nanobody were normalised to the velocity in its absence and plotted against the concentration of nanobody. The experimental data was fitted by 4-parameter logistic non-linear regression to determine the IC₅₀-values.

For determination of inhibition of muPA catalysed plasminogen activation various concentrations of Nb22 (50-0 nM) were incubated with two-chain muPA (1 nM) for 15 min at 37°C. Next, the reactions were initiated by adding 100 nM human plasminogen and 0.5 mM plasmin substrate H-D-Val-Leu-Lys-pNa (S-2251) (Chromogenix, Sweden). S-2251 hydrolysis was monitored for the parabolic increase in absorbance, and the data were transformed to a plot Δ405/Δtime on the ordinate and time on the abscissa for determination of the IC₅₀-value as described in (IV).

In order to evaluate the specificity of Nb22, 5 μM of Nb22 was pre-incubated with various enzymes (2 nM) for 15 min at 37°C, before measuring the initial velocities for hydrolysis of chromogenic substrates. The velocities were compared to samples in the absence of Nb22 to evaluate the effect of Nb22. For the different peptidases the following substrate concentrations was used: tPA, S-2288 750μM; matriptase, S-2288 100 μM; Plasmin, S-2251 750 μM; Thrombin, CS-61(44) 750 μM; Rat, Dog, Rabbit uPA, CS-61(44) 750 μM; human uPA, CS-61(44) 300 μM.

**Analysis of plasmin catalysed cleavage of single-chain muPA and single-chain huPA.**

For Nb22 the analysis was conducted as described in (IV). Briefly, 200 nM single-chain muPA²⁴³ containing a Cys122Ala mutation and the residues GQKALRPRFK before the activation site at Ile16 was incubated in the presence or absence of nanobodies or monoclonal antibodies as indicated in the experiment, before addition of plasmin (5 nM) to start the reactions. The amount of muPA¹⁶⁻²⁴³ generated were evaluated by non-reduced SDS-PAGE and by adding CS-61(44) (750 μM) to measure hydrolysis.

For Nb39, Nb48 and Nb49 single-chain huPA (9.3 μM) was pre-incubated with for 15 min at 37°C in the presence or absence of Nb39, Nb48 or Nb49 (46.5 μM). At time
point 0 plamin (110 nM) were added. The reactions were quenched by adding the serine peptidase inhibitor phenylmethanesulfonylfluoride (PMSF) (1 mM) after 20, 40 and 60 min of incubation. The samples were analysed by reduced SDS-PAGE analysis to visualise the amount of generated two-chain uPA. In addition we also determined the amount of generated two-chain uPA by incubating 100 nM of the nanobodies with 10 nM single-chain huPA for 15 min at 37°C, before adding 0.5 nM plasmin to start the reactions. Quenching the reactions with approtinin (1 µM) at the indicated time points, and measuring hydrolysis of CS-61(44) (0.5 mM) allowed us to determine how much of the generated two-chain uPA that remained active after activation of single-chain huPA by plasmin.

**ELISA for measuring nanobody binding to human uPA variants.** Anti-uPA polyclonal antibodies (5 µg/mL) was immobilised on the solid phase by coating in a solution of 100 mM NaHCO₃ and 100 mM Na₂CO₃ pH 9.6 in a 96-well Maxisorp plate (Nunc, Denmark). Following a block of the wells by PBS containing 5% skim milk powder, 100 µL of conditioned media containing two-chain uPA, single-chain uPA or single-chain uPA alanine mutants were incubated for 1 hour at 22°C. Nb39, Nb48 and Nb49 or the mouse monoclonal anti-uPA-clone6 antibody (binding to the kringle domain of uPA) were added to 200 nM in HBS+5% BSA, and incubated for 1 hour at 22°C. The anti-mouse anti-HA monoclonal antibody (1:2000), recognizing the C-terminal hemagglutinin peptide in the nanobodies, were then added in HBS+5% BSA to detect the amount of bound nanobody to the uPA variants. The ELISAs were developed by adding HRP-conjugated polyclonal rabbit anti-mouse IgG (1:2000) (Dako, Denmark) using OPD tablets according to manufactures instructions measuring the absorbance at 490 nm. As the monoclonal antibody clone6 binds equally well to two-chain and single-chain uPA, and since none of the generated alanine mutants affected the binding of clone6, the signal from the nanobody treated wells were normalized to the uPA capture level by clone6. Between the individual steps in the described ELISA procedure the wells were washed with in PBS containing 0.05% Tween.

**Carbamylation assays.** To analyse the effect of Nb39 and Nb49 on the carbamylation rate of the N-terminal Ile16 α-amino group in two-chain huPA, 1 µM enzyme was
pre-incubated with or without the nanobodies 1 µM in a buffer containing 10 mM HEPES pH 7.4, 140 mM NaCl and 0.1 % polyethylene glycol 8000Da at 22°C for 15 min. After pre-incubation potassium cyanate (0.2 M) or buffer was added and the reactions were incubated at 37°C for 0, 30, 60, 120, 180 and 240 min. To stop the carbamylation reaction, each of the mixtures were diluted 100-fold in HBS+0.1% BSA and the nanobodies were allowed to dissociated for 2 hours at 37°C. The residual activity of two-chain huPA were determined by adding CS-61(44) (1 mM) and measuring the rate of hydrolysis at 405 nm for 1 hour at 37°C in the microplate reader.

Para-aminobenzamidine displacement assays. p-aminobenzamidine (60 µM) were incubated in the presence or absence of muPA (0.23 µM) for 15 min at 22°C in a buffer containing 10 mM HEPES pH 7.4, 140 mM NaCl and 0.1 % polyethylene glycol 8000Da. Nb22 (800 nM) or a control nanobody binding to human uPA (800 nM) were added and after 15 min of incubation fluorescence emission spectrums were recorded at 25°C on a PTI quantamaster spectrofluorometer in a 2 mm x 10mm semi-micro quarts cuvette. An emission scan of 340-400 nm using an excitation wavelength of 335 nm and an integration of 1-2s over a 1.0 nm step resolution was recorded.

X-ray crystallography. Complexes between the catalytic domain of muPA and Nb22 were formed by incubating muPA and Nb22 in a 1:2 molar ratio for 1 hour at 25°C. The complex was purified by size exclusion chromatography on a Superdex 75 gel filtration column, in a buffer containing 50 mM Tris pH 7.4 and 150 mM NaCl. The complex was crystallised by hanging drop vapour diffusion using 6 mg/mL of the complex in a 1:1 drop ratio with 0.2 M Ammonium acetate, 0.1 M Tris pH 8.0, 16 % w/v PEG10,000 in the reservoir. Crystals were cryoprotected in mother liquid supplemented with 20 % (v/v) ethylene glycol and flash-frozen in liquid nitrogen. Datasets were collected at 100 K at a wavelength of 1.54 Å at the Petra III beamline at the DESY research centre (Hamburg, Germany). The data was processed with XDS, and solved by molecular replacement in Phaser (276) using a search model of murine uPA from the crystal structures of muPA in complex with Nb7 (appendix V). The
initial model was build using *phenix.autobuild* (277), and further improved by manual building in *Coot* (278). Refinement was conducted in *phenix.refine*. The structure was validated using *MolProbity* (279). 84.66 % of the residues was in the favoured region in the Ramachandran plot, 10.96 % in the allowed regions, whereas 4.38% was in outliers. All graphic was prepared in PyMol (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC).
Discussion

The discussion is divided into four sections. The first section concerns orthosteric regulation of activity in trypsin-like serine peptidases, and the development of cyclic peptides and nanobodies targeting human and mouse uPA. The second section discusses allosteric regulation in trypsin-like serine peptidases, and the development of allosteric monoclonal antibodies and nanobodies. The third section describes the development of nanobodies for targeting zymogen activation of human uPA. Finally, the last section describes the perspectives and future studies.

Orthosteric regulation of activity in trypsin-like serine peptidases

Orthosteric agents block the active site of the peptidase and competitively inhibits substrate turnover, and most antipeptidase therapeutics currently in clinical use are competitive (91). Development and discovery of orthosteric agents such as small molecule inhibitors are in general easier, as compared to allosteric agents, due to the availability of large chemical compound libraries and the simplicity of the activity based screening. However, cross-reactivity with other peptidases, and the competitive nature of inhibition, which often requires a higher dose of the compound, is major disadvantages of orthosteric agents.

Cyclic peptides against uPA as orthosteric agents. Compared to the small-molecule inhibitors, peptides of 10-20 amino acids form a larger interaction surface with their target peptidase, affording a higher specificity and affinity. The first orthosteric peptidic inhibitor against human uPA, upain-1 (CSWRGLENHRMC), was selected and characterised in 2005 (118). It was found to inhibit uPA activity with a $K_i$ value of 30 $\mu$M. The upain-1 derivative called upain-2 (CSWRGLENHAAC), was designed in both a monocyclic and bicyclic format but without any significant improvements to the affinity (108, 109). However, a N-terminal extension to upain-2 improved the affinity 10-fold (280). The selection of the bicyclic peptides UK504, UK18 and its derivative UK202 and demonstrated that peptides with affinities in the nano-molar range could be constructed based on selection of a scaffold from a peptide phage-display library (116, 117, 281). Mupain-1 (CPAYSRYLDC) is a monocyclic peptide isolated from a
phage-display library, and was found to inhibit muPA activity with a $K_i$ value of 400 nM (119). Substitution of the P1 Arg6 residue in mupain-1 with unnatural amino acids resulted in two peptides, mupain-1-12 and mupain-1-16, with improved affinities (109).

**Increasing peptide flexibility results in increased affinity.** The large interaction surface of peptidic inhibitors with their target peptidase allows rational design, based on structural information, to improve the peptide-peptidase interaction surface. As outlined in the introduction, using a scaffold of a naturally occurring inhibitor such as SFTI-1, this strategy has been applied for development of specific inhibitors towards various trypsin-like serine peptidases. However, our findings with mupain-1 and its derivatives demonstrates that affinity-enhancing changes to the peptide is not always rationally predictable. As such, it was unexpected that removal of one hydrogen bond between Asp9 in mupain-1 and Arg35 in huPA-H99Y, by introducing the D9A mutation, resulted in a 3-fold increase in affinity of mupain-1. The equivalent muPA amino acid to Arg35 in huPA-H99Y is Gln35. Thus, we expected that a similar binding mode of mupain-1 to huPA-H99Y and muPA would also result in an increase in affinity towards muPA upon the D9A mutation. However, the affinity of mupain-1 towards the original target peptidase muPA was not affected by the D9A substitution. This demonstrates that the interaction surface of mupain-1 to huPA-H99Y and muPA in the 37-loop area differs between the two enzymes (figure 29). In fact, mutation of Gln35 to Ala did not affect the affinities of the peptides towards muPA. However, removing Lys41 in muPA by introducing an Ala increased the affinity of mupain-1 D9A towards muPA. A possible explanation of the observed effects was derived from the isothermal titration calorimetry (ITC) experiments demonstrating that the increased affinity of the Ala9 peptides to huPA-H99Y is a result of increased flexibility of the Ala9 peptides in solution, and the bound state, which allows the Ala9 peptides to adopt themselves to surface interactions on the peptidase inaccessible to the original Asp9 peptides (figure 29). This concept of increasing affinity by increasing flexibility is going against conventional attempts to improve affinity by reducing the entropic penalty upon binding of the peptide to the target peptidase. This is the rationale behind the development of bicyclic peptides,
which provides a further constrain on the peptide backbone than for the monocyclic peptides (117). This principle was recently demonstrated with the optimisation of the bicyclic peptidic inhibitor UK18 against human uPA. The hypothesis was that replacing the P1’ Gly13 residue in UK18 with D-amino acids, which contrary to Gly preferentially adopts positive ϕ angles, would result in reduced conformational flexibility of the peptide in solution thus reducing the entropic penalty upon binding to uPA. Accordingly the substitution of Gly13 with D-Ser resulted in a 1.75-fold increase in affinity (281).

The rationale of increasing affinity by increasing peptide flexibility was further strengthened by selection of the mupain-1-IG (CPAYSRYIGC) peptide from the back-flip peptide-peptidase fusion library. Mupain-1-IG displayed a 25-fold affinity improvement as compared to the original mupain-1 peptide. As expected upon the L8I D9G substitution in the peptide, ITC experiments revealed a large entropy penalty upon binding of the IG peptides to muPA. Although the X-ray crystal structure analysis did not reveal any significant difference between the IG and LD peptides when bound to huPA-H99Y, site-directed mutagenesis suggested that the IG substitution in mupain-1 allows it to adapt to different surface interactions in the 37-loop than for the original LD peptides. Although the increase in affinity of mupain-1-D9A and mupain-1-IG is a result of increased flexibility in both peptides,
the binding mechanism of the two peptides to the 37-loop area in muPA must be somewhat different, as the D9A mutation did not affect the affinity of mupain-1 towards muPA but only towards huPA-H99Y, whereas the IG substitution affected the affinity of the mupain-1 towards huPA-H99Y as well as muPA. This is likely to be a result of the IG substitution offering maximal flexibility, which allows the mupain-1-IG peptide to adapt to various surface interactions in the 37-loop. In good agreement with this hypothesis, we also observed increased affinity of mupain-1-IG towards other trypsin-like serine peptidase such as plasma kallikrein.

**S1-P1 interactions of mupain-1 and its derivatives.** Introducing unnatural amino acids as P1 residues is an effective strategy for enhancing target affinity of cyclic peptides. Replacement of the P1 Arg6 residue in mupain-1 with L-4-guanidino-phenylalanine (mupain-1-12) or L-3-(N-amidino-4-piperidyl)alanine (mupain-1-16) improves the affinity 2- to 10-fold respectively. The crystal structures of mupain-1, mupain-1-12 or mupain-1-16 in complex with huPA-H99Y did not reveal any significant changes in the P1-S1 interaction that could explain the observed 10-fold difference in affinity. However, site-directed mutagenesis of residues in the S1 pocket of muPA suggested that the three P1 residues fit differently in the S1 pocket, as they were affected differently by the S190A and V213T substitutions in muPA. However, the affinity-enhancing effect of the unnatural P1 residues was not only influenced by a better fit in the S1 pocket, but also by amino acids positioned further away from the S1 pocket. Especially mutation of Lys143, which is >7Å from the S1 pocket, affected the P1 substitutions differently. This observation suggests that the function of the S1 pocket might be controlled by distal structural elements such as the 140-loop (autolysis loop). The substitution of Arg6 with unnatural amino acids may also come with a cost of reduced specificity. For example, mupain-1-16 was found to inhibit human plasma kallikrein more than 500-fold more potently than the original mupain-1 peptide. However, there was no measurable inhibition of plasma kallikrein by mupain-1-12. This suggests that a change in the P1 residue of the peptide allow the remainder of the peptide to adapt to other interactions on the peptidase surface, and demonstrates the versatile and unique properties of the mupain-1 scaffold. Moreover, it suggests that a certain combination of P1 residue
and affinity-enhancing residues at other positions in the peptide could in principle turn mupain-1 into a specific inhibitor of e.g. human plasma kallikrein.

**Inhibitory mechanism of mupain-1 and its derivatives.** From the crystal structures of the mupain-1 peptides in complex with huPA-H99Y it was evident that the peptides binds in the active site of uPA in a substrate-like manner, with the conformation of the peptides resembling that of the RCL of PAI-1 from the S195A-PAI-1 Michaëlis complex (78). This is opposite to the non substrate-like conformation of upain-1, UK504 and UK18 in complex with human uPA. Upain-1 (CSWRGLNHRMC) and UK504 (CCLGRGCENHRCL) adopts a similar non substrate-like conformation in the active site of uPA, and prevents catalysis by disrupting the charge relay system with Glu7 or Glu8 of the peptide (figure 30). UK18 runs in the opposite direction to upain-1 and UK504, and prevents catalysis by adopting a highly constrained conformation which places the carbonyl group of Arg12 6.9 Å from the oxygen atom of Ser195. The mechanism of inhibition by mupain-1 and its derivatives is different from that of upain-1, UK504 and UK18. Mupain-1 does not disrupt the charge-relay system of uPA by inserting an amino acid between His57 and the oxyanion hole. Instead it rely on an altered distance from the Oγ atom of the catalytic Ser195 to the carbonyl group of the putative scissile bond (3.9Å as compared to 2.7Å for the scissile bond of PAI-1). Also the oxygen atom of the carbonyl group is not probably aligned in the oxyanion hole for catalysis to proceed. Thus mupain-1 is an inhibitor, and not a substrate. However, the substrate-like orientation of mupain-1 in the active site of muPA does not exclude the possibility of hydrolysis of the putative scissile bond between Arg6 and Tyr7 by muPA, although such hydrolysis is likely to proceed with a slow rate.

**Nanobodies against uPA as orthosteric agents.** Monoclonal antibodies or antibody fragments developed by conventional hybridoma-based methods or by screening of phage-display libraries has resulted in antibodies that inhibit their target trypsin-like serine peptidase with high affinity and specificity. This is the case for the antimatriptase antibody fragments A11, S4, E2, the anti-plasma kallikrein antibody DX2930, and the anti-HGFA antibody Ab58. Although not strictly orthosteric in
nature, these antibodies insert one or more of their CDR loops into the active site of the peptidase. This discriminates them from the allosteric or steric antibodies, which exclusively binds to exosites distantly from the active site.

**Inhibitory mechanism of Nb4.** The binding mode of Nb4 to the active site of uPA differ from the previously reported orthosteric antibodies, as the CDR-H3 loop of Nb4 inserts in a complete substrate-like manner utilising the S3, S2, S1, S1’, and S2’ specificity pockets. The carbonyl group of the putative scissile bond in Nb4 is perfectly positioned 2.9Å for a nucleophilic attack by Ser195. Moreover, the oxygen atom of the carbonyl group of the putative scissile bond is stabilised by the NHs of Ser195 and Gly193 in the oxyanion hole. Thus, the CDR-H3 of Nb4 also aligns differently in the active site of uPA than the orthosteric peptidic inhibitors. However, the conformation of the CDR-H3 resembles that of the standard-mechanism inhibitor BPTI. In agreement with this observation, we observed that the reactive peptide bond in Nb4 between Arg100f and Arg100g is hydrolysed slowly by uPA. Thus, the inhibitory potency of Nb4 is dependent on the stability of the Nb4:uPA complex. Contrary to BPTI, the P and P’ side of the reactive bond in Nb4 is not connected by a covalent disulphide bond. Instead, the stability of Nb4 in the active site of uPA relies solely on non-covalent interactions especially between Arg100g and Asp95 of the CDR-H3 as identified by the proteolytic experiments. Although other factors are likely to affect the stability of the Nb4:uPA complex, the weaker P-
P’ side interaction in Nb4 is likely to account for its 800-fold lower inhibitory potency than BPTI towards their target peptidases. The cleaved form of BPTI (BPTI*) remains very tightly bound to trypsin resulting in a very slow dissociation of the complex. The high stability of the BPTI*:trypsin complex makes it more favourable for reformation of the reactive-peptide bond, than for the hydrolysis to proceed. Nb4 also dissociated very slowly from uPA. Thus it is not unlikely that the reactive-peptide bond in Nb4 is reformed after cleavage due to stability of the cleaved Nb4:uPA complex, in good agreement with the notion that the cleavage of Nb4 by uPA seems to reach a steady state after 96h of incubation. This implies that the catalytic cycle is arrested at the acyl-enzyme intermediate, and that the position of the leaving group P1’ Arg100g prevents the catalytic water to enter the active site for catalysis to proceed (figure 31). Instead the leaving group would act as a nucleophile to attack the acyl-enzyme for reformation of the putative scissile bond in Nb4. A similar mechanism has been suggested for the inhibition of trypsin by BPTI (73, 75). Alternatively, the R100gA mutation may provide enough flexibility of the CDR-H3 loop to allow proper alignment into the active site of uPA for a more efficient hydrolysis and dissociation of the leaving group. This suggests that the rigidity of the acyl-enzyme intermediate is more important for the inhibition by Nb4 that the rigidity of the enzyme-inhibitor complex.

Inhibitory mechanism of Nb22. Nb22 also targets the active site of muPA. However, contrary to Nb4 the CDR-H3 of Nb22 does not align into the active site cleft of muPA in a substrate-like manner. Nb22 only utilises the S1 and S3 pockets, whereas the
rest of the CDR-H3 loop aligns improperly to the remainder of the specificity pockets. Nb22 is not cleaved by muPA, as the oxyanion hole (NHs of Ser195 and Gly193) is destabilised due to the interactions between Lys192 and Asp95 of Nb22, which results in a flip the NH of Gly193 towards Lys143 of the 140-loop. The oxygen atom of the carbonyl group of Arg100 in Nb22 is instead stabilised by the NHs of Ser195 and Asp194. Moreover, Nb22 prevents hydrolysis of the putative scissile bond as the P1’ Asp100a disrupts the charge-relay system by forming hydrogen bonds to His57 and Ser195. This mechanism of inhibition resembles that of the peptidic inhibitors upain-1, UK504 and UK18, in which a Glu or Asp residue disrupts the catalytic machinery by interacting with His57 and the NHs of Gly193 and Ser195 in the oxyanion hole (figure 32). However, Asp100a of Nb22 does not interact with the oxyanion hole. Furthermore, the inhibitory and binding mechanism of Nb22 is distinct from that of previously reported orthosteric anti-peptidase antibodies, thus demonstrating the unique inhibitory mechanism of Nb22.

![Image](https://via.placeholder.com/150)

**Specificity enhancing elements of Nb4 and Nb22.** Compared to the orthosteric peptides, the interaction surface between the conventional antibodies and the nanobodies and their target peptidase is in general much larger (approximately 700-800 Å² for peptides versus 900-1200 Å² for antibodies) The enlarged interaction surface contributes to the superior specificity of the monoclonal antibodies and nanobodies. The conventional orthosteric anti-peptidase antibody all buries one of the surface-exposed loops of the peptidase domain into a groove between the Vₜ/Vₜ interfaces or between two CDR loops of the heavy chain. In the case of Nb4 the CDR-
H1 contributes to the specificity by forming a direct interaction with an exosite in the 37-loop of human uPA. The 37-loop is unique to human uPA, hence Nb4 do not inhibit any of the tested human peptidases such as FVIIa, matriptase and plasma kallikrein. However, the 37-loop is conserved between human uPA, dog uPA and rabbit uPA explaining why Nb4 also inhibits the activity of uPA from these species (figure 33 for Nb22 and figure S3 in appendix VI for Nb4). Nb22 on the other hand do not inhibit activity of any of the tested peptidases from different species. Similar to the conventional orthosteric anti-peptidase antibodies Nb22 buries the 140-loop between the CDR-H3 and the CDR-H2 by forming multiple interactions with residues in the 140-loop including Lys143, Ser145, Glu146, Ser147, Tyr149 and Tyr151. This stretch of amino acids is not completely preserved in any of the tested peptidases thus explaining the selectivity of Nb22 for muPA (figure 33). Moreover, the selectivity of Nb22 for muPA is further enhanced, as muPA is the only of the tested peptidase with a Lys residue at position 192, which was also found to be an important residue for the interaction between Nb22 and muPA.

![Sequence alignment of different trypsin-like serine peptidase with muPA](image)

**Allosteric regulation of activity in trypsin-like serine peptidases**

Allosteric regulation of activity in trypsin-like serine peptidases has recently gained much interest by the realisation that most dynamic proteins are amenable to be allosterically regulated by ligands and cofactors (20-23, 32, 38-40, 282-285). The concept of allostery is best described by the conformational selection model. This model describes how dynamic proteins can adopt different conformation, which at equilibrium is populated differently, with one conformation (e.g. the active one) being the predominant. By binding to allosteric sites, ligands or cofactors disturb the pre-existing equilibrium of conformations by stabilising a distinct conformation.
associated with a specific function (e.g. substrate specificity). This results in a population shift towards that specific conformation. The allosteric signal, transmitted through the protein from the allosteric site, can travel through multiple dynamic pathways without any significant conformational change to the secondary structure of the protein (25, 286). Discovery of new allosteric sites and mechanisms is of general interest since these sites and mechanism may represent means of which the proteins are naturally regulated. Moreover, discovery of conserved allosteric sites and mechanisms can be of pharmacological interest in that they may offer new inhibitor interaction sites that potentially can allow development of a framework for designing allosteric inhibitors e.g. by stabilising inactive conformations of the target protein. While orthosteric sites (the active site) topology of homologous proteins is often conserved, allosteric sites are usually less conserved. Thus it is expected that it is easier to obtain specificity with allosteric inhibitors than with orthosteric ones.

**Allostery in active muPA.** The catalytic domain of muPA was demonstrated to exists in a distorted state in which affinity for small peptide substrates at the active site was lower than in the full-length peptidase. The lower affinity for the small peptide substrates was due to a destabilisation of the oxyanion hole as the N-terminal Ile16 was found to be more frequently solvent-exposed than in the full-length peptidase. Interestingly, the disordering could be restored by three means. Firstly, active site binding ligands such as the peptidic inhibitor mupain-1-16, led to a stabilisation of N-terminal Ile16 insertion. Secondly, the intra-domain linker (Ser-16 to Cys1) was found to restore the affinity for the small peptide substrates. Thirdly, the monoclonal antibody mU3, which binds to a previously unidentified allosteric site in the 37- and 70-loop, restored the function of the active site by increasing the affinity for small peptide substrates through an allosteric mechanism, which involves a stably incorporated N-terminal Ile16 in the activation pocket. Moreover, our identification of the allosteric inhibitory monoclonal antibody mU1 and the allosteric nanobody Nb7, which also binds to the 37- and 70-loop of muPA but acts by an opposite mechanism to mU3 by destabilising the oxyanion hole through a lack of N-terminal Ile16 insertion and by affecting the function of the S1 specificity pocket
suggested the following; Ligand free muPA pre-exists in equilibrium between active and inactive conformations. Removal of the intra-domain linker or binding of mU1 to the 37- and 70-loops shifts the equilibrium towards the inactive conformations, whereas binding of mU3 or active site ligands shift the equilibrium towards the active conformations. Furthermore the 37- and 70-loop in muPA is a very delicate allosteric site as binding of mU1, Nb7 or mU3 to an almost identical epitope results in such a different functional effect on the catalytic activity of muPA.

**The allosteric communication between the 37- and 70-loops and the active site in muPA.** The exact route for the propagation of the allosteric signal from the 37- and 70-loop to the active site of muPA remained partly elusive for mU1 and mU3. However, our identification of an allosteric nanobody, Nb7, which also binds to the 37- and 70-loops, may have revealed a common allosteric network exploited by all three allosteric agents. X-ray crystal structure analysis of the muPA:Nb7 complex in the absence or presence of active site ligands suggests that the propagation of the allosteric signal may rely on subtle changes of dynamics in residues along the allosteric trajectory. Although the trajectory in principle could involve multiple allosteric pathways depending on the ligand being an inhibitor or activator (286), our analysis pointed towards one pathway that predominates upon binding of Nb7. In the substrate bound conformation (muPA\(_{Nb7:EGR-cmk}\)) only the tip of the 140-loop (autolysis loop) and a part of the activation loop was dynamic. All other elements are relatively fixed in their positions. Changing the active site binding ligand to the S1 binding probe \(p\)-aminobenzamidine results in a muPA conformation (muPA\(_{Nb7:p\-\text{aminobenzamidine}}\)) in which the dynamics of the N- and C-terminal stem of the 140-loop, and of the nearby 180-loop (oxyanion-stabilising loop) is increased. Without active site ligands (muPA\(_{Nb7}\)) the dynamics of the stems of the 140-loop and of the 180-loop is further increased. Moreover, the 220-loop (S1 entrance frame) also appears more dynamic in the muPA\(_{Nb7}\) conformation. The increased dynamics of the muPA\(_{Nb7}\) conformation, as evaluated by the crystal structures, was confirmed by limited proteolysis experiments revealing the 140-loop as highly flexible. Together these finding suggests that stabilising effects such as binding of EGR-cmk or \(p\)-aminobenzamidine to the S1 pocket restricts the dynamics of residues at the stems.
of the 140-loop. However, the propagation of the allosteric signal is not unidirectional, thus any effect to the dynamics of residues at the stems of the 140-loop is also likely to affect the dynamics of the residues in the S1 pocket. This was also observed for the mupain-1 peptides with unnatural amino acids, which responded differently to mutation of Lys143, even though this residues is positioned >6Å from peptides. Thus, binding of mU1, mU3 or Nb7 to the 37- and 70-loop activates an allosteric pathway, which affects the dynamics of residues in the N- and C-terminal stem of the 140-loop. The increase in dynamics then propagates to the rest of the 140-loop, the 180-loop and the 220-loop affecting the function of the S1 specificity pocket. Furthermore, our analysis indicated that the allosteric signal propagates through Lys143, which interconnects the 140-loop to the 180-loop and the activation loop through hydrogen bonds to Ile16 and Lys192 (figure 34). Lys192 is adjacent to the Cys191-Cys220 disulphide bond, which is important for the function of the S1 specificity pocket. Furthermore, Glu146 at the tip of the 140-loop is also within hydrogen-bonding distance to Cys220 and Glu222 of the 220-loop. Thus any changes to the dynamics at the stems of the 140-loop are likely to affect the function of the S1 specificity pocket. Following this hypothesis mU3 would decrease the dynamic at the N- and C-terminal stem of the 140-loop resulting in a more ordered active site region increasing the affinity of muPA for small peptide substrates, whereas mU1 and Nb7 works by an opposite mechanism increasing dynamics at the stems of the 140-loop.
A similar allosteric pathway was suggested in FVIIa for the allosteric 37- and 70-loop binding peptide E-76 (152). Opposite to Nb7, E-76 stabilises a distinct conformation of the 140-loop, which also results in a disruption of a hydrogen bond between Gln143 and Lys192. However, the structural analysis was performed with FVIIa stabilised in an active conformation with a covalent inhibitor (D-FFRCMK), thus preventing observation of any structural or dynamic changes to the active site.

The Nb7, mU1 and mU3 binding epitope in the 37- and 70-loop is homologous to exosite I in thrombin. Numerous studies with thrombin has demonstrated an allosteric linkage connecting the active site to exosite I and vice versa (40, 287, 288), and the thrombin exosite I binding ligands hirugen and thrombomodulin have been demonstrate to stimulate cleavage of small peptide substrates and protein C respectively. Several studies has addressed the mechanism by which hirugen and thrombomodulin modulates the function of the active site, and these studies using NMR or hydrogen-deuterium exchange (HDX) demonstrate that binding of the ligands to exosite I reduces the dynamics of the surface-exposed loops surrounding the active site region (31, 65). As our analysis pinpoints specific residues as responsible for the observed dynamic allosterity, together these observations suggest a common allosteric regulatory mechanism by which ligands to the 37- and 70-loops regulates the activity of trypsin-like serine peptidases.
Structural insights into the allosteric nature of muPA. Although the NMR technique has demonstrated that dynamic proteins exist in a conformational equilibrium we do not know much about the structural details of these different conformational states (20, 21, 289). Especially for the trypsin-like serine peptidase thrombin, much effort has been directed towards understanding the role of conformational selection and describing the structural features of pre-existing active and inactive conformations (38, 40). As such, our structural identification of a previously unknown inactive conformation of the catalytic domain of muPA (muPA\textsubscript{inactive}) adds to the complexity of allostery in trypsin-like serine peptidases. The structural features of muPA\textsubscript{inactive} can be directly coupled to some of the biochemical observations from our study with mU1 and mU3. The biochemical data demonstrated that the inactive conformations stabilised by mU1 or by removal of the intra-domain linker, was characterised by a more frequently solvent-exposed Ile16. In agreement with this observation electron density for the stretch of the activation loop from residue 16-23 in muPA\textsubscript{inactive} was missing. The lack of the ion-pair between Ile16 and Asp194 results in a destabilisation of not only the oxyanion hole but also the entire oxyanion stabilising loop (180-loop). In fact, the entire C-terminal β-barrel of muPA\textsubscript{inactive} was highly dynamic as electron density of the 140-loop was missing, and as the 170-, the 180- and the 220-loop was completely disorganised as compared to active conformations observed for other trypsin-like serine peptidases. We were only able to crystallise an inactive conformation of muPA as the otherwise highly flexible 170-, the 180-, and the 220-loops were stabilised by crystal contacts. This observation is in good agreement with the notion that once released form the crystal contacts the equilibrium shifts towards the active conformations (muPA\textsubscript{active}). The muPA\textsubscript{active} conformations react with PAI-1, as the surface-exposed loops of the C-terminal β-barrel are free to rearrange themselves. A highly dynamic nature of the C-terminal β-barrel has also been observed in thrombin. Two independent NMR experimental setups indicated that the surface-exposed loops of the activation domain is highly dynamic in the Apo-form of thrombin, and that binding of ligands to the active site or exosite I restricts the dynamics of the surface-exposed loops (31, 32). Our structural observations of an inactive conformation of muPA highlights these
findings and suggests that the highly dynamic apo-forms in trypsin-like serine peptidases is characterised by distinct structural features such as lack of N-terminal Ile16 insertion.

The role of the 70-loop in allosteric regulation of muPA activity. Crystal structures of different conformation of muPA; muPA\textsubscript{inactive}, muPA\textsubscript{Nb7}, muPA\textsubscript{Nb7:EGR-cmk}, muPA\textsubscript{Nb7:p-aminobenzamidine} and muPA\textsubscript{Nb22}, has allowed us to speculate about possible mechanisms as muPA interconverts between active and inactive conformations in solution. Assuming the muPA\textsubscript{Nb22} conformation resembles an active one, which is in reasonable agreement with the superposition of the structure to active human uPA (PDB: 1lmw, RMS all atoms 0.793Å), it was noticeable that the 70-loop, when compared to the muPA\textsubscript{Nb7} conformation, adopts a “closed” conformation occluding the hydrophobic pocket into which the CDR-H3 of Nb7 is inserted. In the “closed” conformation the 70-loop engages in a tight hydrogen-bonding network involving hydrogen bonds from Ser71 to Val24, Ser71 to Leu155, Lys72 to Asn154, Glu73 to Lys153, and from Thr22 to Asn154 (figure 35). Furthermore, a hydrophobic interaction involving Phe30, Phe40, Phe141 and Leu155 strengthens the interconnectivity of the 70-loop with the 140-loop and the activation loop. In the “open” conformation observed in muPA\textsubscript{inactive}, the interconnectivity of the loops is deteriorated due to a 14Å movement of the 70-loop. The only remaining hydrogen bond is from Thr22 to Leu155. The movement of the 70-loop also causes a change to the activation loop, and a minor change to the side chain of Phe141. Interestingly, there was no electron density for most of the 70-loop, the 140-loop and the activation loop in muPA\textsubscript{inactive}, suggesting that these loops are highly flexible, thus supporting the hypothesis of interconnectivity between the loops. Also the hydrophobic patch formed by Phe30, Phe40, Phe141 and Leu155 was disrupted in muPA\textsubscript{inactive}. 
Together these observations suggest that the 70-loop in ligand free muPA exists as an ensemble of conformations. The “open” conformations, of which Nb7 stabilises one, is associated with a high degree of flexibility in the C-terminal β-barrel, and, as a consequence, a lower affinity for substrates at the active site due to high conformational binding entropy. In the “closed” conformations, for which Nb22 stabilises one of them, the C-terminal β-barrel is more ordered due to the re-established hydrogen-bonding network between the 70-loop, the activation loop and the 140-loop. This lowers the conformational entropy of the C-terminal β-barrel resulting in a more favourable substrate binding enthalpy. Thus, changes to the conformational entropy of the 70-loop (“open” vs. “closed”) propagate through an allostERIC network, likely to be similar to the one activated by Nb7, to the C-terminal β-barrel, which ultimately enhances (mU3) or inhibits (mU1 and Nb7) substrate binding at the active site. Reconsidering the epitopes of mU1 and mU3, with respect to their different functional effect on muPA activity it is noticeable that Lys72 is part of the epitope for mU1 but not for mU3. In the muPANb22 conformation Lys72 participate in a hydrogen-bonding network with Thr22 and Asn154. Thus, it is
tempting to speculate that the difference between the inhibitory effect of mU1 and the stimulatory effect of mU3 depends on the stability of this hydrogen-bonding network interconnecting the 70-, the 140-, and the activation loops. Together our finding led to a model of allostery in muPA, which is presented in figure 36.

As mentioned previously identification of allosteric sites and ligands may reveal mechanism by which the allosteric protein is regulation by natural ligands or cofactors. It is tempting to compare the allosteric site in the 37- and 70-loop with exosite I in thrombin. As already discussed exosite I mediates allosteric signals from cofactors, inhibitors and substrates such as thrombomodulin to the active site of thrombin. However, evidence for a biological role of the 37- and 70-loop in uPA remains to identified, but it is not unlikely that the area around the 37- and 70-loop could play a role for uPA-catalysed plasminogen activation. The 140-loop was previously identified as an important exosite for the interaction between tPA and plasminogen (290). Thus it is not unlikely that binding of the uPA substrate plasminogen allosterically shift the equilibrium in uPA towards the active conformations to enhance its own activation. A similar mechanism has been described for thrombin-mediated cleavage of the exosite I binding substrate protease activated receptor I (PAR-1) (58).
Inhibition of zymogen activation.

Targeting zymogen activation has proven a successful strategy for intervention with the catalytic activity of trypsin-like serine peptidase. This was demonstrated with the anti-uPA monoclonal antibody mAb-112. mAb-112 delays plasmin-catalysed single-chain uPA activation and efficiently inhibit metastasis formation in an orthotopic xenograft murine cancer model (182). mAb-112 binds to the 140-loop of human uPA, and delays plasmin-catalysed cleavage of single-chain uPA by preventing the access of plasmin to the Lys15-Ile16 peptide bond. Even after...
proteolytic cleavage, mAb-112 is able to stabilise an inactive conformation of two-chain uPA, by maintaining the 140-loop in an inactive conformation in which the ion-pair between Ile16 and Asp194 is disrupted. This was demonstrated by the crystal structure of the complex between Fab-112 and the active catalytic domain of uPA (167). Contrary to mAb-112, mU1 was found to bind with the same affinity to two-chain and single-chain muPA. mU1 did not delay proteolytic cleavage of the Lys15-Ile16 peptide bond, but maintained activated two-chain muPA in an inactive conformation in which the N-terminus insertion of Ile16 was destabilised. The nanobody Nb49 binds to an epitope similar to that of mAb-112 in the 140-loop of human uPA. Like mAb-112, Nb49 also binds with higher affinity to single-chain uPA than to two-chain uPA, inhibits hydrolysis of a small chromogenic substrate by two-chain uPA, and increases the rate of carbamylation of the N-terminus Ile16 in two-chain uPA. Thus the inhibitory mechanism of Nb49 resembles that of mAb-112 on many parameters.

The nanobodies Nb39 and Nb48 was found to inhibit plasmin-mediated cleavage of single-chain uPA. This is in good agreement with their binding epitope, which comprises residues Ile17 and Phe21 in the vicinity of the Lys15-Ile16 activation peptide bond. Thus, Nb39 and Nb48 sterically hinder the access of plasmin to the Lys15-Ile16 peptide bond in single-chain uPA. When this bond is cleaved in single-chain uPA, Nb39 and Nb48 is no longer able to make the full binding interaction, which is in agreement with the observation that Nb39 and Nb48 does not bind to two-chain uPA. This is similar to the anti-uPA monoclonal antibody mAb-PUK which was found to bind exclusively to single-chain uPA and to prevent its conversion to the two-chain form (181).

**Equilibrium between active and inactive conformations in single-chain uPA.** As outlined in the introduction a large variation to the change in activity is observed after proteolytic activation of zymogens in the trypsin-like serine peptidases. The flexibility of the surface-exposed loops of the activation domain has been demonstrated as central to the relatively high catalytic activity of single-chain tPA. In the crystal structure of single-chain tPA Lys156 of the 140-loop (autolysis loop) was found to form an ion-pair to Asp194 hence stabilising formation of the oxyanion hole
In uPA Lys156 was proposed not to insert into the activation pocket in the single-chain form due to a strong polar interaction with Glu144, which is a His residue in tPA (292). However, Lys143 was proposed as an Ile16 surrogate in single-chain uPA to account for its relatively high intrinsic activity (293). Thus the equilibrium between active and inactive conformations in single-chain uPA is strongly depended on the conformation of the surface-exposed loops of the activation domain especially the 140-loop.

Nb22 binds to single-chain muPA and inhibits plasmin-catalysed proteolytic cleavage of the Lys15-Ile16 peptide bond. This was unexpected based on the observations from the crystal structure that Nb22 binds to the active site of muPA. The most likely explanation for this observation is that Nb22 upon binding to the immature active site of single-chain muPA, either stabilises a active-like conformation around the Lys15-Ile16 activation peptide which is not recognised by plasmin, or by sterically interfering with the interaction between single-chain muPA and plasmin by shielding important exosite interactions. The former mechanism is in agreement with the notion that the conformational equilibrium in single-chain trypsin-like serine peptidases implies that a small (or large in the case of tPA) population of the single-chain enzyme has a functional oxyanion hole. The existence of such equilibrium is in good agreement with the observation that PAI-1 was found to react a 1000-fold slower with single-chain uPA than two-chain uPA (294, 295). This sparsely populated conformation in single-chain muPA may be able to react with Nb22, although with a slower association rate as determined by the SPR analysis. Thus it is reasonable to suggest that Nb22 shifts the equilibrium of conformations in single-chain muPA towards an active-like conformation, which is not recognised by plasmin. The mechanism of Nb22 may be similar to that observed for BPTI in complex with trypsinogen. BPTI was found to stabilise a trypsin-like conformation of rat trypsinogen S195A. However the oxyanion hole was not stabilised by the normal Ile16-Asp194 ion-pair, as the Lys15-Ile16 peptide bond was still intact. Instead the oxyanion hole was stabilised by a water-mediated hydrogen bond between the amide nitrogen of Ile16 and Asp194, (296, 297).
Future studies

The five X-ray crystal structures of muPA has provided valuable glimpse of the dynamic allostery associated with the interconversion between active and inactive conformations of muPA in solution. Furthermore, they have provided detailed information about the inhibitory mechanism of orthosteric and allosteric inhibitors. However, in the case of the allosteric nanobody Nb7 the X-ray crystal structure did not reveal the full conformational change of muPA associated with Nb7 binding. This demonstrates that the “static” lowest energy state trapped in the crystals may in some cases not represent the full allosteric conformational changes associated with ligand binding. Thus, in order to fully understand dynamic allostery in trypsin-like serine peptidases X-ray crystal structures could be supplemented using solution based dynamic techniques such as nuclear magnetic resonance (NMR) and hydrogen deuterium exchange mass spectrometry (HDX-MS) (21). As discussed above allosteric signals may propagates through a protein by redistribution of its internal dynamics with some amino acids showing rigidification and some being more flexible. It would be interesting to use NMR relaxation dispersion of isotopically labelled muPA in the presence or absence of Nb7, which would provide information about the changes in millisecond time scale motions indicative of the allosteric mechanism. Furthermore, HDX-MS experiments with muPA in the presence or absence of the monoclonal antibodies mU1 and mU3 can be used to study changes in backbone dynamics on a millisecond to second time scale.

Single domain nanobodies are easily engineered into multivalent and multispecific constructs (234). Bivalent or bispecific nanobodies are obtained by cloning of two identical or two different nanobodies separated by a short linker. This was demonstrated for bivalent anti-TNF nanobodies, which was 500-fold more potent than their monovalent formats in treatment of rheumatoid arthritis (218). Targeting more than one function of a trypsin-like serine peptidase was recently demonstrated for the anti-uPA aptamer Upanap-126 that besides inhibiting activation of single-chain uPA also prevents binding of uPA to its cell surface receptor uPAR, as well as binding of the uPA:uPAR complex to vitronectin. Upanap-126 was found to inhibit invasion and dissemination of cancer cells in in vivo models.
of tumour dissemination (187, 188). With the identification of Nb4, which inhibits activity of two-chain uPA, and identification of Nb39 and Nb48, which inhibits activation of single-chain uPA it is possible to engineer a bifunctional nanobody construct that targets more than one function of a trypsin-like serine peptidase. At the cell surface uPA will primarily exists in the single-chain form bound to uPAR. Once activated the activity of uPA is believed to be quenched relatively fast by reaction of two-chain uPA with PAI-1. The uPAR:uPA:PAI-1 complex is then cleared by LRP-mediated endocytosis. Thus, an efficient inhibitor of uPA has to associate fast to uPA to quench its activity. A multifunctional nanobody construct consisting of e.g. Nb39 and Nb4 would prevent activation of single-chain uPA by plasmin. Moreover, if miniature amounts of two-chain uPA is generated Nb4 would be in proximity to the active site of uPA in order to associate and quench the activity fast and efficiently. It would be interesting to test such a multifunctional nanobody inhibitor in in vivo models as those used for Upanap-126.

Despite the high potency and specificity offered by peptides their application as therapeutics has been limited due to disadvantages such as low systemic stability and fast clearance from circulation (103). Construction of bicyclic peptides decreases the sensitivity of the peptide for degradation by plasma peptidases. One strategy for prolonging the circulation half-life of peptides is to conjugate the peptide to polyethylene glycol (PEG)(298). Nanobodies may offer an alternative approach to prolong the circulation half-life of peptides. The single domain nanobody is, like peptides, cleared relative fast from the circulation. However, engineering of a bispecific nanobody consisting of the anti-target nanobody and a nanobody binding to serum albumin has been demonstrated to prolong the half-life from 35 min to 2.2 days without affecting the therapeutic activity of the nanobody (218). In a similar manner it would be interesting to recombinantly engineer a nanobody construct with a anti-serum albumin binding nanobody fused to mupain-1-IG in order to prolong the half-life of the peptide. If the strategy works, such an inhibitor could be used in in vivo mouse models of rheumatoid arthritis such as the collagen-induced arthritis (CIA) model. A muPA specific inhibitor could further help elucidating the role of uPA in progression of arthritis.
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APPENDIX 1

Manuscript I

A Cyclic Peptidic Serine Protease Inhibitor: Increasing Affinity by Increasing Peptide Flexibility

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A Cyclic Peptidic Serine Protease Inhibitor: Increasing Affinity by Increasing Peptide Flexibility

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Abstract

Peptides are attracting increasing interest as protease inhibitors. Here, we demonstrate a new inhibitory mechanism and a new type of exosite interactions for a phage-displayed peptide library-derived competitive inhibitor, mupain-1 (CPAYSRYLDC), of the serine protease murine urokinase-type plasminogen activator (uPA). We used X-ray crystal structure analysis, site-directed mutagenesis, liquid state NMR, surface plasmon resonance analysis, and isothermal titration calorimetry and wild type and engineered variants of murine and human uPA. We demonstrate that Arg6 inserts into the S1 specificity pocket, its carbonyl group aligning improperly relative to Ser195 and the oxyanion hole, explaining why the peptide is an inhibitor rather than a substrate. Substitution of the P1 Arg with novel unnatural Arg analogues with aliphatic or aromatic ring structures led to an increased affinity, depending on changes in both P1 - S1 and exosite interactions. Site-directed mutagenesis showed that exosite interactions, while still supporting high affinity binding, differed substantially between different uPA variants. Surprisingly, high affinity binding was facilitated by Ala-substitution of Asp9 of the peptide, in spite of a less favorable binding entropy and loss of a polar interaction. We conclude that increased flexibility of the peptide allows more favorable exosite interactions, which, in combination with the use of novel Arg analogues as P1 residues, can be used to manipulate the affinity and specificity of
this peptidic inhibitor, a concept different from conventional attempts at improving inhibitor affinity by reducing the entropic burden.

Introduction

Peptides are of considerable interest as drug candidates. Peptides binding to specific protein targets can be selected from phage-displayed peptide libraries with a diversity of up to $10^6$ different sequences. The primary structure of the peptides in the libraries can be modified by introduction of disulfide bonds \cite{1} or by chemical cross-linking \cite{2}. Peptides directly selected from phage-displayed peptide libraries usually bind their targets with $K_D$ values in the $\mu$M range, but the affinities can be improved by construction of focused libraries or chemical modification, like introduction of unnatural amino acids. Peptides have predictable absorption, distribution, metabolism, and excretion properties, can be delivered \textit{in vivo} by new formulation methods, and stabilized against proteolytic degradation by various means \cite{3}.

Serine proteases of the trypsin family (clan SA) have many physiological and pathophysiological functions \cite{4–6}. There is therefore extensive interest in generating specific inhibitors for pharmacological intervention with their enzymatic activity. Moreover, serine proteases are classical subjects for studies of catalytic and inhibitory mechanisms \cite{7}. One interesting member of the trypsin family of serine proteases is urokinase-type plasminogen activator (uPA), which catalyses the conversion of the zymogen plasminogen into the active protease plasmin through cleavage of plasminogen's Arg$^{15}$–Val$^{16}$ bond (using the chymotrypsin numbering \cite{8}). Plasmin generated by uPA participates in the turnover of extracellular matrix proteins in physiological and pathophysiological tissue remodeling \cite{9,10}. Abnormal expression of uPA is responsible for tissue damage in several pathological conditions, including rheumatoid arthritis, allergic vasculitis, and xeroderma pigmentosum, and in particular, is a key factor for the invasive capacity of malignant tumors \cite{11}. uPA is therefore a potential therapeutic target.

From a phage-displayed peptide library, we previously isolated a serum-stable, disulfide bond-constrained peptide, CPAYSRYLDC, termed mupain-1, which competitively inhibits murine uPA (muPA). As based on site-directed mutagenesis, mupain-1 gains high specificity for its target by having an extended interaction surface with the target protease, involving a number of exosite interactions. Its affinity for the target is moderate, the $K_i$ value for inhibition of muPA being around 0.5 $\mu$M \cite{12}. Substituting the P1 Arg residue with different non-natural amino acids in a mupain-1 background improved the affinity. Two variants of mupain-1, with the unnatural amino acids L-4-guanidino-phenylalanine or L-3-(N-amidino-4-piperidyl)alanine (Fig. 1) as P1 residues instead of the original Arg, have a 2- to 10-fold improved affinities \cite{13}.  

Competing Interests: The authors have declared that no competing interests exist.
In this study, we aimed at understanding the inhibitory mechanism and binding mechanism of mupain-1 and its derivatives. Why are these peptides protease inhibitors and not protease substrates? Which are the molecular events during the binding of peptides to serine proteases? Why do P1 substitutions increase the affinity? Is the specificity of the peptides among different serine proteases determined by the fit of the P1 residue into the specificity pocket, the exosite interactions, or the solution structures?

To answer these questions, we used X-ray crystal structure analysis, site-directed mutagenesis, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and NMR spectroscopy to study the interaction of mupain-1 and derivatives with recombinant wild type (wt) muPA and engineered variants of muPA and human uPA (huPA). Several recent papers on peptidic protease inhibitors describe how binding affinity can be increased by a more favorable binding entropy following introduction of a more rigid peptide structure by bicyclisation \([2, 14, 15]\). Here, we go in another direction and show how increased flexibility can lead to an increased affinity.

**Materials and Methods**

**Peptides**

Chemicals for peptide synthesis were purchased from Sigma-Aldrich, Iris Biotech GmbH, or Rapp Polymere GmbH, and used without further purification. Fmoc-L-4-guanidino-phenylalanine(\(N, N'\)-di-Boc)-OH and Fmoc-L-Ala-4-piperidyl(Alloc)-OH were commercially available. Analytical HPLC was performed on a Dionex UltiMate 3000, using a Phenomenex Gemini 110 Å C18 column (3 \(\mu\)m, 4.6 \(\times\) 50 mm) with a flow rate of 1.0 ml per min and a linear gradient going from 95% H\(_2\)O, 5% acetonitrile with 0.1% HCOOH to 100% acetonitrile with 0.1% HCOOH over 10 min. Preparative HPLC was performed using a Dionex UltiMate 3000, equipped with a Phenomenex Gemini-NX C18 110 Å column, running at a flow rate of 10.0 ml/min and a linear gradient going from 95% H\(_2\)O/5% acetonitrile with 0.1% TFA to 100% acetonitrile with 0.1% TFA over 30 min. High resolution mass spectra were obtained on a Micromass...
LCT high resolution time-of-flight instrument by direct injection. Ionization was performed in positive electrospray mode.

Solid-phase peptide synthesis was performed using N\(^\text{\textregistered}\)Fmoc-protected amino acids, a HBTU-HOBt activation protocol, and a Tentagel resin with Rink amide linker (0.23 mmol/g); HBTU (3.8 eq.), HOBt-HOAt (4:1, 4 eq.), Fmoc-AA-OH (4 eq.), DIPEA (7.2 eq.) in NMP. Manual peptide syntheses was performed with preactivation for 5 min and single couplings for 90 min. Fmoc deprotections were performed using piperidine/NMP (1:4) for 2+15 min.

Automated peptide synthesis was performed on a Biotage SyroWave. Standard Fmoc-amino acids were coupled in parallel mode 120 min: Fmoc-AA-OH (s5.2 eq.), HBTU (5 eq.), HOBt/HOAt (4:1, 5 eq.), DIPEA (9.8 eq.). Arginine mimics were coupled at 75°C for 10 min: Fmoc-AA-OH (2 eq.), HBTU (1.9 eq.), HOBt/ HOAt (4:1, 2 eq.), DIPEA (3.6 eq.). Fmoc deprotections were performed using piperidine/NMP (2:3) for 3 followed by piperidine/NMP (1:4) for 15 min.

Peptides with Alloc protected amino acids were deprotected to a free amine by treating the fully protected and N-acetylated peptides with a mixture of Pd(PPh\(_3\))\(_4\) (0.05 eq.) and Me\(_2\)NH-BH\(_3\) (0.2 eq.) in degassed CH\(_2\)Cl\(_2\) (30 min) and washed with NMP (5 x). The peptides were then treated with N, N\'-di-Boc-1H-pyrazole-1-carboxamidine (5 eq.) in NMP overnight. Following peptide assembly, the resins were washed extensively with NMP and CH\(_2\)Cl\(_2\), before peptide release with TFA/H\(_2\)O/triethylsilane (95:2.5:2.5). Peptide release proceeded for 2 h before the TFA-peptide mixture was collected by filtration. The resin was additionally washed with TFA (2x) and the TFA mixtures were pooled. TFA was removed under a stream of nitrogen and the peptide was precipitated with diethyl ether. The peptides were dissolved in a minimum amount of H\(_2\)O/acetonitrile (2:1) before being purified by preparative HPLC. The purified peptides were dissolved in H\(_2\)O/acetonitrile (2:1) to a final concentration of 1 mM. The solution was brought to pH 7.5–8 with NH\(_3\) in methanol. The peptides were oxidized to form disulfide bridges by addition of 1.2 eq. of H\(_2\)O\(_2\) (30–60 min). The oxidation was stopped with the addition of acetic acid (0.1 ml) followed by evaporation and HPLC purification. Mass spectrometry: Mupain-1-12 D9A [M+H]\(^+\) 1224.4 [M+2H]\(^2+\) 613.1; mupain-1-16 D9A [M+H]\(^+\) 1234.5 [M+2H]\(^2+\) 617.7.

The concentrations of the peptide variants were determined by measurements of OD\(_{280}\) and the use of sequence-derived extinction coefficients provided by the Protparam tool on the Expasy server (located at http://www.expasy.org).

**Proteases**

cDNA encoding full length muPA, full length huPA and site directed mutants were cloned into the pTT5 or pCDNA3.1 vectors. All variants contained a C-terminal hexa-His tag. The cDNAs were transfected into human embryonic kidney 293 (HEK293) 6E suspension cells, which were cultured in a humidified 5% CO\(_2\) incubator at 37°C. The medium used was F17 medium (Invitrogen) supplemented with 0.1% Pluronic F-68, a nonionic detergent (Invitrogen), 4 mM L-Gln (Lonza), and 25 µg/ml of the selective agent for eukaryotic cells G418.
(Invitrogen). $M_r \sim 25,000$ linear polyethylenimine (400 µg) (Polysciences) was preincubated with cDNA (200 µg) for 15 min and added to 200 mL cells with a density of $1 \times 10^6$ cells/mL. Twenty-four hours post-transfection, Tryptone N1 (Organotechnie SAS) was added to a final concentration of 0.5% (w/v). Conditioned medium was collected 96 h post-transfection, and the recombinant proteins were purified using immobilised metal ion affinity chromatography followed by benzamidine-Sepharose affinity chromatography. The purified proteins were at least 95% pure, as judged by Coomassie Blue-stained SDS-PAGE gels. To ensure that the uPAs purified from the conditioned media were completely in the two-chain form, they were treated with plasmin for 2 hours in a 1:100 ratio.

The cloning, production, and purification of recombinant uPA catalytic domain (residues 159–411), harbouring a H99Y mutation, to be used for crystallisation and isothermal titration calorimetry (ITC), was largely as described previously [16]. Basically, the recombinant catalytic domain of huPA-H99Y was secreted from a stable Pichia pastoris strain (X-33) after induction by methanol and captured by a cation exchange column. The protein was further purified on a gel filtration column (Superdex 75 HR 10/30 column from GE Health Care) equilibrated with 20 mM sodium phosphate, pH 6.5, 150 mM NaCl. The protein was eluted as a single peak under these conditions, with a retention time of approximately 13.6 ml. The recombinant uPA catalytic domain expressed in this way is an active protease with an activity comparable to full-length two-chain uPA [16]. The protein was dialysed in 20 mM potassium phosphate, pH 6.5 overnight and concentrated to 10 mg/ml, using stirred ultrafiltration cells (Millipore and Amicon Bioseparations, Model-5124), prior to protein crystallization. The recombinant catalytic domain of huPA-H99Y to be used for ITC assays was further purified with benzamidine-Sepharose affinity chromatography.

Crystallization and data collection of uPA or uPA H99Y in complex with mupain-1 variants

The crystallization trials were carried out with the sitting-drop vapour-diffusion method. The crystals of the catalytic domain of huPA-H99Y were obtained by equilibrating huPA-H99Y protein against a reservoir solution containing 2.0 M ammonium sulfate, 50 mM sodium citrate, pH 4.6, and 5% polyethylene glycol (PEG) 400 at room temperature. The crystals appeared in about 3 days. The crystals of huPA-H99Y were then soaked for 2 weeks in new soaking buffer (40% PEG 4000, 0.1 M Tris-HCl, pH 7.4), containing 1 mM mupain-1 variants. A solution of 20% PEG 4000, 0.1 M Tris-HCl, pH 7.4 and 20% (v/v) glycerol was used as cryoprotectant for X-ray diffraction data of the crystals at the BL17U beamline, Shanghai Synchrotron Radiation Facility and 3W1A beamline, Beijing Synchrotron Radiation Facility (BSRF). The diffraction data was indexed and integrated by HKL2000 program package [17].
Crystal structure determination and refinement

The crystal structures of the different complexes were solved by molecular replacement [18], using the uPA structure (PDB code: 2NWN) [16] as the search model. The electron density for the peptide was clearly visible in the uPA active sites and was modelled based on the F₀–Fₐ difference map. The structures were refined (ccp4 program package)[18] and manually adjusted (by the molecular graphics program COOT) [19] iteratively until the convergence of the refinement. Solvent molecules were added using a F₀–Fₐ Fourier difference map at 2.5 σ in the final refinement step. Statistics of data collection and final model refinement are summarized in Supporting Table S1. The final structure was analysed by software Pymol [20].

Model of mouse uPA

The sequence of the catalytic domain of muPA (positions 16–243) (Uniprot P06869, EC: 3.4.21.73) was homology modeled onto the X-ray crystal structure of huPA-H99Y, using Molecular Operating Environment [21]. The sequence identity is 71% between muPA and huPA-H99Y (S1 Fig.). The generated molecular model was refined by CNS program package [22].

Determination of $K_M$ values

To determine the $K_M$ values for hydrolysis of S-2444 (pyro-Glu-Gly-Arg-p-nitroanilide) by the different uPA variants used in the present study, a 200 µL 2-fold dilution series of the substrate (4 - 0 mM for huPA variants, 24 – 0 mM for muPA variants) in a buffer of 10 mM HEPES, pH 7.4, 140 mM NaCl (HEPES-buffered saline, HBS), with 0.1% bovine serum albumin (BSA), was incubated 2 min at 37°C, prior to the addition of a fixed concentration of each protease (approximately 2 nM final concentration). The initial reaction velocities ($V_i$), monitored as the changes in absorbance at 405 nm, were plotted against the initial substrate concentration ([S]) and non-linear regression analysis was used to determine the $K_M$ according to equation 1:

$$V_i = \frac{V_{max}[S]}{([S] + K_M)} \tag{1}$$

The $K_M$ values for hydrolysis of S-2444 by the uPA variants employed in the present study are listed in S2 Table.

Determination of $K_i$ values

For routine determination of $K_i$ values for the inhibition of the various enzymes under steady state inhibition conditions, a fixed concentration of purified enzyme or conditioned media from transfected cells (approximately 2 nM enzyme as the
final concentration) was pre-incubated in a total volume of 200 μL HBS with 0.1% BSA at 37°C, with various concentrations of mupain-1 variants for 15 min prior to the addition of the chromogenic substrate in concentrations approximately equal to the $K_M$ value for each particular variant. The initial reaction velocities were monitored at an absorbance of 405 nm. The inhibition constants ($K_i$) were subsequently determined from the non-linear regression analyses of plots for $V_i/V_o$ versus $[I]_0$, using Equation 2, derived under assumption of competitive inhibition:

$$ \frac{V_i}{V_0} = \frac{K_i \times (K_M + [S]_0)}{(K_i \times [S]_0) + (K_M \times (K_M + [I]_0))} \tag{2} $$

where $V_i$ and $V_0$ are the reaction velocities in the presence and absence of inhibitor, respectively; $[S]_0$ and $[I]_0$ are the substrate and inhibitor concentrations, respectively; $K_M$ is $K_M$ for substrate hydrolysis by each protease. In Equation 2, it is assumed that $[S]_{free} \approx [S]_0$ and $[I]_{free} \approx [I]_0$. These conditions were fulfilled, as less than 10% of the substrate was converted to product in the assays and as the assay typically contained a final concentration of each protease of 2 nM and inhibitor concentrations in the μM range.

In cases, in which we observed no measurable inhibition (i.e., <10%) at the maximal inhibitor concentration used, i.e., 400 μM, the accuracy of the assay allowed us to conclude that the $K_i$ value was more than 1000 μM (indicated as “$>1000$ μM” in the tables).

The validity of performing the $K_i$ determinations with uPA-containing conditioned media from transfected cells was verified by controls in which the determinations were performed with conditioned media as well as with purified preparations. These controls were performed with murine uPA wt and human uPA wt, obtaining indistinguishable values with the two types of samples [13].

Surface plasmon resonance (SPR) analysis
To determine the equilibrium binding constants ($K_D$), the association rate constants ($k_{on}$) and dissociation rate constants ($k_{off}$) for peptide binding to uPA, surface plasmon resonance analysis was performed on a Biacore T200 instrument (Biacore, Uppsala, Sweden). A CM5 chip was coupled with the uPA variant (muPA or huPAH99Y) to be analysed, by injecting a concentration of 30 μg/mL uPA in immobilization buffer (10 mM sodium acetate, pH 5.0), aiming for an immobilised level of approximately 500 response units (RU). Immobilisation was followed by surface blocking with ethanolamine. A reference cell was prepared in the same way, without coupling of uPA. Mupain-1 variants in HBS with 0.1% BSA, in a dilution series, were injected at a flow rate of 30 μL per min during 60 s at 25°C. Subsequently, the dissociation was monitored during 600 s. Kinetic constants ($k_{on}$ and $k_{off}$) were calculated with the Biacore Evaluation Software, using the 1:1 kinetic fit. The $K_D$ values were calculated as $k_{off}/k_{on}$.
ITC
For these experiments, we used the catalytic domain of huPA-H99Y expressed in and purified from *Pichia pastoris* strain X-33 (see above). The protein was dissolved in and dialysed against a buffer of 20 mM sodium phosphate, pH 7.4, 140 mM NaCl. The protein concentration was determined by absorbance at 280 nm, using an extinction coefficient of 43810 M$^{-1}$cm$^{-1}$. The peptides were dissolved in the above-mentioned buffer. All isothermal titration calorimetry experiments were performed with a MicroCal™ ITC200 instrument equilibrated to a temperature of 25°C (298oK). The concentration of uPA-H99Y catalytic domain used in the 200 µl sample cell was 5–50 µM, depending on the affinity of the ligand. Titrations were performed by injecting 2 µl of the ligand until the total syringe volume of 40 µl was spent. Titration of ligand into buffer was performed to obtain buffer correction. The equilibrium association constant $K_A$ and the reaction enthalpy $\Delta H$ were calculated by fitting the integrated titration peaks using a one-binding-site model in the ITC ORIGIN7 programme package. The following formulas for Gibbs free energy $\Delta G$ were used to analyse the measured energies

$$\Delta G = -RT \ln K_A$$  \hspace{1cm} (3)

$$\Delta G = \Delta H - \Delta S$$  \hspace{1cm} (4)

where R is the gas constant and T the absolute temperature. $\Delta S$, the entropic change during the reaction, was calculated using equations 3 and 4 and the measured $K_A$ and $\Delta H$ values.

NMR spectroscopy
Peptide samples were dissolved in a buffer of 10 mM sodium phosphate, 140 mM NaCl in D$_2$O/H$_2$O (7:93, v/v). The pH was adjusted to 7.4. For chemical-shift reference and to increase the long-term stability of the samples, 2,2-dimethyl-2-silapentane-5-sulfonic acid (10 µM) and NaN$_3$ (150 µM) were added. The peptide concentrations were 5.0 and 6.7 mM for mupain-1 and mupain-1-16, respectively. NMR experiments were acquired with a Bruker Avance III 500 spectrometer (500.13 MHz; Bruker Biospin, Rheinstetten) equipped with a standard inverse triple-resonance TXI 5 mm probe. Two-dimensional TOCSY data (80 ms mixing time), NOESY data (200 ms mixing time) and natural abundant $^{13}$C HSQC data were acquired for both peptides. The experiments were acquired at 5°C to slow down peptide tumbling, favour lowest-energy conformations, and obtain the highest signal in the NOESY spectra for assignment. Assignments were obtained by standard methods with CCPN software [23]. Visualisation of spectra and integration of NOE peaks were performed in SPARKY [24]. Random coil shifts were calculated by using values
provided by Kjærgaard et al. [25] and corrected by subtraction of correction values from Schwarzinger [26] which contains values for oxidised Cys and for cis-Pro. These correction values were obtained by subtracting Schwartzinger’s values for Cys\textsubscript{red} from Cys\textsubscript{ox} and trans-Pro from cis-Pro for all the different proton types in these residues. The order parameter (S\textsuperscript{2}) was calculated according to the method of Berjanskii and Wishart [27] as implemented within TALOS+ [28].

**Results**

Inhibitory mechanism and binding mode studied by X-ray crystal structure analysis

While being unable to generate crystals of muPA, we did manage to crystallise huPA-H99Y, a murinised version of human uPA which, in contrast to human uPA wt, is able to bind mupain-1, although with a somewhat lower affinity than muPA [12,13]. We determined the structures huPA-H99Y in complex with mupain-1 (CPAYSRYLDC) itself as well as with either of two other inhibitory peptides, namely mupain-1-12 (CPAYS[4-guanidinophenylalanine]YLDC) and mupain-1-16 (CPAYS[L-3-(N-amidino-4-piperidyl)alanine]YLDC). Mupain-1-12 and mupain-1-16 have around 10-fold higher affinities to huPA-H99Y than mupain-1 [13]. X-ray data collection and model refinement statistics are shown in S1 Table. Important features of the structures are illustrated in Fig. 2. The contact distances between the residues of the peptides and residues of huPA-H99Y are shown in S3-S5 Tables. The B-factors are listed in S7 Table.

The analysis showed very similar conformations of the three peptides when bound to huPA-H99Y (Fig. 2A). The RMSD values among these peptides are quite small (0.32–0.35 Å). In the complexes, the inhibitory peptides adopt cyclic conformations with an overall Ω shape. The disulfide bonds are the main structural restraint responsible for this conformation. Beginning from the N-terminus, the cyclic peptides approach the active site of huPA-H99Y from the 99-loop, insert residue 6 into the S1 pocket, and exit the active site towards the 37-loop (Fig. 2A). In each of the structures, the amino acid in position 6 of the peptide, i.e., Arg, 4-guanidinophenylalanine, or L-3-(N-amidino-4-piperidyl)alanine, forms polar interactions to Asp\textsuperscript{189}, Ser\textsuperscript{190}, and Gly\textsuperscript{218} in the S1 pocket (Fig. 1B). In addition, the X-ray crystal structure analysis showed that the huPA-H99Y residues Arg\textsuperscript{35}, Val\textsuperscript{41}, Leu\textsuperscript{97b}, Tyr\textsuperscript{99}, Gln\textsuperscript{192}, Trp\textsuperscript{215}, and Arg\textsuperscript{217} have the largest contact surface area to the peptides (S3-S5 Tables). In particular, Arg\textsuperscript{35} forms polar interactions with peptide residues Tyr\textsuperscript{7} and Asp\textsuperscript{9} (Fig. 2C) and Tyr\textsuperscript{99} forms polar interactions with peptide residue Ser\textsuperscript{4} (Fig. 2D). In the enzyme-peptide structures, the peptides are constrained by two type I tight β-turns (Pro\textsuperscript{2-Ala\textsuperscript{3}}-Tyr\textsuperscript{4}-Ser\textsuperscript{3} and Tyr\textsuperscript{7}-Leu\textsuperscript{8}-Asp\textsuperscript{9}-Cys\textsuperscript{10}) and three intra-peptide H-bonds (Pro\textsuperscript{2} O – Ser\textsuperscript{5} N; Ser\textsuperscript{18} Oγ - Tyr\textsuperscript{7} N; Ser\textsuperscript{5} Oγ - Arg\textsuperscript{6} N; S2 Fig.). The tight β-turns are likely to play an important role in maintaining the conformation and stability of the bound peptide. On the enzyme side, there are no major changes in the conformation of surface loops of the enzyme following peptide binding based on
Fig. 2. X-ray crystal structure analysis of huPA-H99Y in complex with peptidic inhibitors. (A) Overall structure of the complexes between huPA-H99Y and mupain-1 (cyan), mupain-1-12 (salmon), mupain-1-16 (grey), and mupain-1-16-D9A (red). (B) A zoom on interactions of mupain-1’s Arg7 in the S1 pocket; polar interactions are indicated by stippled lines. (C) A zoom on the polar interactions (stippled lines) between huPA-H99Y residue Arg35 and mupain-1 residues Tyr7 and Asp9. (D) A zoom on the polar interaction (stippled lines) between huPA-H99Y residue Tyr99 and mupain-1 residue Ser5. (E) An overlay of the active site areas of the huPA-H99Y – mupain-1 complex and the huPA S195A – PAI-1 Michaelis complex (pdb entry 3pb1; [29]); the P2, P1, and P1’ residues are indicated, those of PAI-1 in salmon. (F) A zoom on the Lys143 – Gln192 area of the huPA-H99Y – mupain-1 complex; distances, in Å, between different residues are indicated. In all parts of the figure, huPA-H99Y are shown in wheat cartoon presentation. In Fig. 1E, huPA S195A is shown in grey cartoon presentation. The peptides are shown in stick representation. huPA-H99Y residues are labelled with black letters, peptide residues with dark red letters.

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the comparison of the structure of the enzyme in the absence or the presence of peptide.

The inhibitory mechanism of these peptides and the reason that they are inhibitors and not substrates readily become evident from the structural analysis. The overall conformation of the peptide backbone on the enzyme surface is quite similar to that of the reactive centre loop of plasminogen activator inhibitor-1 (PAI-1) in its Michaelis complex with uPA S195A, aligning into the active site in a substrate-like manner (Fig. 2E; [29]). However, compared to PAI-1 in its Michaelis complex with uPA S195A, the scissile bond of mupain-1 in its complex with uPA-H99Y is shifted approximately 0.5 Å away from residue 195. The distance from the Ser^{195}Oγ to the carbonyl group of the P1 residue of mupain-1 and derivatives is too large (approximately 3.9 Å) to allow the nucleophilic attack associated with catalysis (Fig. 2E). Moreover, the distance from the oxygen atom of the carbonyl group of the P1 residue to the amido group of the Ser^{195} is too large (approximately 4 Å) for formation of a polar interaction, implying that oxyanion stabilisation cannot take place (Fig. 2E).

In spite of the X-ray crystal structure analysis having a good resolution, no differences could be detected between the complexes of huPA-H99Y with each of the peptides mupain-1, mupain-1-12, and mupain-1-16, the $K_i$ values of which differ around 10-fold. A 10-fold difference in $K_i$ corresponds to a $\Delta(\Delta G)$ for binding of approximately 6 kJ/mol, about the same energy as that of an average hydrogen bond. In this case, it is therefore possible that small differences in hydrophobic interactions and in the length and angles of polar interactions, at the detection limit by the structural analysis, may account for the differences in $K_i$ values. Alternatively, and more likely, the peptide-enzyme complexes, in solution, may sample a number of similar conformations of which only the most stable one is selected during crystallisation.

**Analysis of peptide-huPA-H99Y exosite interactions by site-directed mutagenesis**

We determined the $K_i$ values for inhibition of variants of huPA-H99Y by variants of mupain-1, mupain-1-12, and mupain-1-16. The Ala-substituted residues of the peptide and of huPA-H99Y are those deemed to be important for binding from the X-ray crystal structure analysis (Table 1). In good agreement with the structural analysis, Ala-substitution of mupain-1 residues Pro^2, Tyr^4, Ser^5, and Arg^6 led to strong reductions in affinity, Ala-substitution of Tyr^7 to a moderate reduction in affinity, and Ala-substitution of Leu^8 to a very small change in affinity. The observed effect of Ala-substituting the peptide’s Ser^5 is in good agreement with the fact that the binding of mupain-1 to huPA is dependent on the substitution of its His^{99} with Tyr [12], shown here to be able to form a hydrogen bond with Ser^5 (Fig. 2D). Surprisingly, Ala-substitution of Asp^9 of mupain-1, mupain-1-12, and mupain-1-16 led to a 3–10-fold increased affinity to huPA-H99Y. This observation is in contrast to the expectancies from the X-ray
crystal structure analysis, which predicts a polar interaction between Asp\(^9\) of mupain-1 and Arg\(^{35}\) of huPA-H99Y (Fig. 2C; S3-S5 Tables). Ala-substitution of huPA-H99Y residues Arg\(^{35}\), Val\(^{41}\), Lys\(^{143}\), and Gln\(^{192}\) led to 2–4 fold reductions in affinity to mupain-1, but smaller if any reductions in the affinity to mupain-1-D9A, mupain-1-12, mupain-1-16, mupain-1-12-D9A, and mupain-1-16-D9A. The effects of the R35A and Q192A mutations are in good agreement with the predictions of polar interactions from the X-ray crystal structure analysis (Fig. 2C, Fig. 2F). The V41A mutation may result in loss of hydrophobic interactions. From the X-ray crystal structure analysis, Lys143 is not predicted to make any direct contacts to the peptides, but the observed effect of the K143A mutation may be caused by an indirect effect through a polar interaction between Lys 143 and Gln192 (Fig. 2F). The observed changes following the Ala substitutions in the enzyme were in all cases small, in agreement with the fact that the predicted polar interactions are surface exposed. In general, there was less dependence on the exosite interactions with the peptides with the unnatural P1 residues and the peptides with an D9A substitution. This observation suggests that the effects of the exosite mutations and the D9A substitution is influenced by interactions in the S1 pocket.

The \(K_i\) determinations were supported by determinations of \(K_D\) values with SPR (Table 2; S3 Fig.) and ITC (Table 3). The \(K_D\) values determined by SPR and ITC agreed well with the \(K_i\) values, in particular when considering that \(K_D\) values were routinely determined at 25°C and the \(K_i\) values at 37°C; separate control experiments showed that the \(K_i\) values were 2–3 fold lower at 25°C than at 37°C.

Table 1. Inhibition of huPA-H99Y and huPA-H99Y exosite mutants by mupain-1 variants.

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<tr>
<td>Mupain-1 CPAYSRYLDC</td>
<td>15.3 ± 2.0 (3)*</td>
<td>39.5 ± 2.9 (3)</td>
<td>32.4 ± 5.3 (3)</td>
<td>56.6 ± 8.0 (3)</td>
<td>33.2 ± 4.2 (3)</td>
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<tr>
<td>Mupain-1 P2A CAAYSRYLDC</td>
<td>&gt;1000</td>
<td>nd</td>
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<tr>
<td>Mupain-1 Y4A CPAASRYLDC</td>
<td>&gt;1000</td>
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<tr>
<td>Mupain-1 S5A CPAYARYLDC</td>
<td>&gt;1000</td>
<td>nd</td>
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<tr>
<td>Mupain-1 R6A CPAYSAYLDC</td>
<td>&gt;1000</td>
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<tr>
<td>Mupain-1 Y7A CPAYSRALDC</td>
<td>44.0 ± 3.0 (3)</td>
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<tr>
<td>Mupain-1 L8A CPAYLSRYLDC</td>
<td>19.1 ± 1.1 (3)</td>
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<tr>
<td>Mupain-1 D9A CPAYSLRYLAC</td>
<td>5.99 ± 0.43 (3)</td>
<td>2.68 ± 0.29 (3)</td>
<td>7.20 ± 1.44 (3)</td>
<td>8.74 ± 0.64 (3)</td>
<td>4.85 ± 0.32 (3)</td>
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<tr>
<td>Mupain-1-12 CPAYS[4-guanidino-phenyl-alanine]YLDC</td>
<td>1.86 ± 0.74 (3)*</td>
<td>3.26 ± 1.04 (3)</td>
<td>1.60 ± 0.54 (3)</td>
<td>2.14 ± 0.78 (3)</td>
<td>2.35 ± 0.74 (3)</td>
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<tr>
<td>Mupain-1-12 D9A CPAYS[4-guanidino-phenyl-alanine]YLAC</td>
<td>0.186 ± 0.036 (3)</td>
<td>0.231 ± 0.078 (3)</td>
<td>0.478 ± 0.137 (3)</td>
<td>0.402 ± 0.146 (1)</td>
<td>0.438 ± 0.079 (3)</td>
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<tr>
<td>Mupain-1-16 CPAYS[L-3-(N-amidino-4-piperidyl)alanine]YLDC</td>
<td>2.48 ± 0.07 (3)*</td>
<td>2.50 ± 0.29 (3)</td>
<td>1.95 ± 0.30 (3)</td>
<td>1.13 ± 0.32 (3)</td>
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<tr>
<td>Mupain-1-16 D9A CPAYS[L-3-(N-amidino-4-piperidyl)alanine]YLAC</td>
<td>0.309 ± 0.013 (3)</td>
<td>0.174 ± 0.059 (3)</td>
<td>0.480 ± 0.158 (3)</td>
<td>0.188 ± 0.057 (3)</td>
<td>0.438 ± 0.072 (4)</td>
<td></td>
</tr>
</tbody>
</table>

The \(K_i\) values (in \(\mu\)M) for inhibition of the indicated enzymes by the indicated peptides at 37°C are shown as means ± S.D; the numbers of determinations are indicated in parentheses. *These values are reproduced from previous publications [Andersen et al., 2008; Hosseini et al., 2011] and shown here to facilitate comparison.

doi:10.1371/journal.pone.0115872.t001
Table 3. Isothermal titration calorimetry for binding of peptides to huPA-H99Y.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide sequence</th>
<th>N</th>
<th>$K_D$ (µM)</th>
<th>$\Delta G$ (kJ/mole)</th>
<th>$\Delta H$ (kJ/mole)</th>
<th>$\Delta T \Delta S$ (kJ/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mupain-1 CPAYSRYLDC</td>
<td>1.09 ± 0.15 (4)</td>
<td>5.04 ± 1.61 (4)</td>
<td>-30.4 ± 0.7 (4)</td>
<td>-36.5 ± 3.2 (4)</td>
<td>-6.1 ± 2.6 (4)</td>
<td></td>
</tr>
<tr>
<td>Mupain-1 D9A CPAYSRYLAC</td>
<td>1.03 ± 0.09 (6)</td>
<td>1.17 ± 0.30 (6)</td>
<td>-33.9 ± 0.7 (6)</td>
<td>-37.9 ± 2.4 (6)</td>
<td>-4.0 ± 2.9 (6)</td>
<td></td>
</tr>
<tr>
<td>Mupain-1-12 CPAYS[4-guanidino-phenylalanine]YLDC</td>
<td>0.85 ± 0.08 (4)</td>
<td>0.400 ± 0.016 (4)</td>
<td>-36.5 ± 0.1 (4)</td>
<td>-43.8 ± 5.5 (4)</td>
<td>-7.6 ± 5.6 (4)</td>
<td></td>
</tr>
<tr>
<td>Mupain-1-12 D9A CPAYS[4-guanidino-phenylalanine]YLAC</td>
<td>0.88 ± 0.01 (3)</td>
<td>0.138 ± 0.007 (3)</td>
<td>-39.2 ± 0.8 (3)</td>
<td>-60.1 ± 5.4 (3)</td>
<td>-18.9 ± 5.1 (3)</td>
<td></td>
</tr>
<tr>
<td>Mupain-1-16 CPAYS [L-3-(N-amidino-4-piperidyl)alanine]YLDC</td>
<td>0.87 ± 0.09 (4)</td>
<td>0.380 ± 0.065 (4)</td>
<td>-36.7 ± 0.4 (4)</td>
<td>-33.2 ± 4.2 (4)</td>
<td>3.5 ± 1.0 (4)</td>
<td></td>
</tr>
<tr>
<td>Mupain-1-16 D9A CPAYS [L-3-(N-amidino-4-piperidyl)alanine]YLAC</td>
<td>0.88 ± 0.11 (5)</td>
<td>0.134 ± 0.061 (5)</td>
<td>-39.5 ± 1.1 (5)</td>
<td>-46.6 ± 2.9 (5)</td>
<td>-6.2 ± 2.7 (5)</td>
<td></td>
</tr>
</tbody>
</table>

$^1$The value for the D9A peptide is significantly different from the value for the unmodified peptide (p<0.01). The table shows thermodynamic parameters for the binding of the indicated peptides to huPA-H99Y at 25°C, pH 7.4. Means, standard deviations, and numbers of determinations are indicated.

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(data not shown). The SPR measurements showed that the 9–12-fold increased affinities to huPA-H99Y following the D9A substitution were associated with 3–4-fold increased $k_{on}$ values as well as 2–3-fold decreased $k_{off}$ values (Table 2). The ITC measurements showed that the increased affinity of the Ala$^9$ peptides to huPA-H99Y, as compared to the original Asp$^9$ peptides, was associated with a binding entropy penalty but mainly accounted for by a more favourable binding enthalpy (except with mupain-1 and mupain-1 D9A, in which case the differences were not statistically significant; Table 3). The most ready molecular interpretation of the SPR and ITC data, taken together, is that the D9A substitution renders the peptide more flexible in solution, thereby making the binding entropy less...
favourable, the association activation energy lower, and the \( k_{\text{on}} \) higher, and allows a more favorable binding enthalpy and a more stable bound state, thereby increasing the dissociation activation energy and decreasing the \( k_{\text{off}} \).

Considering the unexpected effect of the D9A substitution, we also crystallised mupain-1-16 D9A in complex with huPA-H99Y (S1 and S6 Tables), but in spite of the much higher affinity of the D9A peptides, the structures of the mupain-1-16 and the mupain-1-16-D9A complex were indistinguishable (Fig. 2A). Noticeably, however, the relative B-factor for mupain-1-16 D9A was higher than that for mupain-1-16 (1.39 versus 1.24; S7 Table).

In summary, while most interactions between the peptides and huPA-H99Y predicted by X-ray crystal structure analysis were largely in agreement with the results of the site-directed mutagenesis, the increased affinity following the D9A substitution in mupain-1 was unexpected and could not be correlated with any structural differences observable by X-ray crystal structure analysis. However, the SPR and ITC analyses indicated a more stable complex following the D9A substitution, in spite of a binding entropy penalty.

Analysis of peptide-muPA exosite interactions by site-directed mutagenesis

Next, we analysed the \( K_i \) values for inhibition of several muPA variants by mupain-1, mupain-1-12, and mupain-1-16, Ala substituting residues of the peptide and the enzyme in positions implicated in peptide-enzyme interaction by the X-ray crystal structure analysis of the huPA-H99Y-peptide complex (see S1 Fig. for alignment of the amino acid sequences of the catalytic domains of huPA and muPA). Previously, we showed that Ala-substitution of mupain-1 residues Pro\(^2\), Tyr\(^4\), Ser\(^5\), Arg\(^6\), and Tyr\(^7\) leads to substantial loss of affinity to muPA, while Leu\(^8\) and Asp\(^9\) could be Ala-substituted with minimal consequences [12]. Thus, the effect of the D9A substitution is different with muPA and huPA-H99Y. Now, we found that Ala-substitutions of muPA residues Lys\(^{41}\), Tyr\(^{99}\) and Lys\(^{143}\) increase the \( K_i \) values 5–50-fold (Table 4). However, in contrast to observations with huPA-H99Y, there were no effects of Ala substitutions of muPA residues in the 37-loop. From a model of muPA in complex with mupain-1, it seems that Lys\(^{41}\) is able to make polar interactions with Tyr\(^7\) (2.3 Å) and Asp\(^9\) (2.8 Å) of mupain-1 (Fig. 3C), explaining the effect of substitution of this muPA residue. From the model, Lys\(^{143}\) is predicted to be more than 6 Å away from the closest part of the peptide and more than 6 Å away from Lys\(^{192}\) (Fig. 3B), leading to the notion that the peptide and/or residue 192 have different conformations in the huPA-H99Y-mupain-1 complex and the muPA-mupain-1 complex. Thus, the observed importance of mupain-1 residues Pro\(^2\), Tyr\(^4\), Ser\(^5\) and Arg\(^6\) and muPA residue Tyr\(^{99}\) suggests that the CCPAYSR stretch of peptides occupies a position on muPA similar to that inferred from the X-ray crystal structure analysis of the peptide-huPA-H99Y complexes, while the stretch YLD of mupain-1 makes different interactions in the two complexes.
Interestingly, the D9A substitution, while having no effects on the affinities of the peptides to muPA wt, muPA-Y99A, and muPA-K143A, strongly increased the affinity to muPA-K41A (Table 2). Thus, the $K_i$ values for inhibition of muPA by the Ala$^9$ peptides depend differently on exosite mutations than those for the original Asp$^9$ peptides. This observation shows than the interactions between the Ala$^9$ peptides and muPA differ from the interactions between the Asp$^9$ peptides and muPA. Importantly, the D9A substitution in all cases reduced the difference in $K_i$ between huPA-H99Y and muPA (Tables 1 and 4): For the Asp$^9$ peptides, the $K_i$ value for inhibition of huPA-H99Y were 28, 7, and 55-fold higher than those for inhibition of muPA, while the corresponding values for the Ala$^9$ peptides were only 7, 1, and 4-fold higher.

The $K_i$ values were in good agreement with the $K_D$ values determined by SPR (Table 2).

### Analysis of S1-P1 interactions by site-directed mutagenesis

In order to characterise the mechanism of the increase in affinity following substitution of the P1 Arg with either of the two unnatural P1 residues, we introduced mutations in the S1 pocket of muPA, i.e., S190A and V213T (Table 5). The X-ray crystal structure analysis of the peptide-huPA-H99Y complexes implicated Ser$^{190}$ in hydrogen bonding to the P1 residues (Fig. 2B; S3–S5 Tables); many serine proteases, including tissue-type plasminogen activator (tPA), have an Ala in this position. Val$^{213}$ forms a hydrophobic patch at the entrance to the S1 pocket (Fig. 3D); we substituted the Val with a Thr, as some serine proteases, including plasma kallikrein, has a Thr in this position. The S190A substitution resulted in a decreased affinity for all the peptides, while the V213T mutation resulted in an increased affinity for all the peptides (Table 5).

In order to visualise the effects of the S1 pocket mutations, the $K_i$ values for the inhibition of the muPA mutants by each of the 6 peptides were plotted logarithmically versus the $K_i$ values for inhibition of muPA wt by the same.

---

### Table 4. Inhibition of muPA wt and muPA exosite mutants by mupain-1 variants.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Sequence</th>
<th>muPA-wt $K_i$ (mM) ± S.D.</th>
<th>muPA-K41A $K_i$ (mM) ± S.D.</th>
<th>muPA-Y99A $K_i$ (mM) ± S.D.</th>
<th>muPA-K143A $K_i$ (mM) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mupain-1</td>
<td>CPAYSRYLDC</td>
<td>$0.550 ± 0.080$ (8)</td>
<td>$4.29 ± 0.15$ (3)</td>
<td>$26.0 ± 5.8$ (3)</td>
<td>$4.88 ± 0.35$ (3)</td>
</tr>
<tr>
<td>Mupain-1 D9A</td>
<td>CPAYSRYLAC</td>
<td>$0.890 ± 0.020$ (4)</td>
<td>$0.270 ± 0.029$ (3)</td>
<td>$27.2 ± 3.3$ (3)</td>
<td>$4.34 ± 0.64$ (3)</td>
</tr>
<tr>
<td>Mupain-1-12</td>
<td>CPAY[4-guanidino-phenyl-alanine]YLDCA</td>
<td>$0.280 ± 0.020$ (5)</td>
<td>$1.72 ± 0.24$ (3)</td>
<td>$5.20 ± 0.06$ (3)</td>
<td>$1.00 ± 0.04$ (3)</td>
</tr>
<tr>
<td>Mupain-1-12 D9A</td>
<td>CPAY[4-guanidino-phenyl-alanine]YLAAC</td>
<td>$0.190 ± 0.010$ (3)</td>
<td>$0.091 ± 0.007$ (3)</td>
<td>$5.89 ± 1.31$ (3)</td>
<td>$1.00 ± 0.03$ (3)</td>
</tr>
<tr>
<td>Mupain-1-16</td>
<td>CPAYS[L-3-(N-amidino-4-piperidyl)alanine]YLDCA</td>
<td>$0.045 ± 0.010$ (4)</td>
<td>$0.30 ± 0.05$ (3)</td>
<td>$1.24 ± 0.44$ (3)</td>
<td>$0.13 ± 0.02$ (3)</td>
</tr>
<tr>
<td>Mupain-1-16 D9A</td>
<td>CPAYS[L-3-(N-amidino-4-piperidyl)alanine]YLAAC</td>
<td>$0.076 ± 0.003$ (3)</td>
<td>$0.014 ± 0.002$ (3)</td>
<td>$1.36 ± 0.33$ (3)</td>
<td>$0.10 ± 0.02$ (3)</td>
</tr>
</tbody>
</table>

The $K_i$ values (in μM) for inhibition of the indicated enzymes by the indicated peptides are shown as means ± S.D.; the numbers of determinations are indicated in parentheses. *These values are reproduced from previous publications [Andersen et al., 2008; Hosseini et al., 2011] and shown here to facilitate comparison.

Besides the muPA mutants reported in the table, we found no significant change in $K_i$ values after the following substitutions: Q35A; N37A; K37aA; G37cA; S37dA; P37eA; P38A; Q60aA; E146A; Y149A. For unknown reasons, muPA K192A could not be expressed.

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peptides (Fig. 4). For data points above the \( y=x \) line, the mutations increase the \( K_i \) value or decrease the affinity; for points above the \( y=x \) line, the mutations decrease the \( K_i \) value or increase the affinity. The slopes of the lines defined by the data points (which may be referred to as an “interdependence factor”) were 0.68 in the case of the S190A mutation and 1.27 in the case of the V213T mutation. The slopes being different from 1 shows that the effects of the S1 pocket mutations vary with the P1 residue: Following the S190A mutation, the \( K_i \) value increases...
Table 5. Inhibition of muPA wt and muPA S1 pocket mutants by mupain-1 variants.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Sequence</th>
<th>muPA wt</th>
<th>muPA-S190A</th>
<th>muPA-V213T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mupain-1</td>
<td>CPAYSRYLDC</td>
<td>0.550±0.080 (8)*</td>
<td>1.60±0.53 (3)</td>
<td>0.246±0.044 (3)</td>
</tr>
<tr>
<td>Mupain-1 D9A</td>
<td>CPAYSRYLAC</td>
<td>0.890±0.020 (4)*</td>
<td>3.02±0.33 (3)</td>
<td>0.373±0.055 (3)</td>
</tr>
<tr>
<td>Mupain-1-12</td>
<td>CPAY[4-guanidino-phenyl-alanine]YLDC</td>
<td>0.280±0.020 (5)*</td>
<td>2.30±0.46 (3)</td>
<td>0.086±0.030 (3)</td>
</tr>
<tr>
<td>Mupain-1-12 D9A</td>
<td>CPAY[4-guanidino-phenyl-alanine]YLAC</td>
<td>0.190±0.010 (3)</td>
<td>3.52±0.50 (3)</td>
<td>0.166±0.075 (4)</td>
</tr>
<tr>
<td>Mupain-1-16</td>
<td>CPAY[L-3-(N-amidino-4-piperidyl)alanine]YLDC</td>
<td>0.045±0.010 (4)*</td>
<td>0.352±0.040 (3)</td>
<td>0.013±0.003 (3)</td>
</tr>
<tr>
<td>Mupain-1-16 D9A</td>
<td>CPAY[L-3-(N-amidino-4-piperidyl)alanine]YLAC</td>
<td>0.076±0.003 (3)</td>
<td>0.529±0.052 (3)</td>
<td>0.014±0.004 (3)</td>
</tr>
</tbody>
</table>

The $K_i$ values (in $\mu$M) for inhibition of the indicated enzymes by the indicated peptides are shown as means ± S.D; the numbers of determinations are indicated in parentheses. *These values are reproduced from previous publications [Andersen et al., 2008; Hosseini et al., 2011] and shown here to facilitate comparison.

doi:10.1371/journal.pone.0115872.t005

Fig. 4. The relationship between the $K_i$ values for inhibition of muPA S1 mutants and the $K_i$ values for inhibition of muPA wt. The figure is based on the $K_i$ values presented in Table 6. The x-axis shows the $K_i$ values for inhibition of muPA wt by the indicated peptides. The y-axis shows the corresponding $K_i$ values for inhibition of muPA S190A (filled dots) or muPA V213T (open dots). The lines resulted from simple linear regression analysis. The slopes of the lines are 0.68 (muPA-S190A) and 1.27 (V213T). The stippled line is the one which would have resulted if the $K_i$ values for inhibition of the muPA S1 mutants had been identical to those for inhibition of muPA wt ($y=x$).

doi:10.1371/journal.pone.0115872.g004
more with mupain-1-12 and mupain-1-16 variants than with the Arg variants, while following the V213T mutation, the \( K_i \) value decreases more with the mupain-1-12 and mupain-1-16 variants than with the mupain-1 Arg variants. For instance, the V213T mutation increases the affinity for the mupain-1 Arg variants around 2.5-fold, but increases the affinity for the L-3-(N-amidino-4-piperidyl-l)alanine] variants around 5-fold. These findings are compatible with the notion that each of the three P1 residues fit differently into the S1 pocket.

To further visualise the importance of the P1 residues of mupain-1, the \( K_i \) values for inhibition of the muPA exosite mutants K41A, Y99A, and K143A by mupain-1, mupain-1-12, and mupain-1-16 and their D9A variants were plotted logarithmically versus the corresponding \( K_i \) values for the wt enzymes (Fig. 5). The above-mentioned difference between the Asp\(^9\) and the Ala\(^9\) peptides with respect to inhibition of muPA K41A was obvious in the plot, the data points in an Ala\(^9\) group falling below the y=x line and the data points for the Aap\(^9\) group falling above the y=x line. There was no such split with the other muPA mutants. With the K41A and the Y99A mutants, the slopes of the lines defined by the data pairs were relatively close to 1. However, with the K143A mutant, the slopes were around 1.4. This slope corresponds to a 9-fold increase in \( K_i \) for mupain-1 following the K143A mutation and an only 2.9-fold increase in \( K_i \) for mupain-1-16 following the K143A mutation and similar differences when comparing the other peptides. This observation shows that the identity of the amino acid in position 143 influences the effect of a P1 substitution and vice versa.

The effects of substituting the P1 residue were also analysed by SPR (Table 2). The increased affinities associated with the unnatural P1 residues were found to be accounted for almost exclusively by lower off-rates.

Taken together, these observations are in agreement with the conclusion that the increased affinity with the two unnatural P1 residues is caused by changes in the binding in the S1 pocket. Interestingly, there seems to be a cross-talk between interactions of the peptide in the S1 pocket and interactions with Lys143.

**Analysis of the importance of the P1 residue by NMR**

For a further characterization of the importance of the P1 residue, mupain-1 and mupain-1-16 were subjected to \(^1\)H liquid-state NMR analysis. Analysis of the backbone amide and \( \alpha \)-proton region of a TOCSY spectrum revealed a doubling of the expected number of peaks of mupain-1 and mupain-1-16. Following standard procedures for analysis of TOCSY and NOESY spectra [30], the NMR data could be assigned to two markedly different resonance forms, as illustrated by two parallel “backbone walks” connecting sequential residues (Fig. 6). Based on analysis of \(^13\)C chemical shifts assigned by natural abundance \(^13\)C-HSQC (difference between C\(\beta\) and C\(\gamma\) chemical shifts) (S8 Table) [31], the two sets of resonances were inferred to be due to cis-trans isomerization around the Cys\(^1\)-Pro\(^2\) peptide bond. By integration of isolated peaks in the TOCSY spectrum, the steady state ratio between the two forms was found to be 1:3 in favor of the trans conformation. Since no exchange cross peaks were observed between the two
Fig. 5. The relationship between the $K_i$ values for inhibition of muPA exosite mutants and the $K_i$ value for inhibition of muPA wt. The figure is based on the $K_i$ values presented in Table 5. The x-axes show the $K_i$ values for inhibition of muPA wt by the indicated peptides. The y-axes shows the corresponding $K_i$ values for inhibition of muPA K41A, Y99A, or K143A. The lines resulted from simple linear regression analysis. The slope of the lines are 1.04 (muPA K41A with Asp$^9$ peptides); 1.15 (muPA K41A with Ala$^9$ peptides); 1.15 (muPA Y99A); 1.40 (muPA K143A). The stippled line shows the line which would have resulted if the $K_i$ values for exosite mutants and muPA wt had been identical ($y=x$).

doi:10.1371/journal.pone.0115872.g005
distinct resonance sets corresponding to the two conformers in the time frame of a 200 ms NOESY experiment, the interconversion between the two conformations was estimated to be slower than 1 s\(^{-1}\) for both mupain-1 and mupain-1-16. The assigned chemical shifts were close to the random coil values, indicating that the peptides are flexible in solution. Both the proton and carbon chemical shifts for the two isomeric forms were substantially different for most of the residues, suggesting that the average conformations for the cis and trans isomers are different. It is notable that the spectral differences due to the cis-trans isomerism propagated throughout the entire peptide chain and not just to the adjacent residues, indicating that the structures are not completely disordered. Furthermore, the chemical shifts for trans-mupain-1 and cis-mupain-1 are very similar to those of trans-mupain-1-16 and cis-mupain-1-16, respectively (as illustrated, e.g., by H\(_a\) in S4 Fig.). This similarity strongly suggests that the structures or average conformations of each of the isomers of each of the two peptides are very similar. Also the similarity in steady state ratio of the cis- and trans-conformations of the two peptides is in agreement with the notion that the two peptides have the same overall structural and dynamic trends.

Importantly, the presence of the peptides in both a trans- and a cis-conformation in solution should be contrasted with the fact that only the trans-conformation was observed in the crystal structures of the peptide-enzyme complexes.
The flexibility of the peptides was probed by deriving a predicted order parameter, $S^2$, based on the chemical shifts, using random coil index implemented within TALOS+ (Fig. 7). Depending on movements on the picosecond to nanosecond time scale, an $S^2$ value of 0 corresponds to a completely disordered peptide and an $S^2$ value of 1 to a completely rigid one. The predicted order parameter was found to be relatively low, as also observed from the TALOS+ classification, in which most of the residues fall in the dynamic category.

In summary, the NMR analysis of the peptides in solution did not reveal differences in solution structures between mupain-1 and mupain-1-16.

Discussion

In this report, we describe studies of the inhibition mechanism and the binding mechanism of derivatives of the serine protease inhibitor mupain-1, which was originally selected from a phage-displayed peptide library for binding to muPA [12]. Besides muPA, we used huPA-H99Y as a model enzyme, because it bound mupain-1 with a reasonable affinity and could, in contrast to muPA, easily be crystallised in complex with the peptides.

Our analyses showed that mupain-1 and derivatives thereof make P1–S1 interactions as well as several exosite interactions with their target proteases. The X-ray crystal structure analysis seemed to yield reliable information about the overall arrangement of the peptide at the enzyme surface and about the inhibitory mechanism of the peptides. There is a good agreement between the X-ray crystal structure analysis and the site-directed mutagenesis analysis as far as the CCPAYS stretch of the peptide is concerned. However, when it comes to the RYLD part of the peptide, it is striking that the effects on the $K_i$ values of substitution of the P1 residues and of Asp are not correlated with corresponding changes in the crystal structures. The most likely explanation for this apparent discrepancy is that the peptide is able to sample a number of conformations while bound to the enzyme but that only one is selected during crystallization. It thus seems that site-directed mutagenesis yield information about the details of peptide-enzyme interactions in solution which is not available from X-ray crystal structure analysis. Anyway, the overall arrangement of peptides in the enzyme surface seem to be largely the same in all cases. The $K_i$ changes observed following site-directed mutagenesis and the differences in $K_i$ between muPA and huPA-H99Y must reflect relatively small local conformational variations.

The reason for the peptides being inhibitors and not substrates readily became evident from the X-ray crystal structure analysis of the peptide-huPA-H99Y complexes. The distance from the Ser195 O\(_\gamma\) to the carbonyl group of the P1 residue of the peptides was found to be too large (>3 Å) to allow the nucleophilic attack associated with catalysis. Moreover, the oxygen atom of the carbonyl group fails to align properly into the oxyanion hole. The conformations of the mupain-1 peptides on the enzyme surface are perpendicular to the previously reported conformation of the peptide upain-1 (CSWRGLENHRMC), a competitive
inhibitor of huPA ([20]). In the upain-1 – huPA complex, Glu^7 of the peptide blocks the oxyanion hole [15, 16, 32]. Thus, the inhibitory mechanism of mupain-1 is different from that of upain-1. An inhibitory mechanism similar to that for upain-1 was observed for two bicyclic peptidic inhibitors of huPA, also with an acidic residue blocking the oxyanion hole [14, 33]. We were unable to crystallise muPA, but the binding mechanism of the peptides to this enzyme was worked out by site-directed mutagenesis. We concluded that the exosite interactions of the CCPAYSR stretch of the peptides are largely the same in their complexes with muPA and huPA-H99Y, respectively, while the exosite contacts made by the YLD stretch of the peptides to each of these two enzymes are likely to differ from those observed by the X-ray crystal structure analysis.

Substituting the P1 Arg of mupain-1 with L-4-guanidinophenylalanine or L-3-(N-amidino-4-piperidyl)alanine resulted in a general increase in affinity to muPA and huPA-H99Y as well as several chimeras between muPA and huPA [13]. *A priori*, the explanation for changes in affinity following the P1 substitution could be sought in an energetically less favorable solution state or an energetically more favorable bound state. We here did a number of observations allowing a distinction between these two possibilities. Firstly, the NMR analysis showed an absence of major differences in the solution structures of mupain-1 and mupain-1 trans and cis conformations. The dots (bottom) represent classification of the residue from TALOS+, as based on the mobility of the backbone, the certainty of the angles of the reference triplets and whether the angles fall into allowed regions in the Ramachandran plot [34]. The colour codes represent good (green), dynamic (yellow), ambiguous (red), and no prediction (blue) for the two different peptides in *cis* and *trans* conformations.

Fig. 7. Predicted order parameter S^2 (top) calculated by TALOS+.
1-16. The NMR analysis showed that both mupain-1 and mupain-1-16 have a cis-trans isomerization around the Cys1-Pro2 peptide bond. In both cases, the bound forms of the peptides had the Cys1-Pro2 peptide bond in the trans conformation, showing that the binding involves a shift in the equilibrium between the conformations towards the trans-form. The peptides are quite flexible in solution, so the binding renders the peptides more rigid. These observations argue against a decisive contribution from a difference in solution states of peptides with different P1 residues. Secondly, our mutational analysis showed that a different fit of the side chains of the three different P1 residues into the S1 pockets of the enzymes contributes to the changed affinities. Thirdly, the affinities are also affected by cross-talk between P1-S1 interactions and exosite interactions in mupain-1, involving in particular Lys143. It can therefore be concluded that the explanation for the increased affinity of the mupain-1 variants with the unnatural P1 residues is an energetically more favorable bound state rather than an energetically less favorable solution state.

The D9A mutation, unexpectedly, increased the affinity of all tested mupain-1 variants to all tested variants of huPA-H99Y, while there was no effect of the D9A mutation on the affinity to the muPA variants except K41A. In fact, the D9A mutation reduced the difference in affinity of the peptides to huPA-H99Y and muPA wt strongly. It thus seems that the D9A substitution allows mupain-1 to assume more favourable interactions with targets other than the one, against which it was selected. Of particular interest is the fact that the D9A substitution strongly increased the affinity of the peptides to muPA K41A without having any effect on the affinity to the other variants. In fact, the D9A peptides even bound stronger to muPA K41A than to muPA wt. The most ready explanation of this observation is that Lys41 of muPA restricts the conformation of the D9A peptide and keeps it from assuming the binding conformation with the lowest energy. Although less striking, the exosite interactions in huPA-H99Y also influenced the effects of the D9A substitution. These observations are in agreement with the notion that the D9A substitution leads to changes in the relative energetic contributions from different exosite interactions. This conclusion is also in agreement with the differential effect of the D9A substitution in muPA and huPA-H99Y, respectively. The D9A mutation makes it easier for the peptide to adopt to the different local environments of muPA and huPA-H99Y in the region around the 37-loop.

The increased affinity following the D9A substitution occurred in spite of an entropy penalty, which was overcome by a more favourable binding enthalpy. The SPR measurements showed that the increased affinity following the D9A substitution was associated with an increased \( k_{on} \) as well as a decreased \( k_{off} \). The most ready interpretation of these observations, taken together, is that the Ala\(^9\) peptides more readily adopt themselves to contacts on the surface of the enzymes than the Asp\(^9\) peptides. The Ala\(^9\) peptides may be able to sample a larger conformational space and visit conformations with peptide-enzyme interactions which are inaccessible to the Asp\(^9\) peptides. Such a larger flexibility of the enzyme-bound Ala\(^9\) peptides, as compared to the Asp\(^9\) peptides, is in good agreement with
the entropy penalty associated with the D9A substitution, assuming that the entropy increase associated with the increased flexibility is larger in the solution state than in the bound state. Also, the relative B-factor in the huPA H99Y complexes was higher for mupain-1-16 D9A than for mupain-1-16, in agreement with the idea of the D9A peptides being more flexible in the complexes.

Our results show that mupain-1 is an unusual peptidic serine protease inhibitor. Usually, attempts at improving affinity of enzyme inhibitors implicate reduction of the entropic burden associated with binding. This approach is also being pursued with peptidic protease inhibitors \([2,15]\). In the present work, another principle was demonstrated. The observation of a strongly increased affinity in spite of an entropy penalty represents a concept going against conventional attempts at improving inhibitor affinity by reducing the entropic burden. Our finding suggests that the D9A versions of mupain-1 may be engineered to target other serine proteases with high affinity. When combined with suitable unnatural amino acids as P1 residues and other measures to assure specificity, the flexibility achieved by the D9A substitution allows it to assume different conformations and make different exosite interactions, resulting in high affinity towards different enzymes. This principle is a new way of engineering inhibitor specificity and affinity.

**Supporting Information**

S1 Fig. Alignment of the amino acid sequences of the catalytic domains of muPA and huPA.

doi:10.1371/journal.pone.0115872.s001 (DOC)

S2 Fig. Conformation of the bound mupain-1 peptide constrained by two tight \(\phi\)-turns and hydrogen bonds.

doi:10.1371/journal.pone.0115872.s002 (DOC)

S3 Fig. SPR analysis of peptide-enzyme binding.

doi:10.1371/journal.pone.0115872.s003 (DOC)

S4 Fig. Secondary chemical shifts for the C\(\alpha\), C\(\beta\), H\(\alpha\), and H\(\beta\) for mupain-1 and mupain-1-16 in cis and trans forms.

doi:10.1371/journal.pone.0115872.s004 (DOC)

S1 Table. X-ray data collection and model refinement statistics.

doi:10.1371/journal.pone.0115872.s005 (DOC)

S2 Table. \(K_M\) values for S-2444 hydrolysis by muPA and huPA variants.

doi:10.1371/journal.pone.0115872.s006 (DOC)

S3 Table. Distances between mupain-1 and huPA-H99Y residues in the crystal structure.

doi:10.1371/journal.pone.0115872.s007 (DOC)

S4 Table. Distances between mupain-1-12 residues and huPA-H99Y residues in the crystal structure.

doi:10.1371/journal.pone.0115872.s008 (DOC)
S5 Table. Distances between mupain-1\textsubscript{-16} residues and huPA-H99Y residues in the crystal structure.
doi:10.1371/journal.pone.0115872.s009 (DOC)

S6 Table. Distances between mupain-1\textsubscript{-16} D9A and huPA-H99Y residues in the crystal structure.
doi:10.1371/journal.pone.0115872.s010 (DOC)

S7 Table. Comparison of B factors in the huPA-H99Y-mupain-1, huPA-H99Y-mupain-1\textsubscript{-12}, huPA-H99Y-mupain-1\textsubscript{-16} structures, and huPA-H99Y-mupain-1\textsubscript{-16}-D9A structures.
doi:10.1371/journal.pone.0115872.s011 (DOC)

S8 Table. Analysis of \textsuperscript{13}C chemical shifts of mupain-1 and mupain-1\textsubscript{-16}.
doi:10.1371/journal.pone.0115872.s012 (DOC)

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Author Contributions

Conceived and designed the experiments: BZ PX LJ TKH JKJ HPS M. Huang PAA. Performed the experiments: BZ PX LJ BP TKH ZL JTN AC M. Hosseini KKS. Analyzed the data: PX LJ BP TKH JKJ HPS JTN NCN KJJ M. Huang PAA. Contributed reagents/materials/analysis tools: TKH JKJ HPS M. Hosseini KKS NCN KJJ M. Huang PAA. Wrote the paper: PX LJ BP JTN KJJ M. Huang PAA.

References


APPENDIX 2

Manuscript II

Selection of High-Affinity Peptidic Serine Protease Inhibitors with Increased Binding Entropy from a Back-Flip Library of Peptide-Protease Fusions.

Hans Peter Sørensen, Peng Xu, Longguang Jiang, Tobias Kromann-Hansen, Jan K. Jensen, Mindong Huang and Peter A. Andreasen

Selection of High-Affinity Peptidic Serine Protease Inhibitors with Increased Binding Entropy from a Back-Flip Library of Peptide–Protease Fusions

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Abstract

We have developed a new concept for designing peptidic protein modulators, by recombinantly fusing the peptidic modulator, with randomized residues, directly to the target protein via a linker and screening for internal modulation of the activity of the protein. We tested the feasibility of the concept by fusing a 10-residue-long, disulfide-bond-constrained inhibitory peptide, randomized in selected positions, to the catalytic domain of the serine protease murine urokinase-type plasminogen activator. High-affinity inhibitory peptide variants were identified as those that conferred to the fusion protease the lowest activity for substrate hydrolysis. The usefulness of the strategy was demonstrated by the selection of peptidic inhibitors of murine urokinase-type plasminogen activator with a low nanomolar affinity. The high affinity could not have been predicted by rational considerations, as the high affinity was associated with a loss of polar interactions and an increased binding entropy.

Introduction

Short peptides, of 10–20 amino acids, are of considerable interest as modulators of protein function, be it as receptor agonists or antagonists or enzyme inhibitors or stimulators. As compared to small molecules, peptides have the advantage of a larger interaction surface with their protein target, affording a specificity approaching that of monoclonal antibodies but still having a size allowing for chemical synthesis and modification. Challenges in the use of peptides for pharmacological intervention are achieving high affinity; their short half-life in the circulation; their susceptibility to proteases, limiting the possibility of making them orally available; and their low membrane permeation, limiting their use against intracellular targets [1]. In this report, we approach the issue of achieving highly efficient peptidic modulators of protein function, using as a model a peptidic inhibitor of a serine protease.

Serine proteases have many physiological and pathophysiological functions and are therefore potential or realized therapeutic targets [2,3]. Moreover, some serine proteases are themselves used as drugs, including blood coagulation factor VIIa [4] or the fibrinolytic enzyme tissue-type plasminogen activator (tPA) [5]. In either case, the possibility of precisely regulating their activities is mandatory. A variety of peptidic modulators have been developed with the purpose of regulating serine protease function, acting by either orthosteric or allosteric mechanisms [6–20].

Peptidic functional modulators can be designed by structure-based residue replacement of naturally occurring peptides; $K_i$ measurements for each in silico designed candidate are feasible but cumbersome. Peptidic modulators can also be isolated directly from phage-displayed peptide libraries by many variations of a basic strategy [21]. In most cases, binding affinity was used as the selection criterion. Nevertheless, the
isolated peptides most often inhibited one or other function of the target protein, the reason probably being that the peptides will tend to bind to areas of the target proteins involved in ligand binding, like enzymes' active sites, allosteric regulatory sites, or receptors' binding cavities [6–22]. In other cases, peptides have been selected from libraries directly on the basis of their ability to affect a specific function of the target protein, a principle used, for instance, for selection of peptides able to activate hepatocyte growth factor [19].

In the new concept described here, peptidic protein modulators were selected according to inhibitory activity of the peptide rather than affinity. Our strategy consists in fusing a peptide covalently at the carboxy-terminus of a serine protease through a long flexible linker, allowing the peptide to flip back to the protease active site, leading to a fusion protein that has a reduced activity toward a chromogenic substrate when the fusion peptide is inhibitory. Variation of the peptide sequence can be used to make a back-flip library, here by expressing the fusion proteins in a human cell line, and it allows identification of peptides with a low $K_i$. Using this strategy, we strongly improved the potency of a disulfide-bridge-constrained peptidic inhibitor, mupain-1 (CPAYSRYLDC), of the serine protease urokinase-type plasminogen activator (uPA) from mouse [murine urokinase-type plasminogen activator (muPA)]. We previously isolated mupain-1 from a phage-displayed library [13]. Mupain-1 is a highly specific competitive inhibitor, which binds to its target with a moderate $K_i$ of around 500 nM [13,23]. We also previously determined its inhibitory mechanism by X-ray crystal structure analysis of an inhibitor--enzyme complex [24,25]. Using the back-flip strategy followed by substitution of the P1 residue with unnatural amino acids, we have now designed an inhibitor of muPA with a $K_i$ of 2 nM, more than 100-fold lower than that for inhibition of any other of the tested serine proteases. This result could not have been predicted by rational considerations: the affinity increase was associated with substitution of the original Asp9 with a Gly, the loss of a polar peptide--enzyme interaction, and an increased binding entropy. Our conceptually new approach should be adaptable to work with other peptidic protein modulators.

![Image](73x61 to 272x496)

**Fig. 1.** Effect of TEV treatment on the muPA-CPAYSRYLDC fusion protein. (a) Schematic illustration of the design of protease–peptide fusion back-flip library. Underlined residues in the sequence of muPA-CPAYSRYLDC (LD at positions 8 and 9) are varied in this study to generate a back-flip library. The peptide is expected to flip back into the active site and reduce the activity of the protease toward a chromogenic substrate. TEV cleavage sites are indicated by arrows. (b) Effect of TEV treatment on migration of muPA-CPAYSRYLDC variants in SDS-PAGE. The muPA-CPAYSRYLDC variants (4 μM) were purified and incubated with TEV protease (0.4 μM) for 120 min at room temperature and then analyzed by SDS-PAGE under reducing conditions. (c) Activity of protease–peptide fusion protein before and after treatment with TEV protease. The muPA-CPAYSRYLDC variants (4 μM) were purified and incubated with TEV protease (0.4 μM) for 120 min at room temperature. The relative amidolytic activities were then measured by incubation with 3 mM S-2444. The fold change in amidolytic activity upon TEV incubation is depicted (means and standard deviations for three independent determinations).
Also, in agreement with our hypothesis, treatment of muPA with the corresponding synthetic peptides (Table S2). The residues present in positions 8 and 9 of the peptides are indicated on the symbols.

**Results**

**Design of a protease–peptide back-flip fusion protein**

We designed a fusion protein, muPA-CPAYSRYLDC, with a covalent link between the proteolytic activity of muPA and the inhibitory activity of mupain-1. The inhibitory peptide was linked to the C-terminus of the catalytic domain of muPA (Fig. 1). Since mupain-1 is a competitive inhibitor [13] and the linker is sufficiently long, the peptide fused to the protease would be expected to be able to flip back into the active site of the protease and compete the binding of substrates. Two cleavage sites for tobacco etch virus (TEV) protease were engineered into the linker to allow controlled release of the inhibitory peptide. The length and composition of the linker between the protease domain and the peptide was designed on the basis of the three-dimensional structure of mupain-1 in complex with the H99Y mutant of human urokinase-type plasminogen activator (huPA) [23,24]. huPA-H99Y is a partly murinized version of huPA, which, in contrast to huPA wild type (wt), binds mupain-1 with a measurable $K_i$ value, although in the micromolar range [13]; we have been unable to crystallize muPA.

As expected, treatment of purified muPA-CPAYSRYLDC with TEV protease reduced its $M_i$ (Fig. 1). Also, in agreement with our hypothesis, treatment with the TEV protease reduced $K_M$ of muPA-CPAYSRYLDC for the chromogenic substrate S-2444 from 21.1 ± 1.4 mM ($n = 3$) to 5.1 ± 1.0 mM ($n = 3$) (Table S1). The latter value is identical with the $K_M$ value for the muPA catalytic domain without a fused peptide [26]. Also, muPA-CPAYSAYLDC, a fusion protein with the P1 Arg6 of the peptide mutated to Ala, had a $K_M$ of 5.1 ± 0.5 nM ($n = 3$) (Table S1).

**Screening of a protease–peptide back-flip library**

A library of muPA-mupain-1 back-flip proteins was generated in order to improve the $K_i$ of the original mupain-1, which inhibits muPA with a $K_i$ of around 500 nM [13]. We chose to randomize positions 8 and 9 of mupain-1 (CPAYSRYLDC), as our X-ray crystal structure analysis had shown that these residues are present in a type I light β-turn and that Asp9 forms a polar interaction with Arg35 of huPA-H99Y [24]. Randomizing these residues, we generated a library of $20^2 = 400$ muPA-CPAYSRYXGC variants. Protein was expressed from each plasmid individually, using human embryonic kidney 293 (HEK293) cells. Supernatants of cells transfected with plasmids of different clones were screened individually in order to identify peptides with improved $K_i$. In order to overcome the difficulty of a considerable variability of the expression level of different plasmids, we designed a screening method on the basis of the difference in velocity of S-2444 hydrolysis by fusion protein preparations before and after treatment with TEV protease. The screening method was first validated using purified fusion proteins. In this setup, the amidolytic activity of muPA-CPAYSRYLDC increased about 7-fold following TEV protease treatment (Fig. 1). As a control, we found that no increase in amidolytic activity occurred when muPA-CPAYSAYLDC was cleaved by TEV protease. muPA-CPAYSRYLDC and muPA-CPAYSAYLDC were always included as controls to reduce interassay variability and for identification of hits. When screening the library by this method, we found a clone representing the fusion protein muPA-CPAYSRYIGC to exhibit a strong induction of amidolytic activity upon treatment with TEV protease. The muPA-CPAYSRYIGC fusion protein was then purified and found to have a $K_M$ for S-2444 hydrolysis of 38.5 ± 3.0 ($n = 3$), considerably higher than the about 21 mM for muPA-CPAYSRYLDC (Table S1). The fusion proteins muPA-CPAYSRYLGC, muPA-CPAYSRYLNC, and muPA-CPAYSRYNGC were also purified and found to have $K_M$ and $K_i$ values between those of muPA-CPAYSAYLDC and muPA-CPAYSRYIGC (Table S1).

Based on the results from the muPA-mupain-1 back-flip library, synthetic peptides corresponding to six sequences were synthesized and their $K_i$ values for inhibition of the amidolytic activity of muPA were determined (Table S2). Mupain-1-IG (CPAYSRYIGC) was found to have a $K_i$ for inhibition of muPA of 20 ± 4 nM ($n = 3$), corresponding to an about 25-fold affinity improvement over the parent peptide.

The $K_M$ values for S-2444 hydrolysis were found to be correlated with the $K_i$ values for inhibition of
non-fused muPA by the corresponding free synthetic peptides in a semilogarithmic plot (Fig. 2). $k_{cat}$ for the muPA-mupain-1 fusion protein was not different from that for the non-fused protease (data not shown). Noticeably, the approximately 200-fold variation in $K_i$ values for the synthetic peptides corresponds to an only approximately 8-fold variation in the $K_M$ values for the fusion protein. The smaller variation makes the $K_M$ values unsuited for predicting accurately the $K_i$ values for the synthetic peptides. Nevertheless, the correlation depicted in Fig. 2 is a strong confirmation of the basic principle of the back-flip strategy.

**Analysis of the affinity of mupain-1-IG and variants to various serine proteases**

Mupain-1-12 and mupain-1-16 are peptides with the unnatural amino acids L-4-guanidino-phenylalanine (12) and L-3-(N-amidino-4-piperidyl)alanine (16), respectively, inserted in position 6 as the P1 residue instead of the Arg of the parent peptide. These peptides have about 2- and 10-fold higher affinity to muPA, respectively, than mupain-1 [23]. When the P1 Arg of mupain-1-IG was substituted with 12 or 16, to generate mupain-1-12-IG and mupain-1-16-IG, the $K_i$ values for muPA dropped from 20 nM to about 10 nM and to about 2 nM, respectively (Table 1).

The IG substitution of mupain-1 also increased the affinity of the peptides to huPA-H99Y and huPA wt, although the affinity in most cases remained lower than that to muPA. Among several other serine proteases tested, the affinities to human plasma kallikrein (hPK) and murine plasma kallikrein (mPK) came closest to those of muPA. mPK was inhibited 1900-fold less potently than muPA by mupain-1-IG, more than 10,000-fold less potently by mupain-1-12-IG, and 110-fold less potently by mupain-1-16-IG (Table 1). Thus, although the IG substitution also increased the affinity to some other serine proteases, mupain-1-IG, mupain-1-12-IG, and mupain-1-16-IG remained highly specific for muPA.

**Surface plasmon resonance analysis**

We performed surface plasmon resonance (SPR) analysis of the binding of the IG peptides to muPA (Table 2 and Fig. S1). The $K_D$ values determined agreed largely with the $K_i$ values. The increased affinity of the IG-substituted peptides was associated with an increased $K_{on}$ and a decreased $K_{off}$.

### Table 2. SPR analysis of the binding of peptides to muPA.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Sequence</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$) $\times 10^{-5}$</th>
<th>$k_{off}$ (s$^{-1}$) $\times 10^{3}$</th>
<th>$K_D$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mupain-1</td>
<td>CPAYSRYLDC</td>
<td>0.914 ± 0.250 (6)$^a$</td>
<td>3.64 ± 1.03 (6)$^a$</td>
<td>0.398 ± 0.046 (6)$^a$</td>
</tr>
<tr>
<td>Mupain-1-IG</td>
<td>CPAYSRYIGC</td>
<td>4.48 ± 0.76 (3)</td>
<td>0.64 ± 0.07 (3)</td>
<td>0.0147 ± 0.0018 (3)</td>
</tr>
<tr>
<td>Mupain-1-12</td>
<td>CPAYS[12]YLDCC</td>
<td>1.56 ± 0.95 (4)$^a$</td>
<td>1.92 ± 0.63 (4)$^a$</td>
<td>0.138 ± 0.034 (4)$^a$</td>
</tr>
<tr>
<td>Mupain-1-12-IG</td>
<td>CPAYS[12]YTCDC</td>
<td>18.0 ± 9.3 (3)</td>
<td>0.797 ± 0.341 (3)</td>
<td>0.00724 ± 0.00422 (3)</td>
</tr>
<tr>
<td>Mupain-1-16</td>
<td>CPAYS[16]YLDCC</td>
<td>0.898 ± 0.545 (3)$^a$</td>
<td>0.671 ± 0.119 (3)$^a$</td>
<td>0.0894 ± 0.0376 (7)$^a$</td>
</tr>
<tr>
<td>Mupain-1-16-IG</td>
<td>CPAYS[16]YTCDC</td>
<td>5.87 ± 0.76 (4)</td>
<td>0.0046 ± 0.0004 (4)</td>
<td>0.00081 ± 0.00001 (4)</td>
</tr>
</tbody>
</table>

The table shows the rate constants and the $K_D$ values for the binding of the indicated peptides to muPA at 25 °C (pH 7.4). Means, standard deviations, and numbers of determinations are indicated. Examples of sensorgrams are shown in Supplementary Fig. S1. 12 is 4-guanidino-phenylalanine; 16 is L-3-(N-amidino-4-piperidyl)alanine.

$^a$ These values were reproduced from a previous publication [23] and shown here to facilitate comparison.
Table 3. ITC for binding of peptides to muPA.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide sequence</th>
<th>N</th>
<th>$K_D$ (μM)</th>
<th>$\Delta G$ (kJ/mol)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$T\Delta S$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mupain-1</td>
<td>CPAYSRYLDC</td>
<td>0.83 ± 0.10 (3)</td>
<td>0.354 ± 0.132 (3)</td>
<td>−37.0 ± 0.9 (3)</td>
<td>−49.6 ± 6.3 (3)</td>
<td>−12.6 ± 7.2 (3)</td>
</tr>
<tr>
<td>Mupain-1 IG</td>
<td>CPAYSRYIGC</td>
<td>0.73 ± 0.08 (3)</td>
<td>0.0293 ± 0.0024 (3)</td>
<td>−43.0 ± 0.2 (3)</td>
<td>−72.4 ± 3.4 (3)</td>
<td>−30.0 ± 3.9 (3)</td>
</tr>
<tr>
<td>Mupain-1-12</td>
<td>CPAYS[12]YDC</td>
<td>1.06 ± 0.03 (3)</td>
<td>0.172 ± 0.028 (3)</td>
<td>−38.8 ± 0.6 (3)</td>
<td>−38.9 ± 0.6 (3)</td>
<td>0.0 ± 0.5 (3)</td>
</tr>
<tr>
<td>Mupain-1-12-IG</td>
<td>CPAYS[12]YIGC</td>
<td>0.56 ± 0.01 (4)</td>
<td>0.033 ± 0.009 (4)</td>
<td>−42.8 ± 0.7 (4)</td>
<td>−60.5 ± 1.9 (4)</td>
<td>−17.6 ± 1.8 (4)</td>
</tr>
<tr>
<td>Mupain-1-16</td>
<td>CPAYS[16]YDC</td>
<td>1.08 ± 0.20 (5)</td>
<td>0.122 ± 0.052 (5)</td>
<td>−38.6 ± 1.2 (5)</td>
<td>−19.8 ± 4.1 (5)</td>
<td>19.9 ± 3.4 (5)</td>
</tr>
<tr>
<td>Mupain-1-16-IG</td>
<td>CPAYS[16]YIGC</td>
<td>0.79 ± 0.07 (3)</td>
<td>0.00405 ± 0.00132 (3)</td>
<td>−48.0 ± 1.0 (3)</td>
<td>−66.8 ± 4.9 (3)</td>
<td>−19.3 ± 4.9 (3)</td>
</tr>
</tbody>
</table>

The table shows thermodynamic parameters for the binding of the indicated peptides to muPA at 25 °C (pH 7.4). Means, standard deviations, and numbers of determinations are indicated. 12 is 4-guanidino-phenyl-alanine; 16 is L-3-(N-amidino-4-piperidyl)alanine.

Fig. 3. X-ray crystal structure analysis of peptides in complex with huPA-H99Y. (a and b) Tyr7-Ile8-Gly9 conformations of mupain-1-IG (a) or mupain-1-16-IG (b) are well-defined by their electron density maps ($2F_o - F_c$ map, contoured at 1.0 σ level). (c) Overall structure of mupain-1-IG (pink sticks) or mupain-1 (cyan sticks) bound to huPA-H99Y (wheat-colored ribbons or sticks). The most relevant polar interactions between the peptides and the enzyme are indicated. Peptide residues have numbers between 1 and 10; the relevant enzyme residues have numbers between 35 and 192.

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Isothermal titration calorimetry

The thermodynamic parameters for peptide-enzyme binding were determined by isothermal titration calorimetry (ITC). The $K_v$ values determined by ITC largely agreed well with the $K_i$ values and the $K_v$ values determined by SPR. The increased affinity following the IG substitution was enthalpy driven and associated with an entropy penalty (Table 3).

The most ready molecular interpretation of the SPR and ITC data, taken together, is that the IG substitution renders the peptide more flexible in solution, thereby making the binding entropy less favorable, the association activation energy lower, and the $K_v$ higher, and it allows a more favorable binding enthalpy and a more stable bound state, thereby increasing the dissociation activation energy and decreasing the $k_{off}$.

X-ray crystal structure analysis

We determined the structures of huPA-H99Y in complex with mupain-1-IG and mupain-1-16-IG, respectively (Fig. 3) to high resolution (2.05 Å and 1.90 Å, respectively; Table S3). In agreement with our previous X-ray crystal structure analysis of peptide:huPA-H99Y complexes [24,25], we find that the amino acids in position 6 of these peptides, that is, Arg, 12, or 16, are inserted into the S1 pocket of the enzyme, while the rests of the peptides are stretched out on the surface of the enzyme, among the 99-loop, Arg217, Gln192, and the 37-loop (Fig. 3c). The mupain-1-IG:huPA-H99Y complex and mupain-1-16-IG:huPA-H99Y complex have overall conformations almost identical with those of the mupain-1:huPA-H99Y complex and the mupain-1-16:huPA-H99Y complex (Fig. 3c). The root-mean-square deviations for all main-chain atoms between the mupain-1:huPA-H99Y complex or mupain-1-16:huPA-H99Y complex on one side and the mupain-1-IG:huPA-H99Y complex or mupain-1-16-IG:huPA-H99Y complex on the other are 0.21 Å and 0.18 Å, respectively. The conformations of the IG segment (Ile8-Gly9) of the peptides were well-defined by the electron density map (Fig. 3a and b). In the huPA-H99Y-bound conformation, Ile8 of mupain-1-IG makes one hydrogen bond to Gln192 and a hydrophobic interaction with Tyr151 of huPA-H99Y. Gly9 does not have any interactions with huPA-H99Y. However, in the mupain-1:huPA-H99Y complex, Leu8-Asp9 does not only have hydrogen bonds and hydrophobic interactions similar to those in the mupain-1-IG:huPA-H99Y complex but also makes one additional hydrogen bond between Asp9 of the peptide and Arg35 of huPA-H99Y. This difference is surprising in view of the much higher affinity of the IG peptides.

We calculated the shape complementarity [27] between the enzyme and the residues 8–9 segment of the peptides (Leu8-Asp9 in mupain-1 and mupain-1-16 and Ile8-Gly9 in mupain-1-IG or mupain-1-16-IG). Ile8-Gly9 in mupain-1-IG or mupain-1-16-IG was found to have a better fit to the enzyme than Leu8-Asp9 in mupain-1 or mupain-1-16: the shape complementarity values of the IG peptides relative to the shape complementarity values of the LD peptides were 1.16 and 1.28, respectively (Table S4). We also compared the mobility ($B$-factors) of the peptides in these structures of the complexes. Mupain-1-IG or mupain-1-16-IG were found to have lower relative $B$-factors than mupain-1 and mupain-1-16 (1.09 and 1.11 versus 1.54 and 1.24, respectively; Table S5), suggesting that the IG peptides bind tighter to the enzyme compared to the original LD peptides. Thus, the Ile8-Gly9 substitution seems to facilitate the exosite interactions by allowing the peptide a better fit to the enzyme.

Ala scanning mutagenesis

To further analyze the effect of the IG substitution, we employed a number of muPA mutants with Ala substitutions of amino acids in positions previously shown to be important for binding mupain-1, that is, Lys41, Tyr99, Lys143, and Ser190. Although the structures of the peptide:muPA complexes are not likely to be exactly identical with those of the peptide:huPA-H99Y complexes [24], the four positions are distributed evenly over the peptide–enzyme interaction surface. We were particularly interested in Lys41 of muPA, which is likely to form a polar interaction with Asp9 of the peptide, similar to that formed with Arg35 of huPA-H99Y [24]. All the tested mutations lead to significant reductions in affinity of mupain-1, mupain-1-12, and mupain-1-16 to muPA (Table S6). In particular, the decreased affinity following the K41A mutation is in agreement with the existence of the polar interaction between Asp9 of the peptide and Lys41 of muPA.

In order to visualize the effects of the IG substitution on the inhibition of muPA wt and mutants, we plotted the $K_i$ values for the IG-substituted peptides ($K_i$ _IG peptides) logarithmically against the $K_i$ values for the corresponding parent LD peptides ($K_i$ _LD peptides) (Fig. 3). All data points are below the $y = x$ line, corresponding to the reduction in $K_i$ and thus an increase in affinity, following the substitution of LD with IG. The reduced affinities of the peptides to the enzyme when going from wt to mutant and when going from mupain-1-16 to mupain-1 are evidenced by a shift of the data points in an upwards, rightwards direction. The data points for muPA wt, muPA-Y99A, muPA-K143A, and muPA-S190A cluster around a common line, suggesting a common mechanism for the change in binding energy following the IG substitution for these four muPA variants. In contrast, with the K41A substitution...
mutation, the IG substitution causes a much stronger reduction in $K_i$.

Assuming that the $K_i$ values are identical with the $K_0$ values, we can calculate the $\Delta(\Delta G)$ values following the IG substitution from the deviation of the lines defined by the data points from the $y = x$ lines; they will obey the general equation

$$\Delta(\Delta G) = 2.30 \cdot R \cdot T \cdot (y_0 + (a-1) \log K_i, LD\ peptides)$$  \hspace{1cm} (1)$$

Here, $y_0$ is the $K_i, LD\ peptides$ when $K_i, LD\ peptides = 1 \mu M$; $a$ is the slope of the line defined by the plot of $\log K_i, IG\ peptides$ versus $\log K_i, LD\ peptides$; $R$ is the gas constant; and $T$ is the temperature.

The differential effect of the IG substitution on $\Delta(\Delta G)$ for muPA K41A and $\Delta(\Delta G)$ for the other muPA variants is in agreement with a notion of the IG substitution leading to changes in the relative energetic contribution from different residues. The deletion of the polar interaction between Asp9 of the peptide and Lys41 of muPA [24] will tend to decrease the affinity, but the final $\Delta(\Delta G)$ will be the resultant of the affinity-deteriorating effect of the loss of the polar interaction and the affinity-enhancing effect of the IG substitution. With the polar interaction already gone in the K41A variant, only the affinity-enhancing effect of the IG substitution will remain and the associated $\Delta(\Delta G)$ will be correspondingly larger. Thus, the use of the type of plot of Fig. 4 has in this case allowed us to distinguish between two different effects of the IG substitution: one consisting in the loss of the Lys41–Asp9 polar interaction and the other one in a less well understood effect.

Strikingly, the $\Delta(\Delta G)$ values vary quite strongly with the $K_i, LD\ peptides$ values. Thus, the increase in affinity following the IG substitution tended to increase in the order muPA-1, muPA-1-12, and muPA-1-16 and in the order muPA-Y99A, muPA-S190A, muPA-K143A, and muPA wt (Fig. 4 and Table S6). This observation may be interpreted as cross-talk between interactions in the S1 pocket and at position 41, 99, or 143 on one side and interactions of peptide positions 8 and 9 on the other side, which could possibly involve changes of the peptide solution conformation.

Fig. 4. The relationship between the $K_i$ values for inhibition of muPA by Ile8-Gly9 versions of mupain-1 and the $K_i$ value for inhibition of muPA by Leu8-Asp9 versions of mupain-1. (a) The figure is based on the $K_i$ values presented in Supplementary Table 6. The x-axes show the $K_i$ values for inhibition of muPA by Leu8-Asp9 versions of mupain-1. The y-axes show the corresponding $K_i$ values for inhibition of muPA by Ile8-Gly9 versions of mupain-1. Each data point is marked with the P1 residue, being either L-Arg, L-4-guanidinophenylalanine (12), or L-3-(N-amidino-4-piperidyl)alanine (16). The lines resulted from simple linear regression analysis. The slope of the lines are 1.30 (muPA wt, Y99A, K143A, and S190A) and 1.64 (muPA K41A). The $y_0$ values are -1.14 (muPA wt, Y99A, K143A, and S190A) and -2.44 (muPA K41A). The stippled line shows the line that would have resulted if the $K_i$ values for Leu8-Asp9 versions and the Ile8-Gly9 versions of mupain-1 had been identical ($y = x$). (b) The two lines represent the $\Delta(\Delta G)$ values for the IG substitution for wt, Y99A, K143A, and S190A together and for K41A, as indicated. As calculated from Eq. (1) in the text and the $y_0$ values and the slopes for the lines (a), the equations describing the two lines are

$$\Delta(\Delta G)_K41A = ((-14.3) + (3.80 \cdot \log K_i, LD\ peptides)) \text{kJ/mol}.$$
Similar observations were performed with huPA-H99Y and the polar interaction between Asp9 of the peptide and Arg35 of the enzyme (Fig. S2).

**Effect of peptidic inhibitors on cell-surface-associated plasminogen activation**

We also studied the ability of the mupain-1 variants to inhibit muPA-catalyzed activation of cell-surface-associated plasminogen activation in cultures of WEHI-3 cells. These cells contain a high level of uPAR, which binds muPA. uPAR-bound muPA catalyzes activation of plasminogen likewise bound to cell surfaces. The presence of α2-antiplasmin results in rapid quenching of the activity of plasmin generated in solution, whereas cell-surface-associated plasmin is protected [28]. The presence of α2-antiplasmin thus ensured that only plasmin generated by uPAR-bound uPA at the cell surface was active.

We found that the IG peptides were very efficient inhibitors of muPA-catalyzed plasminogen activation at the surface of WEHI-3 cells. Based on the fluorescence reads after 10 min, we found IC₅₀ values of 85 nM for mupain-1-IG, around 32 nM for mupain-1-12-IG, and around 7 nM for mupain-1-16-IG (Fig. S3). These values are in good agreement with expectancies from the Kᵢ determinations described above.

**Discussion**

This report presents a strategy for identification of high-affinity/high-specificity peptidic serine protease inhibitors that consist of natural amino acids. The great advantage of this method is that the enzymatic activity the protease−peptide fusion protein correlates with the Kᵢ for inhibition of the protease by the same peptide in chemically synthesized form. The affinity of the protease−peptide fusion protein for substrates can therefore be used for an approximate prediction of the Kᵢ of the synthetic peptide already in the library screening phase. In contrast, from phage-displayed peptide libraries, peptides are selected on the basis of affinity alone. The technique described here should be generally applicable for improvement of peptidic inhibitors of other serine proteases, provided that one of the termini of the protease and one of the termini of the peptide are surface exposed. By the screening procedure employed here, the number of different peptide sequences that can be handled in practice is relatively limited. We randomized only two positions and expressed protein from each plasmid individually in HEK293 cells. We used this small library to illustrate the fundamental principle of a back-flip library. However, the setup may be scaled up at high-throughput facilities for screening of libraries randomized at several positions. The principle is not limited to a mammalian protein production platform but may be directly translated to yeast systems. The fusion protein can also be expressed on cell surfaces, instead of secreted as soluble protein, and the cells can be used directly for activity-based assays and specificity assays. Moreover, the principle presented here should also find application in the study of other protein−peptide interactions or protein−protein interactions. With the two interacting components encoded by the same plasmid, the strategy also allows for randomization of residues in each of them and may thus readily be used for analysis of co-variations of interacting residues in the two components.

In order to elucidate the molecular mechanism behind the discovered affinity-enhancing effect of the IG substitution, we determined the X-ray crystal structure of mupain-1-IG and mupain-1-16-IG in complex with the easily crystalized huPA-H99Y and addressed the peptide−enzyme interaction by site-directed mutagenesis, ITC, and SPR. The ITC analysis showed that the affinity increase is enthalpy driven and associated with an entropy penalty. The entropy penalty is in agreement with expectancies from the fact that the peptide with a Gly must be expected to be able to sample a larger conformational space in solution than the parent peptide due to a larger variability of the backbone torsion angles ψ and φ being allowed for Gly than for other amino acids. The notion of a larger flexibility of the peptide in solution is also in agreement with the SPR analysis, demonstrating an increased kₘₐₜ following the IG substitution, caused by a destabilized solution state and/or a more stable transition state. The SPR analysis also revealed decreased dissociation rates following the IG substitution, a change most readily explained by a stabilization of the bound state. The site-directed mutagenesis analysis showed that the IG substitution led to changes in the relative energetic contributions from different residues and thus to changes in the interaction surface between the peptide and the enzyme. The type of plot used in Fig. 4, which we previously used in other publications [23,24], allowed to analyze the relative contributions from different interactions. Importantly, removal of the polar interaction between Lys41 of muPA and Asp9 of the peptide upon the IG substitution tended to cause a decrease in the affinity. The pure affinity-enhancing effect is represented by the effect of the IG substitution on the binding to muPA K₄1A. Although the X-ray crystal structure analysis did not reveal any major differences between the complexes of huPA-H99Y with each of the peptides mupain-1 and mupain-1-16 on one side and mupain-1-IG and mupain-1-16-IG on the other side, the notion of a changed peptide−enzyme interaction surface is in agreement with the observation that analysis of B-factors and a shape complementarity analysis indicated a better fit of the IG peptides to the enzyme.
Interestingly, we observed that the IG substitution also results in an increased affinity to the plasma kallikreins. Taken together, the results from the biochemical analyses and the X-ray crystal structure analysis suggest that effects of the IG substitution result from the fact that the peptides, when bound to the enzyme, are able to sample a number of similar conformations with subtle variations in the strength of polar and hydrophobic interactions of which only the most stable one is selected during crystallization. The flexibility afforded by the IG substitution seems to enable the peptide to adapt to different enzyme surfaces, resulting in a higher affinity, with the exact energetic contributions of different exosite interactions involved in peptide–enzyme binding varying between peptide–enzyme pairs.

The presently described effect of the IG substitution is reminiscent of our previously reported finding that a D9A substitution enhances the affinity of mupain-1 to huPA-H99Y, but the mechanism in that case must be different, as there was no effect of the D9A substitution on the affinity to muPA [24].

The high affinity and high specificity of the new IG variants of mupain-1 makes them interesting for in vivo use in animal disease models. The mupain-1 series of peptides was previously demonstrated to be stable during incubation in serum [13]. We demonstrated here that the IG variants of mupain-1 potently inhibit plasminogen activation in cell cultures. Their in vivo half-lives and the possibilities of prolonging them remain to be investigated. The IG-substituted mupain-1 variants compare very favorably with other small-molecule or peptidic inhibitors of uPA [29–33] in terms of affinity and documented specificity.

In conclusion, we have established a new concept for identification of peptidic protein modulators with high affinity and high specificity and when combined with the use of unnatural Arg analogues designed an inhibitor of the serine protease muPA with an unprecedented high affinity and high specificity. The affinity-enhancing effect of the IG substitution could not have been predicted rationally, as it implicated deletion of a peptide–enzyme polar interaction and an entropy penalty. The possibility of using the mupain-1 scaffold to achieve larger affinity by a larger flexibility contrasts with the classical concept of improving affinity by reducing the flexibility and the entropic burden.

**Experimental Procedures**

**Urokinase-type plasminogen activator**

cDNAs encoding full-length muPA and muPA-CPAYSRYLDC [muPA<sup>165–243</sup> fused at the C-terminus to two TEV cleavage sites and the sequence CPAYSRYLDC and harboring a C122A mutation (Fig. 1)] were cloned into the pTT5 or pcDNA3.1 vectors. Both variants contained six histidines at the C-terminus. The cDNAs were transfected into HEK293 6E suspension cells and cultured in a humidified 5% CO<sub>2</sub> incubator at 37 °C. The media used was F17 containing 4 mM L-glutamine, 0.1% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 25 μg/mL G418. Linear polyethyleneimine (2.2 mg), cDNA (1.1 mg), and phosphate-buffered saline [10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) and 140 mM NaCl] (110 μL) were preincubated for 15 min and added to 1 L of culture with a density of 1 x 10<sup>6</sup> cells/mL. Tryptone N1 (0.5%) was added to the culture 24 h post-transfection. Culturing was continued for 6 days before harvesting the conditioned medium. Purification from the conditioned media was performed by Ni-Sepharose and benzamidine-Sepharose chromatography, as previously described [34]. The active concentration of the enzyme was determined based on the assumption that 100% of a fraction eluted from benzamidine-Sepharose chromatography was proteolytically active.

Full-length huPA and full-length huPA-H99Y were also produced in and purified from conditioned medium of HEK293 6E suspension cells transfected with the corresponding cDNAs in pcDNA3.1. To ensure that the uPAs purified from the conditioned media were completely in the two-chain form, we treated them with plasmin for 2 h in a 1:100 ratio.

The cloning, production, and purification of recombinant huPA-H99Y catalytic domain (residues 159–411, as numbered from the N-terminus; residues 16–244 by the chymotrypsin template numbering), to be used for crystallization, were largely as described previously [35]. Basically, the recombinant catalytic domain of huPA-H99Y was secreted from a stable *Pichia pastoris* strain (X-33) after induction by methanol and captured by a cation-exchange column. The protein was further purified on a Superdex 75 HR 10/30 column (GE Healthcare) equilibrated with 20 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) and 150 mM NaCl. The protein was eluted as a single peak. The recombinant uPA catalytic domain expressed in this way is an active protease with an activity comparable to full-length two-chain uPA [35]. The protein was dialyzed in 20 mM K<sub>2</sub>HPO<sub>4</sub>/KOH<sub>2</sub>PO<sub>4</sub> (pH 6.5) overnight and concentrated to 10 mg/mL, using stirred ultrafiltration cells (Millipore and Amicon Bioseparations Model-5124), prior to protein crystallization. The recombinant catalytic domain of huPA-H99Y to be used for ITC assays was further purified with benzamidine-Sepharose affinity chromatography.

**Other proteases**

Human Glu plasminogen and human plasmin were from American Diagnostica. Human thrombin was
gift from John Fenton, New York State Department of Health, Albany, NY, USA. Human tPA (Genentech) was converted into 100% active two-chain form with immobilized plasmin [36]. Bovine β-trypsin (Roche Applied Sciences) was further purified by tosyl phenylalanyl chloromethyl ketone treatment of the commercial preparation and chromatography on soybean trypsin inhibitor Sepharose [37]. Murine thrombin, murine tPA, hPK, mPK, and murine plasmin were purchased from Molecular Innovation, Michigan, USA. TEV protease was produced recombinantly in Escherichia coli, essentially as described by Kapust and Waugh [38].

Peptides

All peptides used in the present study were either obtained commercially from the WuXi AppTec Inc., Shanghai, China, or synthesized as previously described [23]. The concentrations of the peptide variants were determined by measurements of absorbance at 280 nm and [38].

Construction of a back-flip library

Libraries were constructed from pTT5 containing the muPA-CPAYSRYLDC encoding insert. Oligonucleotides overlapping the nucleotides corresponding to the peptide with randomization of the nucleotides encoding amino acid positions 8 and 9 of the peptide were designed. These oligonucleotides were used in a classical QuikChange mutagenesis strategy to generate a bacterial library. Plasmid DNA was purified from 400 clones and used to transiently transfect HEK293 6E suspension cells. Conditioned media from these transfections were used as the enzyme source for initial screening assays.

$K_i$ determinations

For routine determination of $K_i$ values for the inhibition of the various uPA variants under steady-state inhibition conditions, a fixed concentration of purified uPA or uPA-containing conditioned media from transfected cells (2 nM uPA as the final concentration) was preincubated in a 200-μL Heps-buffered saline [HBS; 10 mM Heps (pH 7.4) and 140 mM NaCl] with 0.1% bovine serum albumin (BSA) at 37 °C, with various concentrations of mupain-1 variant peptides (0–400 μM) for 15 min prior to the addition of the chromogenic substrate S-2444 in concentrations approximately equal to the $K_M$ value for each particular variant. The initial reaction velocities were monitored at an absorbance of 405 nm. The inhibition constants ($K_i$) were subsequently determined from the non-linear regression analyses of plots of $V/V_0$ versus $[I]_0$ using Eq. (2), derived under assumption of competitive inhibition:

$$
\frac{V_i}{V_0} = \frac{K_i \cdot (K_M + [I]_0)}{(K_i - [S]_0) + (K_M \cdot (K_i + [I]_0))}
$$

where $V_i$ and $V_0$ are the reaction velocities in the presence and absence of inhibitor, respectively; $[S]_0$ and $[I]_0$ are the substrate and inhibitor concentrations, respectively; and $K_M$ is $K_M$ for the uPA-catalyzed hydrolysis of S-2444. In Eq. (2), it is assumed that $[S]_{free} = [S]_0$ and $[I]_{free} = [I]_0$. These conditions were fulfilled, as less than 10% of the substrate was converted into product in the assays and as the assay typically contained a final concentration of uPA variant of 2 nM and inhibitor concentrations in the micromolar range.

The validity of performing the $K_i$ determinations with uPA-containing conditioned medium from transfected cells was verified by controls in which the determinations were performed with conditioned medium and with purified preparations. These controls were performed with muPA wt, huPA wt, and huPA-H99Y, obtaining indistinguishable values with the two types of samples [23].

For routine determination of $K_i$ values for the inhibition of proteases other than uPA under equilibrium inhibition conditions, a fixed concentration of the protease was preincubated in HBS with 0.1% BSA at 37 °C at pH 7.4, unless otherwise stated, with various concentrations of mupain-1 variant (0–50 μM), for 15 min prior to the addition of the appropriate chromogenic substrate. The following protease–substrate combinations were used: human tPA (2.0 nM) and S-2288 (300 μM); human plasmin (2.0 nM) and S-2403 (125 μM); murine plasmin (2.0 nM) and S-2366 (200 μM); human thrombin (0.5 nM) and S-2238 (50 μM); murine thrombin (0.5 nM) and S-2238 (100 μM); bovine β-trypsin (2.0 nM) and S-2222 (50 μM); hPK (4.0 nM) and S-2302 (300 μM); and mPK (4.0 nM) and S-2302 (125 μM). The initial velocities were monitored as changes in the absorbance at 405 nm. As mentioned above, the $K_M$ values for these proteases were determined according to standard Michaelis–Menten kinetics [13]. The $K_i$ values were subsequently determined from the non-linear regression analyses of plots for $V/V_0$ against $[I]_0$, according to Eq. (2).

In cases, in which we observed no measurable inhibition (i.e., <10%) at 400 μM, the maximal inhibitor concentration used, the accuracy of the assay allowed us to conclude that the $K_i$ value was more than 1000 μM (indicated as “<1000 μM” in the tables).

**SPR analysis**

To determine the kinetics of peptide binding to muPA, we performed SPR analysis on a BIACORE
The catalytic domain of huPA-H99Y were obtained by equilibrating huPA-H99Y protein against a reservoir solution containing 2.0 M ammonium sulfate, 50 mM sodium citrate (pH 4.6), and 5% polyethylene glycol (PEG) 400 at room temperature. The crystals appeared in about 3 days. The crystals of huPA-H99Y were then soaked for 2 weeks in new soaking buffer [40% PEG 3350 and 0.1 M Tris–HCl (pH 7.4)], containing 1 mM mupain-1-IG or mupain-1-16-IG. A solution of 20% PEG 3350, 0.1 M Tris–HCl (pH 7.4), and 20% (v/v) glycerol was used as cryoprotectant for obtaining X-ray diffraction data of the crystals at the BL17U beamline, Shanghai Synchrotron Radiation Facility. The diffraction data were indexed and integrated using the HKL2000 program package [39].

The crystal structures of the different complexes were solved by molecular replacement [40], using the huPA structure (PDB code: 2NWN) [35] as the search model. The electron density for the peptide was clearly visible in the uPA active sites and was modeled based on the F₀ – Fc difference map. The structures were refined by CCP4 program package [40] and manually adjusted by the molecular graphics program Coot [41] iteratively until convergence of the refinement. Solvent molecules were added using a F₀ – Fc Fourier difference map at 2.5 σ in the final refinement step. The final structure was analyzed by software PyMOL [42]. We used the CCP4 suite to calculate the value of shape complementarity between the different peptides and huPA-H99Y, respectively [40].

Cell-surface-associated plasminogen activation

Aliquots of WEHI-3 cell suspension (1.5 × 10⁷ cells/mL) were incubated at 37 °C in HBS with 0.1% BSA in the presence of muPA (2.5 nM), plasminogen (100 nM), α2-antiplasmin (200 nM), the fluorogenic plasmin substrate D-Val-Leu-Lys-7-amino-4-methylcoumarin (200 μM), and the indicated concentrations of the indicated mupain-1 variants. The fluorescence of the wells was monitored at 1-min intervals in a fluorescence plate reader, using an excitation wavelength of 390 nm and an emission wavelength of 480 nm. The relative activity in the presence of variable mupain-1 variant concentrations was calculated from the fluorescence observed after 10 min of incubation time. Cells without muPA served as a negative control.

Accession codes

The structures of the mupain-IG:huPA-H99Y and the mupain-16-IG:huPA-H99Y complexes have been deposited in the Protein Data Bank as entries 4ZHL and 4ZHM, respectively.
Acknowledgments

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Author Contributions: H.P.S., M.H., and P.A.A. designed the experiments. H.P.S., P.X., and T.K.H. performed the site-directed mutagenesis and the biochemical analysis. L.J. performed the X-ray crystal structure analysis. K.J.J. contributed with peptide synthesis. H.P.S. and P.A.A. wrote the manuscript.

Appendix A. Supplementary data

Supplementary data about the crystal structures, \(K_d\) determination, SPR analysis, and cell-associated plasminogen activation are provided. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmb.2015.08.005.

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Keywords:
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conformation

\[^{\ddagger}\]http://dg.dk.
\[^{¶}\]http://www.carlsbergfoundation.dk.

Abbreviations used:
BSA, bovine serum albumin; HBS, Hepes-buffered saline; HEK293, human embryonic kidney 293; hPK, human plasma kallikrein; huPA, human urokinase-type plasminogen activator; ITC, isothermal titration calorimetry; mPK, murine plasma kallikrein; muPA, murine urokinase-type plasminogen activator; PEG, polyethylene glycol; SPR, surface plasmon resonance; TEV, tobacco etch virus; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; wt, wild type.

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High-Affinity Peptidic Serine Protease Inhibitors

APPENDIX 3

Manuscript III

Interconversion of Active and Inactive Conformations of Urokinase-type Plasminogen Activator

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Interconversion of Active and Inactive Conformations of Urokinase-Type Plasminogen Activator

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ABSTRACT: The catalytic activity of serine proteases depends on a salt-bridge between the amino group of residue 16 and the side chain of Asp194. The salt-bridge stabilizes the oxyanion hole and the S1 specificity pocket of the protease. Some serine proteases exist in only partially active forms, in which the amino group of residue 16 is exposed to the solvent. Such a partially active state is assumed by the truncated form of the murine urokinase-type plasminogen activator (muPA), consisting of residues 16–243. Here we investigated the allosteric interconversion between partially active states and the fully active state. Both a monoclonal antibody (mU3) and a peptidic inhibitor (mupain-1–16) stabilize the active state. The epitope of mU3 is located in the 37- and 70-loops at a site homologous to exosite I of thrombin. The N-terminus(Ile16) of muPA(16–243) was less exposed upon binding of mU3 or mupain-1–16. In contrast, introduction of the mutations F40Y or E137A into muPA(16–243) increased exposure of the N-terminus(Ile16) and resulted in large changes in the thermodynamic parameters for mupain-1–16 binding. We conclude that the distorted state of muPA(16–243) is conformationally ordered upon binding of ligands to the active site and upon binding of muU3 to the 37- and 70-loops. Our study establishes the 37- and 70-loops as a unique site for binding to compounds stabilizing the active state of serine proteases.

Trypsin-like serine proteases are synthesized inside the cell, secreted as inactive zymogens, and subsequently converted into active enzymes by proteolysis between residue 15 and 16.1 The inactivity of serine protease zymogens results from improper formation of the S1 specificity pocket and the oxyanion hole.2 The zymogen-activation domain, generally composed of residues 142–153, 184–194, and 215–225, shows an altered structure or conformational flexibility in crystal structures of zymogens relative to structures of active proteases. Proteolytic cleavage following residue 15 triggers the nascent N-terminus at position 16 to form a salt-bridge to Asp194, located at the bottom of the activation pocket. This salt-bridge stabilizes the oxyanion hole and the S1 specificity pocket. The activated catalytic domain formed by this transition exhibits a conformation, which changes depending on the binding of substrates and inhibitors to the active site or cofactors to exosites.3 Thrombin, Factor VIIa, and complement factor D represent classical examples of serine proteases, which in the absence of substrates, inhibitors, or cofactors exhibit a resting low activity conformation, which seems to be more flexible than the active conformation.4–7 Ligand-free thrombin rests in a catalytically inefficient state but assumes the fully active conformation upon binding of ligands to exosite I or inhibitors and substrates to the active site. The resting state of thrombin, also called slow-thrombin or E4, seems to feature a more exposed N-terminus(Ile16) than ligand-bound thrombin.8 Another serine protease Factor VIIa is several orders of magnitude less active in the absence of its cofactor, tissue factor.7 Tissue factor converts ligand-free Factor VIIa into its active conformation by an allosteric transition, which can be measured directly by chemical carbamylation of the exposed α-amino group of Ile16 at the N-terminus. Reactivity of the N-terminus(Ile16) to carbamylation is determined by the distribution between a state with the N-terminus(Ile16) inserted into the activation pocket and a state with an exposed N-terminus(Ile16). The presence of trypsin-like serine proteases in resting states in the absence of bound ligands has been corroborated by crystal structures of free enzymes, exhibiting a partial collapse of the 215–217 β-strand into the active site and a disruption of the oxyanion-hole.9 It was recently suggested that the distorted conformations observed in mutants of thrombin are distributed along a continuum and that ligand binding rectifies these molecules to the active conformation, as evidenced by large thermodynamic differences in the binding reactions.10 However, a relationship between the extent of these thermodynamic

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differences and the exposure of the N-terminus\(^\text{(Bk16)}\) has not been demonstrated.

Activation of plasminogen into plasmin, which degrades the extracellular matrix and facilitates dissemination of cancer cells, is catalyzed by urokinase-type plasminogen activator (uPA).\(^1\) Its role in cancer spread has implicated uPA as a therapeutic target. From this point of view, improved understanding of the regulatory mechanisms of allosteric serine proteases is important in the development of new strategies for therapeutic intervention. The catalytic domain represented by the B-chain of uPA is covalently linked to the A-chain by the Cys1-Cys122 disulfide-bond. Cys1 is located C-terminally to the intradomain linker, the epidermal growth factor-like domain and the kringle domain. Specifically, the epidermal growth factor-like domain is responsible for association of uPA to the cell surface by direct noncovalent interaction with the uPA receptor, uPAR.\(^1\) Active uPA associates with a serpin, known as plasminogen activator inhibitor-1 (PAI-1), and forms an inhibited covalent complex.\(^1\)

The monoclonal antibody, mU3 was previously shown to inhibit plasminogen activation catalyzed by murine uPA (muPA).\(^14\) Here we report that mU3 binds to an exosite of muPA in the 37- and 70-loops, a site homologous to exosite I of thrombin. We have addressed how the exosite in the 37- and 70-loops is coupled to events in the active site, the overall conformation of the enzyme, and exposure of the N-terminus\(^\text{(Bk16)}\). Binding of mU3 to the 37- and 70-loops and inhibitors to the active site promoted the conversion from partially inactive distorted states to the active state. Using the active site binding peptidic inhibitor mupain-1\(^\text{16,15}\), we establish for the first time a relationship between the large thermodynamic differences of ligand binding reactions and the increased exposure of the N-terminus\(^\text{(Bk16)}\) in distorted relative to less distorted states of serine proteases.

**Materials and Methods**

**Monoclonal Antibodies and Peptides.** The generation and characterization of mU3 were described previously.\(^1\) The peptide mupain-1\(^-16\) (CPAYS(L-3-(N-amidino-4-piperidyl)-alanine)YLDCl) was prepared by solid-phase synthesis.\(^15\)

**Transfection of HEK293 6E Suspension Cells.** Different cDNAs encoding full-length muPA, muPA\(^\text{16-243}\), and site-directed mutants were cloned into the pTT5 or pCDNA3.1 vectors. All variants contained six histidines at the C-terminus. The muPA\(^\text{16-243}\) variants contained a C122A mutation. The cDNAs were transfected into human embryonic kidney 293 (HEK293) 6E suspension cells and cultured in a humidified 5% CO\(_2\) incubator at 37 °C. The media used was F17 containing 4 mM l-glutamine, 0.1% FP68, 100 units/mL penicillin, 100 units/mL streptomycin, 25 μg/mL G418. Linear polyethylene-imine (PEI) (2.2 mg), cDNA (1.1 mg), and PBS (110 mL) were preincubated for 15 min and added to 1 L of culture with 5% FCS (25 mM L-glutamine, 0.1% FP68, 100 units/mL penicillin, 100 μg/mL streptomycin, 25 μg/mL G418). 2.2 mg of PEI, 1.1 mg of cDNA, and 110 mL of PBS were therefore transfection-attached to the pTT5 or pCDNA3.1 vectors. All variants contained six histidines at the C-terminus. The muPA\(^\text{16-243}\) variants contained a C122A mutation. The cDNAs were transfected into human embryonic kidney 293 (HEK293) 6E suspension cells and cultured in a humidified 5% CO\(_2\) incubator at 37 °C. The media used was F17 containing 4 mM l-glutamine, 0.1% FP68, 100 units/mL penicillin, 100 units/mL streptomycin, 25 μg/mL G418. Linear polyethylene-imine (PEI) (2.2 mg), cDNA (1.1 mg), and PBS (110 mL) were preincubated for 15 min and added to 1 L of culture with a density of 1 × 10\(^8\) cells/mL. Tryptore N1 (0.5%) was added to the culture 24 h post-transfection. Culturing was continued for six days before harvesting the conditioned media.

**Protein Purification.** Full-length tc-muPA and variants were purified from conditioned media of transiently transfected HEK293 6E cells. Briefly, C-terminally 6xhistidine-tagged proteins were captured on nickel-Sepharose, eluted with imidazole, and purified further using Superdex 75 size-exclusion chromatography.

**Plasminogen Activation Assays.** Various concentrations of mU3 (0–25 nM) were incubated with full-length tc-muPA, muPA\(^\text{16-243}\), or mutants hereof (0.5 nM) and preincubated for 30 min at 25 °C in HBS (10 mM Hepes pH 7.4, 140 mM NaCl, 0.1% BSA). Next, the reaction was initiated by addition of 0.5 μM human plasminogen and 0.5 mM plasmin substrate H-Val-Leu-Lys-p-nitroanilide (S-2251). S-2251 hydrolysis was monitored at 37 °C for the parabolic increase in absorbance at 405 nm. To determine the velocity of plasminogen activation, the data were transformed to plot Δ405/Δtime on the ordinate and time on the abscissa.\(^16\) Velocities were calculated from the time interval 5–20 min of these plots and used for calculations of IC\(_{50}\).

**Amidolytic Assay of muPA Proteolytic Activity.** The velocity of muPA catalyzed hydrolysis of H-0-Glu-Gly-Arg-p-nitroanilide (S-2444) was measured at 37 °C in HBS. K\(_{m}\) and K\(_{c}\) were calculated as described previously.\(^2\)

**Surface Plasmon Resonance Measurements.** To determine the equilibrium binding constant (K\(_{d}\)), the association rate constant (k\(_{on}\)) and dissociation rate constant (k\(_{off}\)) for mU3 binding to muPA and variants, surface plasmon resonance analyses were performed on a Biacore T200 instrument (Biacore, Uppsalas, Sweden). A CMS chip was coupled with polyclonal rabbit anti-mouse IgG from the Mouse Antibody Capture kit from GE-Healthcare through amine coupling: A concentration of 30 μg/mL anti-mouse IgG in immobilization buffer (10 mM sodium acetate, pH 5.0) was injected during 7 min at a flow rate of 10 μL/min until a level of 14,000 response units (RU) was reached, followed by surface blocking with ethanolamine. The monoclonal antibody (mU3) in running buffer (30 mM Hepes pH 7.4, 135 mM NaCl, 1 mM EDTA) + 1% BSA was injected at a flow rate of 5 μL/min for 180 s until a capture level of ~1100 RU was reached. A flow cell without injection of mU3 was used as the reference. A dilution series of muPA variants (0–100 nM) in running buffer + 1% BSA was injected at a flow rate of 30 μL/min during 60 s at 25 °C. Subsequently, the dissociation was monitored for 220 s. Binding of mupain-1–16 to muPA variants was measured by immobilization of 20,000 RU of an anti-histidine tag antibody onto a CMS chip as described above and used to capture 3000 RU of muPA or variants. Next, mupain-1–16 (0–1000 nM) was injected in running buffer containing 0.1% BSA at a flow rate of 30 μL/min during 60 s at 25 °C. A flow cell without binding of muPA was used as reference and the response without mupain-1–16 was subtracted from each curve. Kinetic constants (k\(_{on}\) and k\(_{off}\)) were calculated using the Bicore Evaluation Software to generate a 1:1 kinetic fit. Although some data sets could be fitted better to a two-state model, the calculated data were similar when using a 1:1 model. All data were therefore fitted to a 1:1 model. The K\(_{D}\) values were calculated as k\(_{off}\)/k\(_{on}\).

**Carbamylation Assay.** To analyze the effect of mU3 and mupain-1–16 on the carbamylation rate of the N-terminal\(^\text{(Bk16)}\) α-amino group in different muPA variants, 0.5 μM enzyme was preincubated with or without mU3 (1 μM) or mupain-1–16 (1 μM) in HS buffer (10 mM Hepes pH 7.4, 140 mM NaCl) + 0.1% PEG 8000 at 22 °C for 30 min. After preincubation, potassium cyanate (0.2 M) or HS buffer +0.1% PEG 8000 was added and incubated at 22 °C for 0, 30, 60, 120, 180, and 300 min. To stop the carbamylation reaction, each of the mixtures was diluted 100-fold in HBS, and mU3 or mupain-1–16 was allowed to dissociate for 2 h at 22 °C. The residual activity was determined in the presence of S-2444 (750 μM) by measuring the rate of hydrolysis at 405 nm for 1 h at 37 °C in a microplate reader.
Isothermal Titration Calorimetry. All isothermal titration calorimetry (ITC) experiments were performed on a MicroCal VP-ITC instrument equilibrated to a temperature of 25 °C. The muPA variants were dialyzed into a buffer of 20 mM sodium phosphate, pH 7.4, 140 mM NaCl. The peptide mupain-1–16 was dissolved in the same buffer and the concentration was determined using the extinction coefficient calculated using ProtParam located at http://us.expasy.org/tools/protparam.html. In all cases, experiments were designed to provide a fully saturated titration profile with enough signal and curvature to allow precise determination of thermodynamic parameters. The concentration of muPA variant used in the ~1.4 mL sample cell was 0.5–2 µM, depending on the affinity of the ligand. Titration curves were fitted using ORIGIN7 program package. The following formulas for Gibbs energy ΔG were used to analyze the measured energies.

\[ \Delta G = -RT \ln K_A \]  
(1)

\[ \Delta G = \Delta H - T \Delta S \]  
(2)

in which \( R \) is the gas constant and \( T \) is the absolute temperature. \( \Delta S \), the entropic change during the reaction, was calculated by combining eqs 1 and 2 using the measured \( K_A \) and \( \Delta H \). Titration of ligand into buffer was performed to obtain buffer correction.

Determination of Second Order Rate Constants for Binding of muPA to PAI-1. Full-length tc-muPA or muPA(16–243) (0.25 nM) were mixed with 100 nM mU3 or HBS and incubated for 30 min at 25 °C. At time point \( t = 0 \) min, the reaction was started by mixing a diluted series of murine PAI-1 (0–500 nM). The absorbance at 405 nm (\( A_{405} \)) was recorded for 120 min in the presence of 3 mM or 6 mM S-2444 for full-length tc-muPA or muPA(16–243), respectively. The \( k_{obs} \) values for each PAI-1 concentration were determined by fitting the progress curves to the single-exponential function (eq 3).

\[ [P]_t = [P]_0 + [P]_0 e^{-k_{obs} t} \]  
(3)

where \([P]_0\), \([P]_0\), and \([P]_0\) are the product concentrations at time \( t \), time zero, and time infinite, respectively, proportional to the measured \( A_{405} \) values. The calculated \( k_{obs} \) values were then plotted on the ordinate and the PAI-1 concentrations on the abscissa and the \( k_D \) and \( k_{lim} \) values were determined by fitting the data to eq 4.

\[ k_{obs} = k_{lim}[PAI-1]_0 / \left( K_D \left( 1 + \frac{[S-2444]}{K_m} \right) + [PAI-1]_0 \right) \]  
(4)

The second-order rate constant \( k_2 \) was calculated as \( k_{lim}/K_D \).

RESULTS

The Epitope of mU3 Is Located in the 37- and 70-Loops. A total of 29 site-directed alanine substitution mutants of full-length tc-muPA were prepared to identify the epitope of the monoclonal antibody, mU3. The plasminogen activation activities of the full-length tc-muPA mutants G37cA, P37eA, P38A, S74A, Y76A, and N77A were not inhibited by mU3 (data not shown). Contrary, mU3 inhibited the plasminogen activation activities of full-length tc-muPA E23A, Q35A, N37A, K37aA, G37bA, S37dA, K41A, Q60aA, K72A, E73A, S75A, P78A, Y93A, E96A, Y99A, T110A, S110Aa, K143A, E146A, Y149A, L150A, K153A, and R217A (data not shown). The inhibition of these 23 mutants by mU3 was indistinguishable from the inhibition of full-length tc-muPA, suggesting the epitope of mU3 to consist of amino acid residues in the 37- and 70-loops. To further validate the plasminogen activation data, we used surface plasmon resonance analysis to quantitate mU3 binding to variants mutated within and around the epitope. Binding to mU3 was diminished for the mutants of full-length tc-muPA, G37cA, P37eA, P38A, S74A, Y76A, and N77A (Table 1 and Figure 1). Hence, the plasminogen activation activities of these six mutants were not inhibited by mU3 as they did not bind the antibody at the concentrations used in the assay. None of the mutations completely abolished the binding. The most compromised mutant was G37cA (Figure 1B and Table 1). The decreased binding of mU3 to the mutants consistently resulted from an increase in \( K_{cat} \) (Table 1). Full-length tc-muPA N37A, K37aA, G37bA, S37dA, K72A, P78A, Y149A, and K153A were shown to bind to mU3 with similar affinity as full-length tc-muPA (Figure 1C). Hence, mU3 binds to an epitope in the 37- and 70-loops (Figure 1C). Interestingly, binding of mU3 to the 70-loop seems to depend on a certain conformation rather than side-chain interactions, as only mutation of Gly and Pro residues affected binding to the 37-loop.

The Antibody mU3 Decreases \( K_m \) for S-2444 Hydrolysis. The truncated mutant, muPA(16–243) consists of the B-chain of murine uPA. As demonstrated below, muPA(16–243) exists in a distorted state, as its N-terminus is more exposed than in full-length tc-muPA. In all our experiments, mU3 restored the active state of muPA(16–243) (see below). The amidoLytic activity of full-length tc-muPA was not affected by mU3, while the amidoLytic activity of muPA(16–243) was dose-dependently increased up to maximally 2-fold (Figure 2A). Furthermore, mU3 improved the affinity of the substrate S-2444 for the active site, as \( K_m \) for muPA(16–243) was decreased from 5 ± 0.7 mM in the absence of mU3 to the level of full-length tc-muPA at 2.4 ± 0.2 mM in the presence of mU3 (Figure 2B and Table 2). Moreover, \( k_{cat} \) was not affected by mU3, but remained constant at approximately 90 s\(^{-1}\) for both full-length tc-muPA and muPA(16–243) (Figure 2B). Notably, mU3 dose-dependently inhibited plasminogen activity.
activation by both full-length tc-muPA and muPA(16–243) with an IC₅₀ of approximately 0.3 nM (Figure 2C). These data indicate that mU₃ restores the affinity of the active site of muPA(16–243) for S-2444 and sterically inhibits plasminogen activation.

**A Peptidic Substrate and Active Site Inhibitors have Lower Affinity for Distorted muPA Variants than for Full-Length tc-muPA.**

Kₘ for hydrolysis of S-2444 was 2-fold higher for muPA(16–243) than for full-length tc-muPA (Table 2). Mutants with substitutions located close to the epitope of mU₃ and the oxyanion hole were analyzed to identify more distorted variants. The mutants muPA(16–243) F40Y and muPA(16–243) E137A were selected for analysis, as their Kₘ values for S-2444 hydrolysis were higher than for muPA(16–243) (Table 2). Hence, it was hypothesized that mutations away from the active site that increased Kₘ for S-2444 hydrolysis would also induce conformational distortion and as demonstrated below, exposure of the N-terminus(Ile16). Inhibition of muPA(16–243) F40Y and muPA(16–243) E137A with the active site antagonists, p-aminobenzamidine (pAB), amiloride and mupain-1–16 showed Kᵢ values for S-2444 hydrolysis were higher than for muPA(16–243) (Table 2). Hence, it was hypothesized that mutations away from the active site that increased Kᵢ for S-2444 hydrolysis would also induce conformational distortion and as demonstrated below, exposure of the N-terminus(Ile16).

**The Affinity of Distorted Mutants for Inhibitors Is Restored by mU₃.** Because mU₃ improved the binding of S-2444 to the active site of muPA(16–243), we now continued to measure whether this effect occurred for active site inhibitors. All three inhibitors tested, pAB, amiloride and mupain-1–16, had a 2–3 fold lower Kᵢ for muPA(16–243) in the presence of mU₃ than in its absence, corresponding to the values for the full-length tc-muPA (Table 2). Similar effects occurred for the F40Y and E137A mutants of muPA(16–243). Based on these data, we conclude that mU₃ restores the active state of the distorted muPA mutants.

**Binding of PAI-1 to muPA(16–243) Is Slower than to Full-Length tc-muPA and mU₃ Reduces the Rate of Binding.** We measured the second-order rate constants for binding of murine PAI-1 to full-length tc-muPA and muPA(16–243) (Figure 3). First, the second-order rate constant was 2–3-fold slower for muPA(16–243) than for full-length tc-muPA. The reason for this is probably that muPA(16–243) is distorted. Second, mU₃ decreased the second-order rate constant by 8-fold for full-length tc-muPA and 5-fold for muPA(16–243). From these data we deduce that mU₃ delays the binding of PAI-1 to muPA, probably by steric hindrance.

**mU₃ and mupain-1–16 Mediate Insertion of the N-Terminus(Ile16) into the Activation Pocket.** We now measured the rates of inactivation of full-length tc-muPA and muPA(16–243) during modification of the exposed α-amino group of Ile16 by chemical carbamylation. The rate of inactivation by carbamylation is proportional to the fraction of the time the N-terminus(Ile16) is solvent exposed versus the time in which it is salt-bridged to Asp194. The rate of
carbamoylation was found to be faster for muPA(16–243) than for full-length tc-muPA (Table 3 and Figure 4). Hence, the N-terminus(Ile16) of muPA(16–243) is more exposed than in full-length tc-muPA. Next, it was demonstrated that mU3 decreased the carbamoylation rate for muPA(16–243) to the rate measured for full-length tc-muPA (Table 3). Similarly, insertion of the N-terminus(Ile16) was promoted by mupain-1(16–243) (0.5 nM) using S-2251 (0.5 mM) and mU3 at the indicated concentrations of mU3. (C) Activation of human plasminogen (0.5 μM) measured for full-length tc-muPA and muPA(16–243) (0.5 nM) using S-2251 (0.5 mM) and mU3 at the indicated concentrations. All measurements are the average ± standard deviations of at least three independent determinations.

Table 2. $K_m$ for S-2444 Hydrolysis and $K_i$ for Inhibition by Amiloride, pAB and mupain-1–16 for muPA Variants

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM) S-2444</th>
<th>$K_i$ (μM) amiloride</th>
<th>$K_i$ (μM) pAB</th>
<th>$K_i$ (nM) mupain-1–16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$-$mU3</td>
<td>+mU3</td>
<td>$-$mU3</td>
<td>+mU3</td>
</tr>
<tr>
<td>full-length tc-muPA</td>
<td>2.4 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>6 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>muPA(16–243)</td>
<td>5.0 ± 0.7b</td>
<td>2.7 ± 0.4</td>
<td>16 ± 1b</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>full-length tc-muPA F40Y</td>
<td>4.5 ± 0.2b</td>
<td>2.4 ± 0.3</td>
<td>14 ± 3b</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>muPA(16–243) F40Y</td>
<td>11.6 ± 0.9b</td>
<td>4.4 ± 0.4b</td>
<td>53 ± 12b</td>
<td>22 ± 7b</td>
</tr>
<tr>
<td>muPA(16–243) E137A</td>
<td>8.0 ± 0.7b</td>
<td>6.9 ± 0.1b</td>
<td>74 ± 12b</td>
<td>19 ± 2b</td>
</tr>
</tbody>
</table>

All data are the average ± standard deviations of at least three independent determinations. A saturating concentration of mU3 (25 nM) was used.

Significantly different from the corresponding value determined for full-length tc-muPA (p < 0.004) — Student’s t test.

Figure 3. Binding of murine PAI-1 to muPA and muPA(16–243) is delayed by mU3. The second-order rate constants for reaction with murine PAI-1 were: full-length tc-muPA; (1.75 ± 0.48)·10⁶ M⁻¹·s⁻¹, full-length tc-muPA+mU3; (2.93 ± 1.41)·10⁵ M⁻¹·s⁻¹, muPA(16–243); (6.85 ± 2.28)·10⁵ M⁻¹·s⁻¹, muPA(16–243)+mU3; (1.29 ± 0.47)·10⁵ M⁻¹·s⁻¹. All measurements are the average ± standard deviations of at least three independent determinations.

Table 3. Rates (min⁻¹) of Inactivation by Chemical Carbamylation

<table>
<thead>
<tr>
<th></th>
<th>ligand-free</th>
<th>+mU3</th>
<th>+mupain-1–16</th>
</tr>
</thead>
<tbody>
<tr>
<td>full-length tc-muPA</td>
<td>1.5 ± 0.7</td>
<td>1.0 ± 0.7</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>muPA(16–243)</td>
<td>9.6 ± 1.0b</td>
<td>1.2 ± 0.7</td>
<td>0.5 ± 0.4</td>
</tr>
</tbody>
</table>

Data are the average ± standard deviations of three independent determinations. Significantly different from the value determined for full-length tc-muPA (p < 0.0003) — Student’s t test.
conformational distortion in different muPA mutants we used isothermal titration calorimetry (ITC) and quantitated the ordering occurring in a distorted mutant when its active state is restored through binding of mupain-1–16. Hence, a more negative ΔH reflects formation and disruption of more noncovalent bonds and a more negative ΔTΔS parameter reflects more ordering upon binding of mupain-1–16. The ΔH for binding of mupain-1–16 is more negative for muPA(16–243) than for full-length tc-muPA, indicating the formation of more noncovalent interactions on a net basis (Table 4 and Figure S1). Because of a compensatory more negative ΔTΔS parameter, the ΔG for binding of mupain-1–16 was comparable for full-length tc-muPA and muPA(16–243). The ΔTΔS values determined by ITC did not deviate measurably from the Kf values determined by surface plasmon resonance analysis (Table 4 and Figure S1). The negative ΔTΔS for binding of mupain-1–16 to muPA(16–243) suggests that this variant is flexible but becomes more ordered upon binding of mupain-1–16. Interestingly, we observed that ΔH and ΔTΔS for binding to mupain-1–16 were alike for full-length tc-muPA and muPA(16–243) when they were in complex with mU3 (Table 4). Based on this, it appears that mU3 restores the active state of muPA(16–243), which seems to resemble the state assumed by full-length tc-muPA. The TΔS value for binding to mupain-1–16 was more negative for muPA(16–243) F40Y and muPA(16–243) E137A than for muPA(16–243), indicating that these two mutants are in even more distorted states but become ordered upon binding to the peptide. For muPA(16–243) F40Y, the koff for binding to mupain-1–16 was similar to muPA(16–243), whereas the koff rate was significantly increased. For the muPA(16–243) E137A mutant, the koff for binding to mupain-1–16 was lower than for muPA(16–243), whereas the koff rates were comparable. The large differences in the thermodynamic parameters (ΔH and ΔTΔS) for binding to mupain-1–16 among the mutants were not a result of changes in the ΔG for which the variation among the tested mutants was about 3% (Table 4).

### DISCUSSION

In this study, we report new data about the allosteric communication between an antibody epitope in the 37- and 70-loops, the N-terminus (Ile16) in the activation pocket, and inhibitors or substrates in the active site of muPA. Overall, our data contribute to a functional elucidation of the interconversion between distorted states of uPA and its active conformation.

It is tempting to compare the epitope of mU3 to exosite I of thrombin, which is also located in the 37- and 70-loops. Exosite I mediates allosteric signals from cofactors, inhibitors and substrates to the active site of thrombin. However, evidence for a biological role of the 37- and 70-loops of uPA remains to be identified. When thrombomodulin binds to exosite I of thrombin, the ability to cleave fibrinogen is lost and the

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**Table 4. Thermodynamic Parameters for Binding of mupain-1–16 to muPA Variants Determined by ITC and k_{on}, k_{off} and K_D Determined by Surface Plasmon Resonance Analyses**

<table>
<thead>
<tr>
<th>Isothermal titration calorimetry</th>
<th>Surface plasmon resonance</th>
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<tbody>
<tr>
<td>ΔH (kJ/mol)</td>
<td></td>
</tr>
<tr>
<td>TΔS (kJ/mol)</td>
<td></td>
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<tr>
<td>ΔG (kJ/mol)</td>
<td></td>
</tr>
<tr>
<td>Kf (nM)</td>
<td></td>
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<tr>
<td>k_{on} (M⁻¹·s⁻¹)</td>
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<tr>
<td>k_{off} (s⁻¹)</td>
<td></td>
</tr>
<tr>
<td>K_D (nM)</td>
<td></td>
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</tbody>
</table>

All data are the average ± standard deviations of at least three independent determinations. n.d.: Data were not determined. For each surface plasmon resonance determination, six different concentrations of mupain-1–16 were used (see Figure S1). Significantly different from the value determined for full-length tc-muPA (p < 0.01) — Student’s t test. Significantly different from the value determined for muPA(16–243) (p < 0.03) — Student’s t test.

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Figure 4. Carbamylation of muPA variants. (A) Time-dependent inactivation by carbamylation of muPA(16–243) in the presence and absence of mU3 or mupain-1–16. (B) Carbamylation of full-length tc-muPA, muPA(16–243), F40Y and muPA(16–243) E137A. All measurements are the average ± standard deviations of at least three independent determinations. The carbamylation rates for muPA(16–243) F40Y was 14.2 ± 1.7 min⁻¹ and 24.2 ± 4.0 min⁻¹ for muPA(16–243) E137A. All other rates are shown in Table 3.
specificity switches toward cleavage of protein C. Exosite I regulates the conformation of thrombin through binding to different ligands, all inducing the active conformation of the enzyme. The mechanism through which muU3 restores the active state of distorted muPA variants could to some extent be analogous to that of thrombin ligands binding to exosite I.

The current work has led to new fundamental knowledge on the relationship between the thermodynamic events occurring upon binding of ligands to muPA. Other serine proteases exhibiting similar distorted conformations may be regulated by the same mechanisms. As it was suggested previously, a continuum of distorted structures seems to be represented in different mutants and ligand-bound states of thrombin. First, as observed when binding ligands to the active site or exosite I of thrombin, we measured a more negative ΔH and a more negative TΔS for binding of mupain-1Δ16 to muPA16-243 and its F40Y and E137A mutants than for binding to full-length tc-muPA. Both ΔH and TΔS became more negative, when the N-terminus16 was more exposed as measured by chemical carbamylation of Ile16, whereas ΔG was largely unchanged (Figure S1). The decrease in TΔS for mupain-1Δ16 binding to mutants in more distorted states reflects the conversion from distorted states to the active state. Hence, a mutant in a more distorted state undergoes more extensive ordering upon binding of mupain-1Δ16 to muPA16-243 occurs as these mutants are in even more distorted states and undergo more extensive ordering. Consequently, binding of mupain-1Δ16 to the active site of muPA induces the active conformation and the N-terminus16 is inserted into the activation pocket. Induction of the active conformation upon binding to mupain-1Δ16 decreases ΔH depending on the number of noncovalent bonds that must be disrupted and formed for the transition to occur. This lowering of ΔH compensates for the drop in TΔS, since the affinities (∆G) of the muPA mutants for mupain-1Δ16 are similar. Based on these data, we suggest that the larger thermodynamic differences for the binding of ligands to distorted states of serine proteases, as demonstrated here for muPA, seem to correlate with increased duration of solvent exposure of the N-terminus16. One of the most important arguments supporting these conclusions is that muPA18-243 is completely rectified into the active state by muU3, which promotes insertion of the N-terminus16 and decreases the ordering associated with binding of active site inhibitors and substrates. Accordingly, the distorted muPA mutants bind mupain-1Δ16 by an induced-fit mechanism, in which the enzyme changes conformation to fit the ligand.

ASSOCIATED CONTENT

Supporting Information

ITC graphs and surface plasmon resonance sensorgrams showing the binding of mupain-1Δ16 to different muPA variants are presented in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

ABBREVIATIONS USED

uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; muPA, murine uPA; tc, two-chain; HEK, human embryonic kidney; PEI, polyethyleneimine; S-2444, H-Glu-Gly-Arg-p-nitroanilide; S-2251, H-Val-Leu-Lys-p-nitroanilide; RU, response units; ITC, isothermal titration calorimetry; pAb, p-aminobenzamidine

ADDITIONAL NOTE

“Numbering is according to the chymotrypsin template.”

REFERENCES

APPENDIX 4

Manuscript IV

Allosteric Inactivation of a Trypsin-like Serine Protease by an Antibody Binding to the 37- and 70-loops

Tobias Kromann-Hansen, Ida K. Lund, Zhuo Liu, Peter A. Andreasen, Gunilla Høyer-Hansen and Hans Peter Sørensen

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Allosteric Inactivation of a Trypsin-Like Serine Protease by An Antibody Binding to the 37- and 70-Loops

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Supporting Information

ABSTRACT: Serine protease catalytic activity is in many cases regulated by conformational changes initiated by binding of physiological modulators to exosites located distantly from the active site. Inhibitory monoclonal antibodies binding to such exosites are potential therapeutics and offer opportunities for elucidating fundamental allosteric mechanisms. The monoclonal antibody mU1 has previously been shown to be able to inhibit the function of murine urokinase-type plasminogen activator in vivo. We have now mapped the epitope of mU1 to the catalytic domain’s 37- and 70-loops, situated about 20 Å from the S1 specificity pocket of the active site. Our data suggest that binding of mU1 destabilizes the catalytic domain and results in conformational transition into a state, in which the N-terminal amino group of Ile16 is less efficiently stabilizing the oxyanion hole and in which the active site has a reduced affinity for substrates and inhibitors. Furthermore, we found evidence for functional interactions between residues in uPA’s C-terminal catalytic domain and its N-terminal A-chain, as deletion of the A-chain facilitates the mU1-induced conformational distortion. The inactive, distorted state is by several criteria similar to the E* conformation described for other serine proteases. Hence, agents targeting serine protease conformation through binding to exosites in the 37- and 70-loops represent a new class of potential therapeutics.

The serine protease urokinase-type plasminogen activator (uPA) can, while bound to urokinase-type plasminogen activator receptor (uPAR) at cell surfaces, catalyze the conversion of the zymogen plasminogen to the active serine protease plasmin. Plasmin catalyzes the degradation of extracellular matrix proteins and up-regulation of uPA is implicated in several physiological and pathophysiological conditions related to tissue remodeling, including wound healing, fibrinolysis, inflammation, embryogenesis, and angiogenesis.1,2 The uPA system is pleiotropic in the neoplastic processes affecting cancer cell proliferation, tumor angiogenesis, adhesion, invasion, and migration.1,2 uPA is a potential therapeutic target in cancer, arthritis, and other diseases.3,4 uPA consists of an N-terminal A-chain and a C-terminal B-chain. The A-chain of uPA is composed of an N-terminal uPAR-binding growth factor-like domain, a kringle domain, an intradomain linker, and residues 1–15 of the catalytic domain. The B-chain encompasses residues 16–250 of the catalytic domain. The A- and B-chains are linked by a disulfide bond between Cys1 and Cys122 (Figure 1). Like other serine proteases, uPA is secreted from cells as a single-chain zymogen, also known as pro-uPA. Cleavage of single-chain uPA between Lys15 and Ile16 can be catalyzed by plasmin and a few other serine proteases.5,6 In general, cleavage of zymogens between amino acid residues 15 and 16 allows for insertion of the liberated N-terminal Ile16 into the activation pocket, a hydrophobic cleft, in which its amino group forms a stabilizing salt-bridge to Asp194 next to the catalytic Ser195. Conformational rearrangements

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induced by single-chain zymogen cleavage organize both the S1 specificity pocket and the oxyanion hole of the active site by a mechanism common to all serine proteases.\(^7\)–\(^10\) Still, in the cleaved as well as uncleaved states, active and inactive forms are in equilibrium. The equilibrium is strongly shifted toward the inactive zymogen in the single-chain form and strongly shifted toward the active protease in the two-chain form. For uPA, the enzyme efficiency (\(k_{\text{cat}}/K_M\)) for hydrolysis of plasminogen by the single-chain form is at least 250 times lower than that of the two-chain form.\(^6\) The relative enzymatic efficiencies of single- and two-chain forms vary among different serine proteases. While a number of three-dimensional structures of the catalytic domain of human uPA have been solved by X-ray crystallography,\(^11\)–\(^12\) structures of human and murine single-chain uPA remain to be determined.

Sufficient specificity of small molecule protease inhibitors is difficult to achieve because of the highly conserved active site topologies among related proteases, as clearly illustrated by the difficulties in obtaining potent and selective uPA inhibitors\(^13\) and the severe side-effects and inefficiency of matrix metalloprotease active site inhibitors in clinical trials.\(^14\) These complications have caused increased interest in reagents such as monoclonal antibodies, binding to the less conserved exosites and allosteric sites. Because of their potency and specificity, antibodies are widely used as therapeutic agents, and a total of more than 35, directed against a variety of targets, monoclonal antibodies have been approved as drugs by the US Food and Drug Administration.\(^15\)–\(^16\) Antibodies are able to specifically inhibit serine proteases by simple steric hindrance,\(^17\) and even greater efficiency may be achieved by antibodies that conformationally regulate the active site by allosteric mechanisms.\(^18\) Detailed studies of allosteric antibodies against the serine proteases hepatocyte growth factor activator (HGFA), hepsin, and human uPA have elucidated distinct inhibition mechanisms with binding sites as far as 15−20 Å away from the active site.\(^19\)–\(^21\)

A murine monoclonal antibody, mU1, binding to murine uPA (muPA), can inhibit plasminogen activation in vitro, rescue mice treated with muPA-activatable anthrax pro-toxin, and impair muPA-mediated fibrinolysis as well as delay wound healing in vivo.\(^22\)–\(^25\) Here, we present the molecular mechanism responsible for mU1-mediated inhibition of muPA. Our analysis revealed that mU1 distorts the active site of muPA, leading to a reduced affinity for substrates and inhibitors, although it binds 20 Å away. The distortion is associated with increased solvent exposure of the amino group of the N-terminal Ile16 of the B-chain. Surprisingly, interactions between the B-chain and Phe(−2) and Lys4 of the A-chain counteract transition into the inactive conformation. The inactive and apparently distorted conformation resembles the inactive E\(^\infty\) conformation described for other serine proteases. We have hereby discovered previously unknown communication routes within the catalytic domain of serine proteases that are of general interest, when designing and developing specific and potent serine protease inhibitors.

**EXPERIMENTAL SECTION**

**Monoclonal Antibodies.** The generation and characterization of mU1 and mAb-112 were described previously\(^22\)–\(^24\).

**muPA Production in HEK293 6E Suspension Cells.** cDNAs encoding muPA (NP_032899.1, 413 residues without the signal peptide), domain deletion mutants, and site-directed mutants were cloned into the pTT5 or pCDNA3.1 vectors. All variants contained a glycine and six histidines at the C-terminus.\(^13\) All variants contained a glycine and six histidines at the C-terminus.\(^13\) The muPA(16−243) and single-chain muPA(2−243) variants contained a C122A mutation, whereas the two-chain muPA(16−250) variant lacks the amino terminal fragment of muPA (Figure 1). The vectors were transfected into HEK293 6E suspension cells, which were cultured in a humidified CO\(_2\) incubator at 37 °C. The medium used was Freestyle F17 (Gibco) supplemented with 4 mM L-glutamine, 0.1% Pharonic F68 (Gibco), 1% penicillin/streptomycin, 25 μg/mL G418. Linear polyethyleneimine (PEI) (2.2 mg) in cDNA (1.1 mg) in phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, 140 mM NaCl) (110 mL) were preincubated for 15 min and added to 1 L of culture with a density of 1 × 10^6 cells/mL. Tryptone N1 (0.5%) was added to the culture 24 h post-transfection. The culture was continued for 6 days before harvesting the conditioned media.

**muPA Purification and Characterization.** Full-length two-chain muPA wt and variants hereof (i.e., single-chain muPA(2−243), muPA(16−243), two-chain muPA(16−250), and two-chain muPA(16−250)(−2)(−243)A-K4G) were purified from conditioned media of transiently transfected HEK293 6E cells. The following alanine substitution muPA mutants were prepared similarly: G37cA, P37eA, P38A, K72A, S74A, Y76A, N77A, and P78A. The proteins, as mentioned above with a glycine and six histidines at the C-terminus, were captured on a nickel−Sepharose column directly from the conditioned media, supplemented with 25 mM imidazole and 50 mM NaCl. The column was washed with 20 mM sodium phosphate, pH 7.4,
0.3 M NaCl, and 25 mM imidazole. The bound protein was eluted from the column with 50 mM bicine, pH 8.0, 0.3 M NaCl, and 0.4 M imidazole. To convert the purified single-chain muPA into its two-chain counterpart, it was incubated at a concentration of 2 μM with 5 nM plasmin for 20 h at 22 °C. Benzamidine-Sepharose chromatography was used to isolate the active fraction, by capturing the proteins on a benzamidine-Sepharose column, which was then washed with 20 mM sodium phosphate, pH 7.4, 0.3 M NaCl, and eluting bound protein with 20 mM sodium citrate, pH 3.0. Protein concentrations were determined using measurements of the absorbance at 280 nm and the theoretical extinction coefficient calculated using the ProtParam server located at http://web.expasy.org/protparam/. All preparations were more than 90% pure, as determined by SDS–PAGE (data not shown). The active enzyme concentration, \( [E]_{\text{TOT}} \), was calculated from the absorbance at 280 nm, assuming that the benzamidine-Sepharose-purified product was 100% active. Glutamyl-glycyl-arginyl-chloromethyl ketone (EGR-cmk), at a concentration of 1 mM, was covalently coupled to muPA (at a concentration of 1 μM) was incubated for 3 h at 22 °C in PBS, followed by extensive dialysis to remove excess EGR-cmk. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry of intact proteins in solution was conducted using reduced preparations (Alphalyse A/S, Odense, Denmark).

**Amidolytic Assay of muPA Proteolytic Activity.** The velocity of muPA-catalyzed hydrolysis of the chromogenic substrate i-proglutamyl-glycyl-i-arginine-p-nitroanilide hydrochloride (S-2444) (Aniara Diagnostica LLC, USA) was measured at 37 °C in HEPES-buffered saline (HBS; 10 mM HEPES, pH 7.4, 140 mM NaCl) with 0.1% bovine serum albumin (BSA) and enzyme and substrate concentrations as specified for each experiment. When antibody inhibition of the catalytic activity of muPA was measured, the enzyme (0.5 nM) was preincubated with various concentrations of muU1 (0–5 μM) in HBS at 22 °C for 15 min before adding S-2444, in concentrations as indicated for each single experiment. In all experiments, the initial reaction velocity was monitored at an absorbance of 405 nm (microplate reader).

**Analysis of Plasmin-Mediated Cleavage of Single-Chain muPA.** To analyze plasmin-catalyzed cleavage of single-chain muPA in the absence or presence of muU1, the single-chain muPA \( ^{\text{12–243}} \) (200 nM) variant, containing the residues GQKALRPRFK before the activation site at Ile16, was preincubated for 15 min at 22 °C with or without muU1 (1 μM) or control antibody mAb-112 (1 μM). At time 0, plasmin (5 nM final concentration) was added and the mixtures were incubated at 22 °C for 60, 30, 15, 10, 5, 2, or 0 min, respectively, after which the activity of plasmin was quenched by addition of aprotinin (1 μM final concentration). The amount of active two-chain muPA, generated by plasmin, was estimated by adding S-2444 (750 μM final concentration) and measuring the absorbance of S-2444 by monitoring the absorbance at 405 nm for 1 h at 37 °C. In addition, samples were taken from the incubation mixtures at each of the time points mentioned above and analyzed by nonreducing SDS–PAGE. The gel was stained with Coomassie Blue. The density of the stained bands were quantified by densitometry using the TotalLab Quant software, and the percentage of single-chain muPA \( ^{\text{12–243}} \), which had been converted to the two-chain muPA form, was plotted against the incubation time.

**Surface Plasmon Resonance Measurements.** To determine the equilibrium binding constants (\( K_D \)), the association rate constants (\( k_{\text{on}} \)), and dissociation rate constants (\( k_{\text{off}} \)) for antibody binding to muPA variants, surface plasmon resonance (SPR) analysis was conducted with a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). Polyclonal rabbit antimouse IgG from the Mouse Antibody Capture Kit (GE Healthcare, Uppsala, Sweden) was immobilized on a CMS chip by amine coupling. Polyclonal rabbit anti-mouse IgG, at a concentration of 30 μg/mL in immobilization buffer (10 mM sodium acetate, pH 5.0), was injected over 7 min at a flow rate of 10 μL/min, until a level of ~14 000 response units (RU) was reached, followed by surface blocking with ethanolamine. Antibody (muU1) in a running buffer (30 mM HEPES pH 7.4, 135 mM NaCl, and 0.1% bovine serum albumin) was then injected at a flow rate of 5 μL/min for 180 s until a capture level of ~410 RU was reached. A flow cell without injection of muU1 was used as the reference. A dilution series of muPA variants (0–0.2 μM) in running buffer, purified on a benzamidine-Sepharose column immediately before the Biacore analysis, was injected over both flow cells at a flow rate of 30 μL/min during 240 s at 22 °C. Subsequently, the dissociation was monitored for 15 min. Kinetic constants (\( k_{\text{on}} \) and \( k_{\text{off}} \)) were calculated using the Biacore evaluation software to generate a 1:1 kinetic fit. The \( K_D \) values were calculated as \( [E]_{\text{TOT}} k_{\text{off}} / k_{\text{on}} \).

**Measurement of Binding \( p \)-Aminobenzamidine to muPA by Fluorescence Spectroscopy.** The binding of the fluorescent probe \( p \)-aminobenzamidine (\( \mu \)PAB, Sigma) to the active site of full-length two-chain muPA was measured by modification of a previously described method.\(^{25}\) Fluorescence emission spectra were recorded at 25 °C on a PTI Quantamaster spectrophuorometer in a 2 mm × 10 mm semimicro quartz cuvette in HBS (10 mM HEPES, pH 7.4, and 140 mM NaCl) with 0.1% polyethylene glycol 8000. Excitation was at 335 nm, and the emission was scanned from 340 to 400 nm. Equilibrium binding reactions were performed over a range of muU1 concentrations (0–1 μM) with 0.1 μM two-chain muPA in the presence of 20 μM \( \mu \)PAB. Emission spectra were collected after 30 min of incubation with the use of an integration of 1–2 s over a 1.0 nm step resolution.

**Quantitation of Incorporation of \([H]-\text{Diisopropylfluorophosphate (DFP)}\) into muPA.** \([H]-\text{DFP (50 μM)}\) and C-terminally 6x histidine-tagged muPA \( ^{\text{16–243}} \) (100 nM) were incubated in the absence or presence of muU1 (0–1 μM) and C-terminally 6x histidine-tagged green fluorescent protein at a concentration of 1 mg/mL in HBS buffer. After 3 h at 22 °C, 100 μL of 50% Ni-Sepharose slurry was added to the 30 μL reaction mixture. The mixture was incubated for 30 min at 4 °C and washed four times in HBS buffer. The mixture was then transferred to scintillation liquid and bound \([H]-\text{DFP)}\) counted. Incubation of muPA \( ^{\text{16–243}} \) and \([H]-\text{DFP}\) for 3 h in the absence of antibodies resulted in app 25% incorporation and was within the range of linearity between incorporation and time.

**Carbamylation Assay.** To analyze the effect of muU1 on the carbamylation rate of the N-terminal \( \alpha \)-amino group of Ile16 in different muPA variants, the enzyme (0.5 μM) was preincubated with or without muU1 in HBS buffer, supplemented with 0.1% polyethylene glycol 8000, at 22 °C for 30 min. The full-length two-chain muPA variants were analyzed using a muU1 concentration of 1 μM. For muPA \( ^{\text{16–243}} \) analysis, a muU1 concentration of 0.5 μM was used. After preincubation, potassium cyanate (0.2 M) or HBS buffer supplemented with 0.1% polyethylene glycol 8000 was added and incubated at 22 °C for 0, 30, 60, 120, 180, and 300 min. To stop the
carbamylation reaction, each of the mixtures was diluted 100 times in HBS supplemented with 0.1% BSA. mU1 was allowed to dissociate from muPA for 2 h at 22 °C. The residual activity was determined from the rate of hydrolysis of S-2444 (750 μM), by measuring absorbance at 405 nm at 37 °C for 1 h.

**RESULTS**

**Mechanism of mU1 Inhibition of Activation of Single-Chain pro-muPA.** It was previously shown that the monoclonal anti-muPA antibody mU1 inhibits plasmin-catalyzed activation of the inactive single-chain form of muPA. We now wished to dissect the step in which the activation reaction is inhibited by the antibody. We therefore studied the effect of mU1 on plasmin-catalyzed cleavage of the Lys15–Ile16 peptide bond of purified single-chain muPA, using either full-length single-chain muPA (results not shown) or a variant of muPA (i.e., single-chain muPA(2–243)) (Figure 2).

Initially, more than 95% of the purified protein appeared to be in the single-chain form, as judged by SDS–PAGE (Figure 2), and the specific activity ($k_{cat}/K_{M}$) was approximately 260 times lower than that of the two-chain activated form. In agreement with the previous results, the activation of single-chain muPA(2–243) by plasmin, as measured using the peptidic S-2444 chromogenic substrate, was inhibited by mU1, whereas a control antibody, mAb-112, which is specific for human uPA, had no effect (Figure 2A).

Unexpectedly, however, we observed no effect of the antibody on the cleavage of the single-chain form into the two-chain form by SDS–PAGE analysis (Figure 2B). Thus, mU1 inhibits activation (Figure 2A) but not cleavage of single-chain muPA (Figure 2B) (i.e., in the presence of mU1, cleavage of single-chain pro-muPA does not lead to the catalytically active form). To find out why mU1 inhibits plasmin-catalyzed activation of single-chain muPA, whereas plasmin-catalyzed cleavage of single-chain muPA was unaffected, we investigated whether mU1 could inhibit the catalytic activity of two-chain muPA with the chromogenic substrate S-2444. The measurements showed that mU1 inhibits the amidolytic activity of full-length two-chain muPA with an IC$_{50}$ value around 50 nM (Figure 3A). The observed mU1-induced inhibition of active two-chain muPA fully explains why the single-chain proform, in a complex with mU1, does not become active after plasmin-catalyzed cleavage.

This finding may seem to conflict with Lund et al. who reported that mU1 does not inhibit active two-chain muPA. However, the maximal mU1 concentration tested by Lund et al. was 10 nM, at which concentration they observed a small, 10% inhibition. This result is fully in agreement with our result of a 50 nM IC$_{50}$ (Figure 2). Also, Lund et al. reported an IC$_{50}$ of around 1 nM for mU1 inhibition of pro-muPA activation. This observation may also seem to conflict with the conclusion that pro-muPA activation is inhibited because mU1 inhibits the activity of the two-chain form, with an IC$_{50}$ of 50 nM. However, once bound to pro-muPA with a high affinity, mU1 will dissociate very slowly, even from two-chain muPA (see below), implying that the equilibrium established during incubation of antibody and pro-muPA will determine the IC$_{50}$ value even after conversion to the two-chain form. Again, the present data are fully in agreement with those of Lund et al.

**Kinetic Analysis of mU1 Inhibition of the Amidolytic Activity of Active Two-Chain muPA.** To determine $K_{M}^{app}$ and $k_{cat}^{app}$ for S-2444 hydrolysis, initial velocities were measured in the presence or absence of mU1 and with 0–24 mM substrate.

Figure 2. mU1 inhibits activation but not cleavage of single-chain muPA(2–243). (A) Rate of hydrolysis of S-2444 (750 μM) by 200 nM single-chain muPA(2–243) in the absence or presence of indicated antibodies (1 μM) after treatment with plasmin (5 nM) for the indicated time periods. (B) Nonreduced SDS–PAGE and Coomassie Blue staining of single-chain muPA(2–243) treated with plasmin under the same conditions as in (A). The migrations of single-chain and cleaved muPA(2–243) are indicated to the left. The migration of a Mr marker is indicated to the right. The densities of the stained bands were quantified by densitometry. The percentage of the single-chain muPA(2–243), which had been converted to the two-chain form, was plotted against the incubation time. All data are shown as means ± standard deviations of at least three independent determinations.
and 0.5 nM muPA. The data were fitted to the following equation:

$$v = \frac{[E][S][K_{cat}][S]_0}{[S]_0 + K_{M}^{app}}$$

The calculated $K_{M}^{app}$ values varied by 5–10% between different preparations of the same enzyme, whereas $K_{cat}^{app}$, for unknown reasons, varied by 50–75% (results not shown). The measurements also showed that mU1 increased the $K_{M}^{app}$ value for hydrolysis of S-2444 by full-length two-chain muPA, whereas $K_{cat}^{app}$ was unaffected (Table 1). Therefore, these data suggest, as a first approximation, that mU1 is a competitive inhibitor of two-chain muPA. However, a more detailed analysis indicated a more complex mode of inhibition. First, plots of steady state velocities of the amidolytic activity against the concentration of mU1 demonstrated that full-length two-chain muPA was only inhibited by about 90% at saturating concentrations of mU1 (i.e., 5 μM) (Figure 3A). The residual activity was not due to contaminating proteases, as a specific peptidic inhibitor of muPA, mupain-1-16,26 inhibited the activity completely (data not shown). The residual activity therefore seems to represent a true activity of the two-chain muPA–mU1 complex. Second, a truncated variant of muPA, muPA(16−245) (Figure 1) was previously demonstrated to assume a conformationally distorted state with a higher $K_{M}^{app}$ for S-2444 hydrolysis than full-length two-chain muPA.27

Surprisingly, we now found that the amidolytic activity of muPA(16−245) was inhibited with an IC$_{50}$ value of only about 5 nM mU1, considerably lower than the IC$_{50}$ value of about 50 nM determined for full-length two-chain muPA (Figure 3A). It was not possible to explain these findings by a competitive, uncompetitive, or a noncompetitive mode of inhibition of S-2444 hydrolysis by mU1.

For a quantitative treatment of these data, we considered a model in which two-chain muPA can equilibrate between two different states, an active one (E) and a distorted state (E*). The two forms are assumed to be in rapid equilibrium (equilibrium constants $K_1$ and $K_2$), and both forms are assumed to bind mU1 (here referred as I), but with different affinities (equilibrium dissociation constants $K_3$ and $K_4$):

Establishing the equilibrium during a preincubation with the antibody, the enzyme activity is determined in an ensuing incubation with a chromogenic substrate. During the activity measurement, the antibody binding equilibrium is presumed to remain unchanged, due to the slow rates of antibody–muPA dissociation (Table 3). The reactions taking place are then defined by the following scheme:

On the basis of the data in Table 1, $k_{cat}^{app}$ does not vary with the mU1 concentration, whereas $K_{M}^{app}$ does. The $K_{cat}^{app}$ in these schemes is presumed to be identical for the different forms of the enzyme, whereas the $K_{M}^{app}$ values are presumed to be variable. For these reaction schemes, one can derive (as described in the Supporting Information) the following equation for the dependence of the rate of substrate hydrolysis $v$ on the antibody concentration $[I]$:

$$v = \frac{v_0K_{D}^{app} + v_{\infty}[I]}{K_{D}^{app} + [I]}$$

Table 3. The reactions taking place are then defined by the following scheme:
Table 1. \(K_{Dapp}^{mU1}\) and \(k_{off}^{mU1}\) for Full-Length Two-Chain muPA-Catalysed S-2444 Hydrolysis in the Presence of Varying Concentrations of mU1

<table>
<thead>
<tr>
<th>[mU1] (nM)</th>
<th>(K_{Dapp}^{mU1}) (mM)</th>
<th>(k_{off}^{mU1}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.4 ± 0.2</td>
<td>124 ± 26</td>
</tr>
<tr>
<td>25</td>
<td>2.8 ± 0.1(^b)</td>
<td>120 ± 30</td>
</tr>
<tr>
<td>50</td>
<td>3.6 ± 0.3(^b)</td>
<td>125 ± 27</td>
</tr>
<tr>
<td>100</td>
<td>4.9 ± 1.1(^b)</td>
<td>130 ± 25</td>
</tr>
</tbody>
</table>

“The \(K_{Dapp}^{mU1}\) and \(k_{off}^{mU1}\) values were determined by incubating full-length two-chain muPA (0.5 nM) with various concentrations of mU1 (0–100 nM) for 15 min at 22 °C before the addition of different concentrations of S-2444 (0–24 mM). Data were analysed according to Michaelis–Menten kinetics. The table shows means ± standard deviations of at least three independent determinations. \(^b\)Significantly different from the value for 0 nM mU1 (p < 0.001 by Student’s t-test).

Table 2. \(K_{Dapp}^{mU1}\) for S-2444 Hydrolysis and \(k_{off}^{mU1}\) for mU1 Inhibition for muPA Variants

<table>
<thead>
<tr>
<th>muPA variant</th>
<th>(K_{Dapp}^{mU1}) (mM)</th>
<th>(k_{off}^{mU1}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>full-length two-chain muPA</td>
<td>2.4 ± 0.2</td>
<td>57 ± 14</td>
</tr>
<tr>
<td>muPA(^{16–243})</td>
<td>5.0 ± 0.7(^b)</td>
<td>8.3 ± 2.8(^b)</td>
</tr>
<tr>
<td>full-length two-chain muPA F(−2)A-K4G</td>
<td>5.3 ± 0.2(^b)</td>
<td>9.3 ± 0.6(^b)</td>
</tr>
<tr>
<td>two-chain muPA(^{16–243})</td>
<td>2.3 ± 0.3</td>
<td>44 ± 12</td>
</tr>
<tr>
<td>two-chain muPA(^{16–250}) F(−2)A-K4G</td>
<td>4.1 ± 0.1(^b)</td>
<td>7.5 ± 1.1(^b)</td>
</tr>
</tbody>
</table>

“The \(K_{Dapp}^{mU1}\) values were determined by measuring the rate of S-2444 hydrolysis by muPA variants (0.5 nM) at different concentrations of S-2444 (0–24 mM) and analysing the data according to Michaelis–Menten kinetics. The \(K_{Dapp}^{mU1}\) values were determined by measuring the rate of S-2444 (750 μM) hydrolysis in the presence of varying mU1 concentrations (0–5 μM) and analysing the data according to eq 2. The table shows means ± standard deviations for at least three independent determinations. \(^b\)Significantly different from the value for full-length two-chain muPA (p < 0.01). All p-values were calculated by Student’s t-test.

It is indicated that the \(K_{Dapp}^{mU1}\) for binding to full-length two-chain muPA and two-chain muPA\(^{16–250}\) are around 50 nM, whereas that for muPA\(^{16–243}\) is about 10 times lower.

The \(K_{Dapp}^{mU1}\) values determined in this way, by inhibition of enzyme activity, are in good agreement with those determined by surface plasmon resonance (SPR) analysis (Table 3) on the basis of a 1:1 model. Importantly, reproducible fits to a 1:1 binding model in the case of two-chain muPA required purification of the enzyme on benzanilide–Sepharose immediately before the SPR. Interestingly, the variations in \(K_{D}\) values were almost totally accounted for by variations in \(k_{on}\) (Figure 4 and Table 3). Also, the \(K_{D}\) value for the binding of mU1 to single-chain muPA\(^{2–243}\) is 7 times lower than the \(K_{D}\) value for binding to muPA\(^{16–243}\) and 20–60 times lower than binding to the longer muPA variants. Again, this variation is easily understood on the basis of the model proposed above and eq 3. A lower \(K_{Dapp}^{mU1}\) value could result from a lower \(K_{D}\) and/or a larger decrease of \(K_{D}\) than of \(K_{Dapp}\). Alternatively, the presumed E* form and thezymogen may have different conformations, but both with a higher affinity to mU1 than the fully active (E) form.

The Epitope of mU1 is Located in the 37- and 70-Loops. Another anti-muPA monoclonal antibody, mU3, binds to an epitope in the 37- and 70-loops. Another anti-muPA monoclonal antibody, mU3, binds to an epitope in the 37- and 70-loops. Figure 4. Surface plasmon resonance determination of the kinetics of binding of mU1 to muPA\(^{16–243}\) and two-chain muPA\(^{16–250}\). Representative surface plasmon resonance sensorgrams for binding of mU1 to muPA\(^{16–243}\) (A) and two-chain muPA\(^{16–250}\) (B). The experimental data (red curves) were fitted to a 1:1 binding model (black curves) with the Biacore evaluation software. The calculated \(k_{on}\) \(k_{off}\) and \(K_{D}\) values are listed in Table 3.
binding to full-length two-chain muPA,\textsuperscript{22} the epitopes of mU1 and mU3 are likely to overlap. To determine the epitope of mU1, we analyzed the mU1 concentration dependence of inhibition of muPA-catalyzed S-2444 hydrolysis, using full-length two-chain muPA alanine substitution mutants in the 37- and 70-loops. As reported above, the $K^\text{app}_{\text{IP}}$ determined from the antibody concentration dependence of inhibition of full-length two-chain muPA by mU1 was about 50 nM (Table 2). However, the following 8 out of 30 mutants, namely G37cA, P37eA, P38A, K72A, S74A, Y76A, N77A, and P78A, all exhibited a reduced or completely abolished susceptibility to inhibition by 500 nM mU1 (Table 4), indicating that these

<table>
<thead>
<tr>
<th>muPA variant</th>
<th>$K^\text{IP}_{\text{m}}$ (nM)</th>
<th>$K^\text{IP}_{\text{b}}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>full-length two-chain muPA</td>
<td>57 ± 14</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>full-length two-chain muPA G37cA</td>
<td>&gt;500 b</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>full-length two-chain muPA P37eA</td>
<td>&gt;500 b</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>full-length two-chain muPA P38A</td>
<td>&gt;500 b</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>full-length two-chain muPA K72A</td>
<td>&gt;500 b</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>full-length two-chain muPA S74A</td>
<td>&gt;500 b</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>full-length two-chain muPA Y76A</td>
<td>&gt;500 b</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>full-length two-chain muPA N77A</td>
<td>&gt;500 b</td>
<td>3.7 ± 0.2 b</td>
</tr>
<tr>
<td>full-length two-chain muPA P78A</td>
<td>&gt;500 b</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

*“Full-length two-chain muPA variants (0.5 nM) were preincubated with mU1 (0–500 nM) for 15 min at 22 °C, before measuring the S-2444 (750 μM) hydrolysis. Besides the values indicated in the table, the full-length two-chain muPA mutants E20A, F21A, T22A, E23A, Q35A, N37A, K37aA, G37cA, P37eA, P38A, K72A, S74A, Y76A, N77A, and P78A were also tested and showed no measurable binding to mU1 ($K_0 > 500$ nM). Altogether, these mutagenesis data mapped the epitope of mU1 to the 37- and 70-loops, located at least 20 Å from Lys72 C\textsubscript{α} to Asp189 C\textsubscript{α} in the S1 specificity pocket. Notably, mutation of a glycine (Gly37) and three prolines (Pro37e, Pro38, and Pro78) affected the binding, indicating that binding of muPA depends on the conformation of the 37- and 70-loops. Overall, the residues implicated in the epitope cover a relatively large interaction surface of 923 Å\textsuperscript{2} (Figure 5) compared to a typical antibody epitope, which is 680–880 Å\textsuperscript{2}.\textsuperscript{21,28}*

**Table 4. Determination of the Epitope for mU1, As Measured by Determination of $K^\text{IP}_{\text{b}}$ Values for Full-Length Two-Chain muPA**

Phe(−2) and Lys4 in the A-chain Stabilize the Active Conformation of muPA. As described above, the truncated form of two-chain muPA, muPA\textsuperscript{16-250}, encoding only the B-chain (i.e., residues 16–250), has a much higher affinity for mU1 than full-length two-chain muPA and two-chain muPA\textsuperscript{16-250}. In addition, the $K^\text{IP}_{\text{m}}$ value for S-2444 hydrolysis by muPA\textsuperscript{16-250} is about 2 times higher than the $K^\text{IP}_{\text{m}}$ value for full-length two-chain muPA and two-chain muPA\textsuperscript{16-250} (Table 2). The residues responsible for these differences must be localized between residue −16 and 15. Alignment of residue −2 to 15 revealed strong interspecies conservation of particularly Phe(−2) and Lys4 (Figure 6A). Furthermore, in three-dimensional structures of human uPA, these two residues form contacts to the B-chain (Figure 6B,C). Substitution of Phe(−2) with Ala and of Lys4 with Gly led to an about 2 times increase in the $K^\text{IP}_{\text{m}}$ for S-2444 hydrolysis and a 5–10 times decrease in $K^\text{IP}_{\text{m}}$ for inhibition of S-2444 hydrolysis by mU1 (Table 2). The $K^\text{IP}_{\text{m}}$ for S-2444 hydrolysis was similar for the complex between mU1 and full-length two-chain muPA (Table 1) and full-length two-chain muPA F(−2)A-K4G double mutant in the absence of mU1 (Table 2). These data support the hypothesis of functional interactions between Phe(−2) and Lys4 in the A-chain and residues in the B-chain maintaining a maximally active state of the catalytic domain.

To verify that the part of the A-chain containing Phe(−2) and Lys4 was intact in the preparations used to draw these conclusions, we analyzed muPA(16–250) (Figure 1), in which the A-chain was short enough to allow accurate mass determinations with MALDI mass spectrometry. Sequencing of two-chain muPA(16–250) by Edman degradation revealed that the N-terminus was GSSLSK, as expected. Analysis of two-chain muPA(16–250) and two-chain muPA(16–250) F(−2)A-K4G by MALDI mass spectrometry under reducing conditions revealed an average mass of the B-chain for both proteins of 29.2 kDa, identical to the theoretical mass based on the sequence. For two-chain muPA(16–250), the monoisotopic mass of the A-chain was found to be 25.91 kDa, corresponding to the sequence KPSSSVDQQFQQCGQ KALRPRFK. For two-chain muPA(16–250) F(−2)A-K4G, the monoisotopic mass of the A-chain was found to be 30.04 kDa, corresponding to the sequence GSSLSKPPSSSVDQQFQQCGQ KALRPRFK. It therefore appears that two-chain muPA(16–250), but not two-chain muPA(16–250) F(−2)A-K4G, was trimmed by removal of the N-terminal amino acids GSSLSK during the cleavage of the single-chain form by plasmin. Nevertheless, the MALDI analysis did show that the chain from residue −12 to 15 is indeed intact in both two-chain muPA(16–250) and two-chain muPA(16–250) F(−2)A-K4G. The analysis also confirmed that the planned mutations were indeed introduced into the latter variant. We conclude that Phe(−2) and Lys4 stabilize the active conformation of two-chain muPA.

**mu1 Induces Exposure of the N-terminus of the Catalytic Domain.** Susceptibility to chemical carbamylation was previously used to measure exposure of the amino group of the N-terminal Ile16 of the catalytic domain of FVIIa and murine uPA.\textsuperscript{27,28,30} The idea behind this analysis is that the amino group of Ile16 of a two-chain serine protease, such as full-length two-chain muPA, becomes susceptible to carbamylation, if it is even temporarily expelled from the activation pocket. The carbamylated Ile16 cannot reinsert, and the enzyme becomes permanently inactivated at a rate corresponding to the frequency of Ile16 exposure.

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As recently demonstrated, full-length two-chain muPA is slowly inactivated by carbamylation, whereas muPA (Ile16−243) is modified faster. The presence of mU1 enhanced the rate of Ile16 carbamylation of both full-length two-chain muPA and muPA (Ile16−243) (Figure 7 and Table 5), indicating a shift in the equilibrium toward a state with the amino group of the Ile16 being exposed (Table 4). Hence, mU1 binding induces a distorted state with an exposed N-terminus Ile16. However, mU1 induces a more frequently exposed N-terminus in muPA (Ile16−243) than in the full-length two-chain muPA, which indicates a more distorted state of the former.

The Active Site is Inaccessible Upon mU1 Binding. Fluorescence spectroscopy was used to measure the binding of pAB to full-length two-chain muPA in the absence or presence of mU1 or the control antibody mAb-112. A mU1 concentration-dependent decrease in fluorescence, indicative of pAB displacement from the active site was observed (Figure 8A), whereas the control antibody (mAb-112) had no effect (data not shown). Hence, the mU1-induced conformational changes in muPA hinder binding of pAB to the active site of muPA.

Disopropyl fluorophosphosphate (DFP) binds covalently to the hydroxyl group of Ser195 of active two-chain uPA. Formation of the covalent bond requires an ordered oxyanion hole, as found only in the active state. Irreversible binding of [3H]-DFP to the oxyanion hole of muPA (Ile16−243) was inhibited in a dose-dependent manner by mU1, whereas the control antibody (mAb-112) did not affect the incorporation (Figure 8B).

These data indicate that both the S1 specificity pocket and the oxyanion hole of two-chain muPA are inaccessible in the complex with mU1.

**DISCUSSION**

Previous experiments demonstrated that mU1 can inhibit muPA in vivo. We initiated this study to explain the mechanism by which mU1 inhibits activation of the single-chain zymogen form of muPA. We found that instead of abrogating the plasmin-catalyzed cleavage between Lys15 and Ile16 and the formation of the two-chain form, mU1 maintains the generated two-chain muPA in an inactive conformation. Nevertheless, our findings are in complete agreement with the original observation that the mU1 prevents plasmin-catalyzed activation of single-chain pro-muPA, although the mechanism appears to be more complicated than originally assumed. Another potentially regulatory point (i.e., the binding of muPA to its cell surface receptor muPAR) has previously been shown not to be affected by mU1 neither in SPR analysis nor in cell binding assays.

The fact that there is a distance of 20 Å between the epitope of mU1 and the active site of muPA suggests that mU1 inhibits the enzyme activity through a conformational transition. In the inactive state induced by mU1, the N-terminal Ile16 is solvent exposed, and the affinities for substrates and inhibitors are much lower than in the absence of mU1. Our present data are compatible with an equilibrium between an inactive and an active state, which is shifted toward the inactive state in the presence of mU1 and also, although less so, in the absence of residues −16 to 15.

Derivatisation of the various muPA variants with EGR-cmk or the presence of a high concentration of pAB reduced the affinity to mU1 maximally 2 times (data not shown). This observation is in good agreement with previous observations with human uPA. Thus, human single-chain pro-uPA could be labeled with S-(dimethylamino)-1-naphthalenesulfonyl (dansyl)-EGR-cmk in the presence of dipeptides mimicking the activating residues 16 and 17 of the catalytic domain, but upon removal of the dipeptide, the dansyl-EGR-cmk-derivatized molecule again assumed a zymogen conformation. In this respect, uPA seems to differ from FVIIa, with which peptide-cmk...
derivatives can affect the $K_D$ for binding to conformation-specific antibodies more than 100 times.35,36 On the basis of these observations and our previous study,27 we suggest that the herein demonstrated mU1-induced distorted state resembles the distorted $E^*$ conformation observed in three-dimensional structures of other serine proteases. In the absence of ligands, an equilibrium between active and distorted inactive $E^*$ forms has been identified in several serine proteases. 37 In thrombin, the $E^*$ state is characterized by a collapse of the 215−217 segment, with Trp215 closing the S1 entrance frame. 38 Collapsed structures with a blocked active site have been observed not only in thrombin but also in complement factor I42 and D,43,44 prostate specific kallikrein,45 prostasin,46 o1-tryptase,47 DegP,48 and HGFA.49 Similarly, collapsed forms are also observed in zymogens of pro-granzyme K,50 chymotrypsinogen,51 plasminogen,52 complement profactor D,53 prokallikrein 6,54 complement pro-factor C1r,55 and prethrombin-I.56

Binding of mU1 to full-length two-chain muPA leads to an increase in the $K_M$, whereas $k_{cat}$ remains unchanged (Table 1).

A previously described monoclonal antibody, mAb-112, binds...
to human uPA and induces azymogen-like conformation.\textsuperscript{21,24} In contrast to mU1, mAb-112 is an apparently noncompetitive inhibitor, reducing \( K_{\text{cat}} \) without affecting \( K_{\text{m}} \). As based on the enzyme kinetics, the mU1-induced state of two-chain muPA therefore seems to be different from the mAb-112-induced state of human uPA. In addition, the epitope for mU1 is located in the 37- and 70-loops, whereas that of mAb-112 is localized in the autolysis loop (residues 142–152). But similarly to mAb-112, the variations in affinity of mU1 to specific conformations (full-length two-chain muPA, muPA(16–243), and single-chain muPA\textsuperscript{(ff)}(16–243)) are associated with variations in \( k_{\text{on}} \) but not \( k_{\text{off}} \) as measured by SPR (Table 3 and Figure 4). This observation is in good agreement with expectancies from a model, in which the antibodies bind to a pre-existing but variably populated conformation in the various enzyme preparations. In addition, X-ray crystal structure analysis of the complex between two-chain human uPA and a Fab fragment of mAb-112 showed that the S1 pocket of the enzyme is occluded, owing to a 9.7 Å displacement of the peptide segment Trp215–Arg217 and burial of the Arg217 side chain in the pocket,\textsuperscript{21} similar to the E\textsuperscript{a} states referred to above. Understanding the exact relation of the mU1-full-length muPA complex and muPA\textsuperscript{(ff)}(16–243) to the E\textsuperscript{a} conformations and the zymogen conformation will, however, have to await X-ray crystal structure analysis of an mU1-muPA complex. In general, the exact relationship between the E\textsuperscript{a} conformation and zymogen conformation is a matter of debate.\textsuperscript{57,58}

The fact that mU1 has a higher affinity for the target in the absence of the –16 to 15 residue stretch may give clues to its mechanism of action. According to our data, Phe\textsuperscript{(-2)} and Lys4 stabilize the structure of the catalytic domain in the active conformation. Residues in the region from position –2 to 15 of uPA are highly conserved among mammalian species, particularly Phe\textsuperscript{(-2)} and Lys4 (Figure 6A). Several three-dimensional structures of the catalytic domain of human uPA have been solved from crystals of proteins containing this stretch of residues (Figure 6B). Phe\textsuperscript{(-2)} engages in a hydrophobic interaction with Pro49 and Pro114 in human uPA, and both residues are conserved in muPA. Lys4 points inward, away from the solvent, forming a hydrogen bond to Glu137. Moreover, in active human single-chain and two-chain tissue-type plasminogen activator (tPA), but tPA as well as HGFA (Figure 6C), Arg4 forms a similar hydrogen bond.\textsuperscript{49,59–61} Accordingly, the quenching of these interactions by our mutations of muPA gives rise to a conformational change of the catalytic domain, which increases the affinity to mU1. One may therefore hypothesize that the residues contacting Phe\textsuperscript{(-2)} and Lys4 may be involved in the communication between the mU1 epitope and the active site. There is an extended hydrogen bonding network encompassing the activation loop, the 70-loop, the autolysis loop, and Asp194. A hydrophobic cluster composed of Phe30, Phe40, and Phe141 may also contribute to the coupling between the A-chain, the active site, and the autolysis loop. Disruption of the Lys4–Gly137 interaction could therefore affect the integrity of the activation pocket.

The inhibitory mechanism suggested here is completely different from the mechanism that can be derived from the crystal structure of the allosteric antibody hH35 in complex with hepsin\textsuperscript{20} and the crystal structure of the allosteric antibody Fab40 in complex with HGFA.\textsuperscript{19} The antibody hH35 binds to the 170-loop of hepsin and inhibits noncompetitively, whereas Fab40 binds to HGFA in the vicinity of the 99-loop and acts through a partially competitive inhibition mechanism. The functional epitope of mU1 is located more than 20 Å away from the active site of muPA compared to the distance of 15–20 Å reported for the structural epitope of hH35 on hepsin.\textsuperscript{20} Hence, its mode of inhibition and the long distance from the epitope to the active site makes the inhibitory mechanism of mU1 distinct from those of hH35 and Fab40.

Epitope mapping revealed overlapping binding sites for mU1 (Figure 5) and mU3,\textsuperscript{27} which is intriguing in light of the different functional effects of these two antibodies. The difference can be explained by the assumption that the enzyme exists in active and inactive distorted states in equilibrium with each other. The epitopes of the two antibodies may have different conformations in the different states, and the two antibodies lock the active and distorted, inactive states, respectively. Binding to one of the antibodies, mU1, shifts the equilibrium in the direction of the inactive, distorted state, whereas binding of the other antibody, mU3, shifts the equilibrium toward the active state. This assumption is in agreement with the observation that the two antibodies have slightly different epitopes. Lys72 and Pro78 were only a part of the mU1 epitope and not of the mU3 epitope. Furthermore, residues in the 37-loop are relatively more important for binding of mU3 than residues in the 70-loop. Compared to the inhibition mechanism of mU1, the slightly different epitope of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{The S1 specificity pocket and oxyanion hole are inaccessible in the presence of mU1. (A) Binding of pAB (20 μM) to full-length two-chain muPA (100 nM) in the presence of different concentrations of mU1 (0–500 nM), as determined by fluorescence spectroscopy. The result is a representative of at least three independent determinations (B) Incorporation of \(^{3}\text{H}\)-DFP (50 μM) into muPA\textsuperscript{(ff)}(16–243) (100 nM) for a 3 h incubation with the indicated concentrations of antibodies. Data are represented as the fractional binding compared to the sample without mU1. Data are means ± standard deviations of at least three independent determinations.}
\end{figure}
mU3 seemingly causes an opposite effect, in which the enzyme is stabilized in the active conformation instead of the distorted one, implying stabilization of the N-terminal Ile16 in the activation pocket and ordering of the active site. A similar situation has been reported with two antibodies (mAb-112 and mAb-12E6B10) binding to an overlapping epitope in human uPA. Although both mAb-112 and mAb-12E6B10 inhibited uPA-catalyzed plasminogen activation, only mAb-112 induced azymogen-like conformation in the enzyme.

Certain naturally occurring mutations in the region of thrombin with homology to position −2 to 15 of uPA cause serious bleeding phenotypes in patients, whereas mutation of Arg4 affects the proteolytic activity of thrombin. According to our data, proteolytic degradation of the region encompassing Phe(−2) and Lys4 or yet to be identified naturally occurring mutations of these residues in muPA and most likely other serine proteases, would favor a distorted state. Although such regulatory mechanisms remain to be identified, they may exist in vivo, under normal or pathological conditions. The presence of this distorted conformation in vivo is not only interesting from a mechanistic point of view but also because it improves the possibilities of therapeutic targeting of uPA. Conversion to the E* state is a new mode of pharmacological intervention with serine protease activity. mU1 is the first antibody described to stabilize this distorted state in a serine protease by targeting the 37- and 70-loops.

**ASSOCIATED CONTENT**

Supporting Information Derivatisation of formulas describing mU1 inhibition of murine urokinase-type plasminogen activator (uPA) are described in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

**ABBREVIATIONS**

DPF, diisopropyl fluorophosphate; EGR-cmk, glutamyl-glycyl-arginyl-chloromethyl ketone; HBS, HEPES-buffered saline; HGFA, hepatocyte growth factor activator; MALDI, matrix assisted laser desorption/ionization; muPA, murine urokinase-type plasminogen activator; pAB, p-aminobenzenamide; PBS, phosphate-buffered saline; PEI, polyethyleneimine; RU, response unit; SPR, surface plasmon resonance; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor

**ADDITIONAL NOTE**

“Template numbering which is based on chymotrypsin倾听. Residues N-terminal to Cys1 are negatively numbered, for example Phe(−2).

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Unraveling an Allosteric Regulatory Mechanism in Trypsin-like Serine Proteases by the use of a *Camelid* Derived Antibody Fragment

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Unraveling an allosteric regulatory mechanism in trypsin-like serine proteases by the use of a *Camelid* derived antibody fragment

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Abbreviations. uPA, urokinase-type plasminogen activator; EGR-cmk, H-Glu-Gly-Arg-chloromethylketone; Nb7, nanobody-7; r.m.s.d, root mean square deviation; V8, endoproteinase Glu-C; PAI-1, plasminogen activator inhibitor-1; HEK293, human embryonic kidney 293 cells; PBS, phosphate buffered saline; HBS, HEPES buffered saline; BSA, bovine serum albumin; CS-61(44), pyro-Glu-Gly-Arg-para-nitroaniline-HCl; TLCK, Tosyl-Lys-chloromethylketone; KCNO, potassium cyanate; FVIIa, Coagulation Factor VIIa.
Summary

Allosteric enzymes exist in equilibrium between maximally active and less active conformations. By differential binding to different conformations, ligands can shift the equilibrium and thus regulate activity. In this report, we have unravelled details of an allosteric regulatory mechanism for trypsin-like serine proteases. Solving the crystal structures of the catalytic domain of murine urokinase-type plasminogen activator (muPA) in the absence or presence of an inhibitory Camelid derived antibody fragment and in the absence or presence of active site ligands, we demonstrate that this protease can exist in a plethora of different conformations. The catalytic domain on its own crystallises in an inactive previously unknown conformation, in which the S1 entrance frame and the oxyanion stabilising loop have exchanges positions. In the presence of a antibody fragment, binding between the 37- and 70-loops, it crystallises in a conformation with strongly increased temperature factors of the active site; this conformation is converted to a normal active conformation by active site ligands. Our analysis revealed allosteric transitions involving disordering of surface-exposed loops distantly from the antibody and ligand binding sites. Moreover, it revealed glimpse of changes in conformational entropy in the enzyme along the allosteric trajectory. The antibody binding site overlaps with previously reported binding sites for regulatory agents; not only for muPA, but also for thrombin and coagulation factor VIIa. Thus, our findings suggest a common type of allosteric regulatory mechanism for trypsin-like serine proteases and provide a perspective to previously unknown conformations in trypsin-like serine proteases.

Keywords: Trypsin-like proteases, Allosteric regulation, Nanobodies, X-ray crystallography
Introduction

Allostery provides a conceptual framework for understanding the regulation of protein function. The prerequisite of allosteric regulation is that the allosteric protein exists in equilibrium between multiple conformational states and that protein function is regulated by a population shift towards active or less active states. The perturbation of this pre-existing equilibrium is often caused by ligands, which stabilises specific conformational states with distinct activities and functions (1-4). Recent studies, using NMR, have highlighted the importance of internal dynamics (conformational entropy) in regulation of protein activity, and demonstrated how binding of ligands to allosteric sites (i.e. a site distant from the active site) modulates the function of remotely located sites by redistribution of internal dynamics through the protein (5, 6). Questions arises about the molecular mechanisms of redistribution of internal dynamics upon binding of an allosteric ligand as it leads to distinct conformational changes from an inactive to an active state in an allosteric protein in a pre-existing conformational equilibrium (7, 8). Thus a prerequisite for understanding how allostery works at the molecular level is structural information about the active and inactive conformations.

Trypsin-like serine proteases are allosterically regulated enzymes involved in critical physiological processes, including digestion, blood coagulation, tissue remodelling, complement activation, and fibrinolysis (9-12). Trypsin-like serine proteases are secreted from cells as immature zymogens, which are activated by proteolytic cleavage between residues 15 and 16 (chymotrypsin numbering). A subsequent rearrangement of four surface-exposed loops (the N-terminal activation loop, the 180-loop, the 140-loop and the 220-loop), jointly referred to as the activation domain, is associated with the formation of an activation pocket in which the new N-terminus (residue 16) inserts itself and forms a salt bridge to Asp194 of the active site (13). This salt bridge is critical as it leads to a correctly assembled S1 specificity pocket and maturation of the active site. The structural architecture of the trypsin fold is that of two six-stranded β-barrels (residue 16-121 and 122-243) which comes together in order to form the catalytic triad (His57, Asp102 and Ser195) at their interface. The strands of the N- and C-
terminal β-barrels are connected by several surface-exposed loops, an ideal arrangement for transmitting long-range allosteric signals between distant surface-loops and across the two β-barrels. In fact trypsin-like serine proteases are characteristic examples of allosteric proteins in which a pre-existing conformational equilibrium is well established. Exemplified by the most studied trypsin-like serine protease thrombin, X-ray crystal structure analysis has only identified two distinct conformations (an active and inactive), although solution based techniques such as NMR suggests that thrombin exists as a conformational ensemble (14-16). Thus, in order to obtain information about alternative active and inactive conformations experimental data with other trypsin-like serine proteases is needed.

In this report we describe the allosteric trajectory as the murine trypsin-like serine protease urokinase-type plasminogen activator (muPA) interconverts between inactive and active conformations. First, we solved the X-ray crystal structure of a previously unidentified inactive conformation of muPA. Next, we used an allosteric inhibitory Camelid derived antibody fragment against muPA to facilitate crystallisation of muPA in the presence or absence of active site ligands. Our analysis revealed that the active conformation converts to the inactive conformation through redistribution of internal dynamics of the surface-exposed loops surrounding the active site. The allosteric mechanism presented in this report may represent a conserved allosteric regulatory mechanism in trypsin-like serine proteases as the antibody-binding site is overlapping with previously reported allosteric binding sites for thrombin and coagulation factor VIIa. Moreover, the information reported here is likely to broaden the unified view and understanding of allostery.
Results

**Structure of muPA crystallised in the absence of ligands.** Attempts to crystallise the catalytic domain of murine uPA (muPA) in the presence of the active site ligand Glu-Gly-Arg-chloromethylketone (EGR-cmk) did not result in crystal suitable for X-ray diffraction experiments. However, the catalytic domain of muPA crystallised readily in the absence of ligands. The X-ray crystal structure was determined to a resolution of 2.85 Å ($R_{\text{free}}=0.2678$ and $R_{\text{work}}=0.2221$) by molecular replacement, with four molecules in the asymmetric unit (table S1). It is evident from the crystal structure that several surface-exposed loop including the oxyanion stabilising loop (180-loop) and the 170-loop are involved in the formation of crystal contacts (figure S1). The structure of muPA revealed an unusual inactive conformation of the catalytic domain. Starting with the N-terminal β-barrel, the N-terminal activation loop (residue Ile16-Glu23), the 70-loop (residue Lys72-Asn77) and the autolysis loop (140-loop) (residue Lys143-Glu146), are highly disordered as electron density of these loops were completely missing. Only chain D could be described in details. We were only able to trace the N-terminal activation loop until Val24/D. This suggests that the N-terminal part of the activation loop is highly flexible and that Ile16 is not inserted into the activation pocket. The remainder of N-terminal β-barrel is in a conformation similar to that found in the ligand free form of human uPA (PDB:4dva) (17) (figure 1). In the C-terminal β-barrel, we observed major rearrangement of the surface-exposed loops surrounding the active site. Firstly, the tip of the 140-loop was not visible in the electron density indicating flexibility. Secondly, we observed a 180-degree swap of the β9-strand connecting the 170-loop and the 180-loop. The result of the β9-strand swap is a >20 Å repositioning of the 170- and 180-loops (Glu175/D Cα and Pro185a/D Cα) and a >7 Å relocation of the Cys191/D-Cys220/D disulphide bond (Cys191/D Cα), as compared to their position in human uPA. The β9-strand swap and the subsequent movement of the Cys191/D-Cys220/D disulphide bond are detrimental to the catalytic machinery of muPA. The Gly193-Ser195 segment moves > 9 Å (Asp194 Cα) into a non-catalytic conformation, which is incompatible with formation of the oxyanion hole and the salt-bridge between Aps194 and Ile16. Furthermore, the S1 specificity pocket,
jointly created by residues from Asp189-Lys192 and Ser214-Gly219, collapses completely as Asp189/D and Arg217/D moves >14 Å and >7 Å respectively. Thus, the observed conformation of muPA in the crystal structure is completely incompatible with substrate binding.

On the basis of the structural information we investigated, if the observed inactive conformation of muPA can convert into an active one, by investigating the activity of muPA from the crystals to form a covalent complex with plasminogen activator inhibitor 1 (PAI-1). Formation of such a complex requires a fully functional catalytic machinery. After dissolving the crystals, we analysed the complex-formation by SDS-PAGE. More than 90% of muPA from the crystals was found to be able to form a covalent complex with PAI-1, demonstrating that the inactive conformation in the crystal can convert to a fully active conformation (figure S2).

**Development and characterisation of an anti-muPA nanobody.** In order to obtain tools for crystallisation of muPA and to obtain muPA inhibitors with novel mechanisms of inhibition, a nanobody library was generated by immunization of an alpaca (*Vicugna pacos*) with recombinant muPA. Screening the library with muPA as bait, we recovered a nanobody (Nb7) that inhibits the proteolytic activity of muPA towards its macromolecular substrate plasminogen (figure 2a). Nb7 also inhibited the amidolytic activity of muPA towards the chromogenic peptide substrate pyro-Glu-Gly-Arg (CS-61(44)) in a mixed type of inhibition with a significant competitive component as only a slight decrease in $V_{\text{max}}^{\text{app}}$ was observed (table S2). Next, we mapped the binding epitope of Nb7 on muPA by generating a panel of alanine mutants of muPA. Subsequent differences between mutant and wild-type $IC_{50}$ values for Nb7 inhibition of CS-61(44) hydrolysis revealed the 37- and 70-loop as the Nb7 epitope (figure 2b and table S3).

The epitope of Nb7 is located >20 Å from the active site of muPA. Nevertheless, binding of Nb7 affects the active site as hydrolysis of CS-61(44) was inhibited. Thus Nb7 is an allosteric inhibitor. To analyse the allosteric effect of Nb7 we first used fluorescent spectroscopy to monitor changes in the S1 specificity pocket of muPA upon Nb7 binding (figure 2c). The reversible S1-binding ligand p-aminobenzamidine emits a low intensity fluorescent signal with emission $\lambda_{\text{max}}$ at
The binding of \( p \)-aminobenzamidine in the S1 specificity pocket of muPA results in a high intensity fluorescent signal with emission \( \lambda_{\text{max}} \) at 368nm. Adding Nb7 caused a reduced fluorescent intensity and a red shift in \( \lambda_{\text{max}} \) from 368nm to 375nm indicating a displacement of \( p \)-aminobenzamidine. Thus, binding of Nb7 to the 37- and 70-loop in muPA affects the function of the S1 specificity pocket more than 20 Å from the Nb7 epitope.

Next, we analysed the effect of Nb7 on the integrity of the active site architecture of muPA by measuring the susceptibility to chemical modification of the N-terminal Ile16 in the presence or absence of Nb7. If the N-terminal Ile16 is temporarily expelled from the activation pocket, it can be carbamylated by potassium cyanate (KCNO) leading to irreversible inactivation of the enzyme, as the modified Ile16 cannot reinsert. Hence, inactivation of muPA in the presence of KCNO is an indirect measurement of the integrity of the Ile16-Asp194 salt-bridge. The analysis revealed that the Ile16 is more frequently solvent-exposed in the presence of Nb7 as the activity of muPA decreases to 12% at 5 \( \mu \)M Nb7 (figure 2d).

**Crystal structures of muPA in complex with Nb7 and active site binding ligands.** The X-ray crystal structure of the catalytic domain of muPA in complex with Nb7 was solved to a 2.45 Å resolution (\( R_{\text{free}}=0.2309 \) and \( R_{\text{work}}=0.1880 \)) by molecular replacement with one muPA:Nb7 complex in the asymmetric unit (table S1). Nb7 (Chain B) covers 923.7 Å\(^2\) of accessible surface area on muPA (Chain A) and binds in a cavity created by the 37-, 70-, and the 140-loops in good agreement with the mutagenesis data (figure 3a, figure S3 and table S4). Comparing the conformation of muPA from the complex with Nb7 (muPANb7) to that of previously reported active conformations of human uPA (PDB:1lmw) (there is no structure of active muPA available) reveals that the 37-loop and 70-loop in muPANb7 undergoes a 4.4 Å and 14 Å conformational change. The 14 Å “outward” movement of the 70-loop exposes a hydrophobic cavity into which the CDR-H3 loop of Nb7 becomes inserted (figure 3b). Inspection of the catalytic machinery revealed that the active site architecture of muPANb7 is preserved as compared to human uPA (figure 3b). As such, the Ile16/A-Asp194/A salt-bridge, and the S1
specificity pocket was formed indicating that the muPA_{Nb7} is in an active conformation as far as the S1 pocket and the oxyanion hole is concerned.

To resolve if the muPA_{Nb7} conformation is able to bind active site ligands, we redetermined the X-ray crystal structures of the muPA:Nb7 complex after soaking with the irreversible active site binding inhibitor EGR-cmk or the reversible S1-binding inhibitor p-aminobenzamidine. The crystal structures were solved to 2.4Å (R_{free}=0.2280 and R_{work}=0.1921) and 2.4Å (R_{free}=0.2352 and R_{work}=0.2143) resolution by molecular replacement (table S1). Electron density for EGR-cmk or p-aminobenzamidine was clearly visible in the two structures (figure 3c and 3d). EGR-cmk was covalently linked to His57/A and Ser195/A, and binds in a conventional substrate-like mode at the S1 and S2 binding sites similar to that observed for human uPA in complex with EGR-cmk (PDB:1lmw). However, we observed electron density for a sulphate ion, which mediated the binding of the Glu-moiety of EGR-cmk to Arg217/A and Gly218/A at the S3 site. Similarly, we also observed a sulphate ion in the S3 pocket after soaking with p-aminobenzamidine, which binds into the S1 specificity pocket forming a salt-bridge to Asp189/A.

To detect any differences between overall highly similar conformations of muPA from the three crystal structures, we compared the individual temperature factors for the Ca-atoms in search of structural elements, which are disorder in the muPA_{Nb7} conformation, but become more ordered as EGR-cmk or p-aminobenzamidine binds in the active site of muPA. The analysis highlights four regions: the N-terminal activation loop (residues 16-22), the 140-loop (residue 142-152), the 180-loop (residue 184-194) and the 220-loop (residue 216-223). These surface-exposed loops are exactly the regions originally defined as the activation domain in trypsin-like serine proteases. Whereas the ordering of the 180- and 220-loops was predictable, due to the stabilising effects of the ligands, we furthermore observed an ordering of more remote surface-exposed loop such as the N-terminal activation loop and the 140-loop. The temperature factor analysis identified a cluster of amino acids, which interconnects the loops of the activation domain. Especially Lys143 at the N-terminal stem of the 140-loop and Lys192 in vicinity of the active site and S1 pocket is highlighted by our analysis. Lys143 connects the 140-loop to the N-terminal activation loop and to the 180-
loop by hydrogen bonds to Ile16 and Lys192. Lys192 is adjacent to the Cys191-Cys220 disulphide bond, which connects the 180-loop to the 220-loop (figure 4 and figure S4). Thus, a local change in dynamics at the S1 pocket by e.g. the reduction of conformation entropy by the stabilising effects of EGR-cmk and p-aminobenzamidine propagates to the N-terminal stem of the 140-loop through the Cys191-Cys220 disulphide bond to Lys192, which is in direct contact with Lys143. However, the allosteric signal is not unidirectional, and changes in dynamics to the N-terminal stem of the 140-loop are likely to affect the function of the S1 pocket utilising the same allosteric pathway.

**Limited proteolysis.**

We evaluated the observed disorder of the surface-exposed loops in muPA by performing limited proteolysis of muPA by endoproteinase Glu-C (Endo Glu-C) (figure 5) (18). After incubation of muPA with Endo Glu-C in the absence or presence of Nb7 and EGR-cmk, SDS-PAGE analysis revealed a single primary cleavage site in muPA, resulting in two major cleavage products with molecular weights of 15kDa and 12kDa receptively. N-terminal sequencing of the two cleavage products revealed Glu146 in the 140-loop as the Endo Glu-C cleavage site. The 15kDa fragment starts at the native N-terminus Ile16 (I16VGGEFT22), whereas the 12kDa fragments starts at Ser147 (S147DYLY151) in the 140-loop. In the presence of Nb7 the Glu146-Ser147 peptide bond becomes more frequently exposed as cleavage by Endo Glu-C is accelerated. In contrast Nb7 had no effect on the cleavage by Endo Glu-C when using EGR-cmk active site inhibited muPA. This observation is in good agreement with the structural observations that Nb7 increases the flexibility of the 140-loop, and with the notion that EGR-cmk reverse the effect of Nb7 by reducing the flexibility of the 140-loop.
Discussion

The ensemble view of allostery has been adopted to account for the trypsin-like serine proteases. Solution based techniques such as NMR has demonstrated how the trypsin-like serine protease thrombin in its ligand free form exists as a conformational ensemble between zymogen-like and proteinase-like conformations (15, 19). However, X-ray crystal structure analysis using wild-type or thrombin mutants has only identified two distinct proteinase-like conformations; an active and an inactive, the so-called E and E* conformations (16). The structural distinction between the E and E* conformation is a >6 Å movement of the 214-217 segment which occludes the S1 pockets in the E* conformation. Our present results add to the hypothesis of allosteric regulation of trypsin-like serine proteases depending on an ensemble of different conformations and provide molecular details about the interconversion between two representatives of these conformations. Here we report the structure of a previously unidentified E* conformation of muPA, which seems to constitute a sparsely populated conformation in equilibrium with a fully active E conformation represented by the crystal structures of muPA in complex with Nb7 in the absence or presence of active site ligands. We have previously presented evidence that muPA exists in equilibrium between active and inactive conformations in solution. One of the major distinctions between the active and inactive conformations was that the N-terminal Ile16 was more frequently solvent exposed in the inactive ones. Active site ligands or the monoclonal antibody mU3 shifted the equilibrium towards the active state whereas destabilising mutations or the monoclonal antibody mU1 shifted the equilibrium towards the inactive state (20, 21). Our structural identification of a strongly disordered E* conformation in muPA, in which Ile16 is not inserted in the activation pocket is likely to represent one such inactive state. In agreement with the hypothesis of a pre-existing equilibrium, this E* conformation interconverts with a fully active E conformation, as The E* conformation from dissolved crystals were able to form a covalent complex with PAI-1. Moreover, this observation suggests that the equilibrium is strongly shifted towards the active states in solution, whereas the sparsely populated inactive conformational state is favoured in the crystal, stabilised by crystal contacts involving the 170- and the 180-loops.
The X-ray crystal structure of muPA in complex with the *Camelid* derived antibody fragment Nb7 reveals that Nb7 traps an “open” conformation of the 37- and 70-loops, in which the 70-loop is displaced by 14 Å. The consequence of this loop movement, as evaluated biochemically, was a conformation of muPA in which the 140-loop is highly flexible and the oxyanion hole destabilised due to lack of incorporation of the N-termius Ile16 in the activation pocket. Moreover, the S1 specificity pocket was also found to be non-functional. These Nb7-stabilised structural changes were not fully revealed by the crystal structure of muPA in complex with Nb7, because crystal-packing effects caused by two Nb7 molecules from adjacent asymmetric units, resulting in ordering of the C-terminal β-barrel (figure S5). Nevertheless, the properties of Nb7 allowing crystallisation of muPA also provided the unique opportunity to follow subtle changes to the enzyme in the presence or absence of active site binding ligands. As evaluated by the temperature factor analysis, we observed a tight interconnectivity between the surface-exposed loops of the activation domain. Binding of EGR-cmk or *p*-aminobenzamidine into the S1 specificity pocket affected not only the 180- and 220-loops through stabilising hydrogen bonds; the stabilising effect also propagated to the N-terminal activation loop and the 140-loop. We believe that this redistribution of internal dynamics (conformational entropy) is not unidirectional. Thus any changes to the dynamics of the N-terminal activation loop or the 140-loop is also likely to affect the 180- and 220-loops of the S1 specificity pocket. Moreover, our analysis identified the stems of the 140-loop as a key element for the interconnectivity of the N- and C-terminal β-barrel.

This dynamic model provides an interesting perspective to allosteric regulation of trypsin-like serine proteases, as a prerequisite for the existence of the structurally observed E* conformation of muPA is an unusually high flexibility of the surface-exposed loops of the activation domain. It is tempting to consider our observations with Nb7 in order to propose a mechanism for the propagation of the allosteric signal as muPA interconverts between the E and E* conformation (figure 6). Based on the assumption of a pre-existing equilibrium of conformations, we believe that the 70-loop of muPA samples various conformational states, analogues to suggestions made on the basis of observation with the homologous exosite I of thrombin (19). In the more “closed” conformations, which
is observed in the ligand free form of human uPA (PDB:4dva), we expect the 70-loop to restrain the dynamics of the activation domain as it engages in both polar and non-polar interactions with Lys153 and Phe141 at the stems of the 140-loop. However, in the more “open” conformations, the dynamics (conformational entropy) at the stems of the 140-loop is increased and is likely to propagate throughout the activation domain. Although the propagation of the allosteric signal is likely to be a coherent sequence of events traveling through multiple pathways (22), our analysis highlighted Lys143 as a key residue for the propagation. Disruption of the Lys143-Lys192 and Lys143-Ile16 hydrogen bonds would weaken the insertion of Ile16 into the activation pocket and the connectivity between the 140-loop and the 180-loop, thus allowing the 180° swap of the β9-strand and the concomitant rearrangement of the 170-loop, the 180-loop and the Cys191-Cys220 disulphide bond as observed in the E* conformation of muPA.

It is appealing to place our findings in the context of ligand recognition, as allosteric regulation of activity in trypsin-like serine proteases by natural and non-natural ligands to the 37- and 70-loops has been described previously (19-21, 23, 24). Solution-based and computational techniques demonstrate how the ligands control enzyme activity by changing the dynamics of the surface-exposed loops surrounding the active site (19, 25). Only in a single case has a plausible structural mechanism underlying these long-range communications been proposed, by a crystal structure of the thrombin mutant D102N in complex with the exosite I binding extracellular fragment of protease activated receptor 1 (PAR-1) (26). In contrast to Nb7, PAR-1 pre-selects binding to an active E conformation of thrombin D102N in which the 70-loop is in the “closed” conformation. The active site was found to be accessible to substrates, thus PAR-1 is believed to allosterically regulates its own cleavage by stabilising an active E conformation of thrombin. As the free form of thrombin D102N crystallises in an inactive E* conformation, a direct comparison revealed how PAR-1, through the 140-loop, restores the catalytic machinery of thrombin by organising the 180- and 220-loops. The 140-loop is also central to the allosteric mechanism of the peptidic inhibitor E-76, which binds to the 37- and 70-loops of coagulation factor VIIa (23). However, changes to other surface-exposed loops might have been obliterated by the presence of the active site ligand D-FFRCKMK used in the crystallisation. Together
with the results presented here these similar findings argues for a conserved allostERIC mechanism by which ligands to the 37- and 70-loop regulates the activity of trypsin-like serine proteases. Although the exact biological role of the 37- and 70-loop exosite in uPA remains to be identified, our previous and present findings demonstrate the possible existence of such role, as non-natural ligands to the 37- and 70-loops is able to stabilise both active as well as inactive conformations (20, 21).

In conclusion, we have reported the underlying mechanisms along the allostERIC trajectory as muPA interconverts between two structurally determined representatives active (E) and an inactive (E*) conformation. Our analysis revealed that the allostERIC signal is likely to propagate through the surface-exposed loops of the activation domain by redistribution of internal dynamics eventually resulting in a conformational change associated with the allostERIC transition. The detection of such subtle changes to the dynamics was only possible due to the availability of an allostERIC inhibitory nanobody, which allowed the crystallisation of muPA at different ligation states. Together our results provide a perspective of how altered distribution of internal dynamics can contribute to protein function through distinct conformational changes.
Online Methods

Production and purification of muPA. Full-length muPA was produced in HEK293 6E suspension cells and purified by nickel-Sepharose chromatography as described previously (21). For production of the catalytic domain, cDNA encoding from residue Gly2 to Gly244 with a C122A mutation was subcloned into a T7 derived expression vector with six histidines at the N-terminus, and expressed as inclusion bodies in E.coli BL21(DE3). For refolding of muPA E.coli cells were resuspended in sonication buffer (50 mM Tris pH 8.0; 0.5M NaCl; 10% glycerol; 1 mM beta-mercaptoethanol; 1 mM EDTA) and sonicated on ice (pulses of 0.8, amplitude 100). The lysed cells were centrifuged 10000 RPM, 4 °C for 10 min and the inclusion bodies were washed in sonication buffer supplemented with 1% Triton X-100. Two additional washes were performed with 0.25% and 0% Triton X-100 respectively. Finally the inclusion bodies was resuspended in denaturation buffer (50 mM Tris pH 8; 100 mM NaCl; 10 mM b-mercaptoethanol; 6 M urea; 1 mM EDTA) and denatured by slow stirring at 4 °C. Next the protein concentration was adjusted to below 0.2 mg/mL in denaturation buffer and dialysed against 10 L of refolding buffer (50 mM Tris pH 8; 1 mM b-mercaptoethanol; 3 M urea; 10 % glycerol) at 4 °C for 22 h. Urea was removed by dialysis against 2x10 L of buffer containing 50 mM Tris pH 8 and 10% glycerol at 4 °C for 22 h. After solubilisation and refolding muPA was subsequently captured on nickel-sepharose and eluted in 50 mM Bicine pH 8; 500 mM NaCl and 400 mM imidazole and dialyzed extensively against PBS (10 mM Na2HPO4; 1.8 mM KH2PO4; 2.7 mM KCl; 137 mM NaCl; pH 7.4). The protein concentration was adjusted to 0.5 mg/mL and incubated with 2.5 µg/mL plasmin at 22 °C for 22h. The incubation with plasmin ensures correct cleavage between Lys15 and Ile16 to generate the catalytic domain. Plasmin was removed by passing the sample over a CnBr (GE Healthcare) activationd aprotinin Sepharose column. To remove non-activated or misfolded protein benzamidine-Sepharose (GE Healthcare) chromatography was applied. Finally the active catalytic domain was purified by size-exclusion chromatography on a Superdex 75 equilibrated with PBS supplemented with 300 mM NaCl. Protein purity was verified by SDS-PAGE gel analysis.
**Generation of anti-muPA nanobodies.** The immunization and construction of the nanobody phage library was conducted as described previously (27). Selection of anti-murine nanobodies was performed by immobilizing muPA (100 µg/mL) in 96-well MaxiSorp immunoplates (Nunc). After adding the nanobody phage library bound phages were eluted with triethyleamine (100 mM) and neutralized with 1 M Tris pH 8.2. Recovered phages were amplified in *E.coli* TG1 cells. During three subsequent selection rounds, the stringency of plate washing was gradually increased. muPA binding nanobodies were identified by a polyclonal phage ELISA by randomly picking single colonies. Positive clones were sequenced and unique clones were transformed into *E.coli* WK6 (su⁻) cells and produced as described previously (28).

**Enzymatic assays.** All enzymatic assays were performed in HEPES buffered saline (HBS; 10 mM HEPES pH 7.4; 140 mM NaCl) with 0.1% bovine serum albumin (BSA). For the plasminogen activation assay various concentrations of Nb7 (4-0 µM) was preincuated with muPA (1 nM) for 15 min at 37 °C. Human plasminogen (100 nM), purified from plasma, was added and the mixture were incubated for 30 min before quenching the activity of muPA with the peptidic muPA inhibitor mupain-1-16 (25 µM) (29). The chromogenic plasmin substrate S-2251 (chromogenix) was added to 0.5 mM. Evaluation of the inhibition mechanism was conducted by preincubating muPA (2 nM) with various concentrations of Nb7 (1-0 µM) for 15 min at 37 °C. CS-61(44) (24-0 µM) was added to initiate reactions. $V_{max}^{app}$ and $K_M^{app}$ were determined by fitting the initial reaction velocities to standard Michaelis Menten kinetic equations. For inhibition of Pyro-Glu-Gly-Arg-pNa-HCL (CS-61(44), Hyphen Biomed), various concentration of nanobodies (4-0 µM) were preincubated with muPA (2 nM) or muPA alanine mutants for 15 min at 37 °C. CS-61(44) were added to 750 µM.

In all cases the initial velocities was monitored at an absorbance of 405 nm for 1 hour at 37 °C in a kinetic microplate reader (Multiscan Go, Thermo Scientific). The $IC_{50}$ values were calculated by fitting the progress curves to the 4-parameter logistic model by nonlinear regression.
**Fluorescent assay.** muPA (0.23 µM) was mixed with Nb7 (3 µM) and p-aminobenzamidine (60 µM) and incubated for 15 min at 22 ºC. Fluorescence emission spectrums were recorded at 25 ºC on a PTI quantamaster spectrofluorometer in a 2 mm x 10 mm semi-micro quarts cuvette. An emission scan of 340 – 400 nm using an excitation wavelength of 335 nm and an integration of 1-2 s over a 1.0 nm step resolution was used. The buffer used was HBS supplemented with 0.1 % polyethyleneglycol 8000. The active site binding peptidic inhibitor mupain-1-16 was used as a positive control to indicates full displacement of p-aminobenzamidine.

**Carbamylation.** muPA (0.5 µM) was incubated with Nb7 (5-0 µM) or a control nanobody (5-0 µM) for 15 min at 22 ºC in HBS supplemented with 0.1% polyethyleneglycol 8000. Potassium cyanante (0.4 µM) (KCNO) or buffer was added as indicated in figure 2b and the mixtures were incubated for 4 h at 22 ºC. The carbamylation reaction was stopped by diluting the samples 300-fold in HBS supplemented with 0.1% BSA. Dissociation of Nb7 was allowed for 2 h before addition of CS-61(44) (750µM) to measure residual muPA activity.

**Crystallography.** For crystallization of the ligand free form of muPA 10 mg/mL of the protein was mixed in a 1:1 drop ratio with 100 mM HEPES, pH7.4 and 1.8 M Li₂SO₄. The muPA:Nb7 complex was prepared in a 2-fold molar excess of Nb7. The complex was purified on a Superdex 75 and verified by SDS-PAGE analysis. The complex was crystallised using 6 mg/mL of the complex in a 1:3 drop ratio with 100 mM HEPES pH 7.4 and 1.6 M Li₂SO₄. Crystals of active site occupied muPA was prepared by soaking muPA:Nb7 crystals with 1 mg/mL H-Glu-Gly-Arg-chloromethylketone (EGR-cmk) or p-aminobenzamidine for 24 h before harvesting the crystals. All crystals were grown by hanging-drop vapour diffusion Crystals were cryoprotected in mother liquid supplemented with 20 % (v/v) ethylene glycol and flash-frozen in liquid nitrogen. Datasets were collected at 100 K at a wavelength of 1.54 Å at the I911-2 beamline at MAX-lab (Lund, Sweden). Individual datasets were merged and processed with XDS, and solved by molecular replacement in Phaser (30) using a search model of human uPA (PDB: 4dva) (17). The initial model was build using phenix.autobuild, (31) and
further improved by manual building in Coot (32) and refined in phenix.refine using auto defined TLS refinement. The structures were validated using MolProbity (33). For ligand free muPA 92.9 % was in the favoured region in the Ramachandran plot, whereas 5.3% was in the allowed region and 1.8% was outliers. For 96.3% was in the favoured region, 3.17% was in the allowed region and 0.53% was outliers. For muPA:Nb7, muPA:Nb7:EGR-cmk and muPA:Nb7:p-aminobenzamidine the values were 96.3%, 3.17%, 0.53% and 97.35%, 2.12%, 0.53% and 97.62%, 1.85%, 0.53%, respectively.

**SDS-PAGE analysis.** Protein crystals of ligand free muPA were dissolved by moving the crystals from the hanging drop to a 2 µL drop containing mother liquid. After two consecutive washes in drops containing 2 µL mother liquid, the crystals were finally dissolved by transferring the crystals to a buffer containing 30 mM HEPES pH 7.4, 140 mM NaCl. For the SDS-PAGE analysis muPA (2 µg), either from the dissolved crystals or from the stock solution used for crystallisation, was incubated for 1 h at 22 °C with 2-fold molar excess of PAI-1. The mixtures were separated by 11 % non-reduced SDS-PAGE analysis.

**Limited Proteolysis.** muPA (0.3 mg/mL) were preincubated with or without Nb7 (0.3 mg/mL) for 15 min at 22 °C. Endoprotinase Glu-C (Roche, Switzerland) was added to 0.03 mg/mL. The reactions were stopped at indicated timepoints with Tos-Lys-chloromethylketone-HCL (Bachem, Switzerland) (1 mM). The buffer used was 25 mM ammonium carbonate pH 7.8. The digestion products were analysed by reducing 18% SDS-PAGE analysis and the density of the band was quantified by densitometry using the GelEval software. The two major cleavage products at 15kDa and 12kDa was N-terminal sequenced at the Department of Molecular Biology and Genetics, Aarhus.
References


Figure 1. The crystal structure of an inactive conformation of muPA. a. The overall conformation of the catalytic domain of muPA (grey) in the absence of ligands. b. The overall conformation of human uPA in the absence of ligands (pdb: 4dva). The black dashed line indicates the N- and C-terminal β-barrels. Red spheres highlight residues and areas discussed in the main text. The β9-strand is coloured red to highlight the swap of this strand between the two conformations.
Figure 2. Biochemical characterisation of the effect of the inhibitory nanobody Nb7 on muPA. a. Inhibition of plasminogen (100 nM) activation by muPA (1 nM) in the presence of Nb7 (4-0 µM). The $IC_{50}$-value was determined to 121 ± 20 nM by non-linear regression. Error bars represent standard errors for three independent experiments. b. The Nb7 binding epitope was mapped by measuring the inhibition of CS-61[44] (750 µM) hydrolysis of various muPA alanine substitution mutants (2 nM) in the presence or absence of Nb7 (4-0 µM). The epitope is shown on a homology model of muPA. Blue residues could be mutated to Ala without affecting the $IC_{50}$. Mutation of red affected the $IC_{50} > 50$-fold, Orange > 5-fold, Green < 0.5-fold. The active site residues His57 and Ser195 is indicated in yellow. c. Fluorescent spectroscopy of p-aminobenzamidine (60 µM) in the presence or absence muPA (0.5 µM) and Nb7 (3 µM) as indicated. The active site binding peptidic inhibitor mupain-1-16 (3 µM) was included as a positive control. The data is representative for three independent experiments. c. Analysis of the susceptibility of the N-terminus Ile16 of muPA (0.5 µM) to chemical modification by potassium cyanate (0.2 M) (KCNO) in the presence or absence of Nb7 (4-0 µM) or an irrelevant control nanobody.
Figure 3. Crystal structures of muPA in complex with Nb7 and active site ligands. 

a. Overall structure of the complex between muPA (wheat) and Nb7 (red) 
b. Alignment of the conformation of the 37- and 70-loops, and residues from the catalytic machinery from the muPANb7 conformation with the corresponding structural elements in active human uPA (green) (PDB: 1lmw). 
c. and d. Alignment of the muPA:Nb7 complex (wheat) to the d. muPA:Nb7 complex after soaking with EGR-cmk (yellow and cyan) and to c. muPA:Nb7 complex after soaking with p-aminobenzamidine (orange and magenta). The 2mF0-DFc electron density map (blue) for EGR-cmk, p-aminobenzamidine, waters and sulphate ions is contoured at σ=1. Waters are coloured blue and represented as spheres. Hydrogen bond are shown as red dashed lines, and the sulphate ions are shown as sticks.
Figure 4. Temperature factor analysis of Nb7:muPA complexes. The b-factor for the Cα atoms in the three complexes were scaled to the average b-factor for all Cα atoms for each individual structure. The scaled b-factors were compared between a. muPA:Nb7 and muPA:Nb7:EGR-cmk or b. muPA:Nb7 and muPA:Nb7:p-aminobenzamidine and plotted as relative scaled b-factors for each residue. Arrows and labels indicate areas and residues highlighted in the cartoon representation of the b-factor for individual Cα atoms in c. muPA:Nb7 and d. muPA:Nb7:EGR-cmk and e. muPA:Nb7:p-aminobenzamidine.
Figure 5. Limited proteolysis of muPA in the absence or presence of Nb7. a. muPA (0.3 mg/mL) was incubated with endoproteinase Glu-C (0.03 mg/mL) in the presence or absence of Nb7 (0.3 mg/mL). The reactions were quenched with TLCK (1 mM) and analysed by Reducing SDS-PAGE. The N-terminal sequence of the 12kDa and 15kDa band is indicated to the right. b. Quantification of the intact muPA band at 28kDa at different time points from different experiments as indicated. Error bars represent standard errors for three independent experiments.
Figure 6. Simplified cartoon of conformational equilibria in muPA. It is suggested that muPA exists in a conformational equilibrium between active (E) and inactive states (E*), with the equilibrium shifted towards the E state. The E and E* states should not be considered as one distinct conformation but as an ensemble of conformations interconverting on a fast time scale (dashed lines). Our representative of an inactive conformation is characterised by more frequently solvent-exposed Ile16 a disordered S1 specificity pocket. Substrates (E:S, red circle) reduce the flexibility of the E conformation, but the surface-exposed loops such as the 70-loop remains flexible. Nb7 (red cartoon) bind to all of these conformations in which the 70-loop is in the “open” conformation, and shifts the equilibrium towards the E* conformation. However, if the Nb7 stabilised conformation is similar to the inactive conformation of muPA observed in the crystal structure remains elusive. The number in parentheses indicated that a representative of that muPA conformation has been solved by X-ray crystallography.
Supporting Information

Unraveling an allosteric regulatory mechanism in trypsin-like serine proteases by the use of a *Camelid* derived antibody fragment

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### Table S1. Crystallographic data for the muPA, muPA:Nb7, muPA:Nb7:EGR-cmk, muPA:Nb7:p-aminobenzamidine.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>muPA</th>
<th>muPA:Nb7</th>
<th>muPA:Nb7:EGR-cmk</th>
<th>muPA:Nb7:p-aminobenzamidine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
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<td>P3_2:1</td>
<td>P3_2:1</td>
<td>P3_2:1</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td>a, b, c (Å)</td>
<td>194.6, 194.6, 36.9</td>
<td>94.45, 94.49, 121.3</td>
<td>94.24, 94.24, 122.98</td>
</tr>
<tr>
<td></td>
<td>α, β, γ (°)</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
<td>90, 90, 120</td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>38.66-2.85(2.927-2.85)</td>
<td>19.63-2.45(2.538-2.45)</td>
<td>38.73-2.401(2.487-2.401)</td>
<td>49.11-2.4(2.486-2.4)</td>
</tr>
<tr>
<td><strong>Rmerge</strong></td>
<td>0.09945(1.375)</td>
<td>0.09901(1.748)</td>
<td>0.1028(2.142)</td>
<td>0.1064(2.775)</td>
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<tr>
<td><strong>I / σI</strong></td>
<td>18.36(1.90)</td>
<td>18.48(1.69)</td>
<td>17.61(1.56)</td>
<td>17.65(1.29)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>98.75(99.19)</td>
<td>99.7(100)</td>
<td>99.8(99.7)</td>
<td>99.4(99.8)</td>
</tr>
<tr>
<td><strong>Redundancy</strong></td>
<td>3.8(3.8)</td>
<td>10.4(10.5)</td>
<td>6.8(6.8)</td>
<td>7.0(7.1)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td>Resolution (Å)</td>
<td>38.66-2.85</td>
<td>19.63-2.45</td>
<td>38.73-2.401</td>
</tr>
<tr>
<td></td>
<td>No. reflections</td>
<td>35924(3549)</td>
<td>23482(2302)</td>
<td>25164(2458)</td>
</tr>
<tr>
<td></td>
<td>Rwork / Rfree</td>
<td>0.2221/0.2678</td>
<td>0.1880/0.2309</td>
<td>0.1921/0.2280</td>
</tr>
<tr>
<td></td>
<td>No. atoms</td>
<td>7289</td>
<td>3082</td>
<td>3155</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>7124</td>
<td>2933</td>
<td>2958</td>
</tr>
<tr>
<td></td>
<td>Ligand/ion</td>
<td>125</td>
<td>30</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>40</td>
<td>119</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>B-factors</td>
<td>Protein</td>
<td>82.5</td>
<td>66.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ligand/ion</td>
<td>159.9</td>
<td>102.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water</td>
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<td>63.6</td>
</tr>
<tr>
<td></td>
<td>R.m.s. deviations</td>
<td>Bond lengths (Å)</td>
<td>0.006</td>
<td>0.008</td>
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<tr>
<td></td>
<td></td>
<td>Bond angles (°)</td>
<td>1.42</td>
<td>1.18</td>
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</table>

*Data collected for two crystals. *Values in parentheses are for highest-resolution shell.
**Table S2.** The effect of Nb7 on $V_{max}^{app}$ and $K_M^{app}$ for hydrolysis of the chromogenic substrate CS-61(44).

<table>
<thead>
<tr>
<th>Nb7 (nM)</th>
<th>$V_{max}^{app} \times 10^{-7}$ (M/s)</th>
<th>$K_M^{app}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.62 ± 0.28</td>
<td>3.6 ± 1.3</td>
</tr>
<tr>
<td>31.25</td>
<td>1.50 ± 0.27</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>62.5</td>
<td>1.41 ± 0.28</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>125</td>
<td>1.36 ± 0.28</td>
<td>6.3 ± 1.5*</td>
</tr>
<tr>
<td>250</td>
<td>1.15 ± 0.25*</td>
<td>7.4 ± 1.0*</td>
</tr>
<tr>
<td>500</td>
<td>1.05 ± 0.25*</td>
<td>8.9 ± 1.8*</td>
</tr>
<tr>
<td>1000</td>
<td>0.97 ± 0.17*</td>
<td>13.5 ± 1.0*</td>
</tr>
</tbody>
</table>

* Significantly different from the value at 0 nM Nb7 (p < 0.01). All p-values were calculated with Student’s t-test. The data are reported as the means ± standard deviations of four independent experiments.
Table S3. *IC*$_{50}$*-*values for inhibition by Nb7 of hydrolysis of the chromogenic substrate CS-61(44) by muPA alanine mutants. n.d., not determined. The data are reported as the means ± standard deviations of three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (nM)</th>
<th>$IC_{50}^{mutant}$/IC$_{50}^{wt}$</th>
<th>$K_M$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine uPA</td>
<td>71 ± 1</td>
<td></td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>E70A</td>
<td>97 ± 28</td>
<td>1.4 ± 0.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>F21A</td>
<td>29 ± 4</td>
<td>0.41 ± 0.06</td>
<td>n.d.</td>
</tr>
<tr>
<td>T22A</td>
<td>49 ± 5</td>
<td>0.68 ± 0.07</td>
<td>n.d.</td>
</tr>
<tr>
<td>E23A</td>
<td>232 ± 52</td>
<td>3.2 ± 0.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>F30A</td>
<td>7 ± 2</td>
<td>0.10 ± 0.03</td>
<td>4.4 ± 2.2</td>
</tr>
<tr>
<td>K37aA</td>
<td>263 ± 62</td>
<td>3.7 ± 0.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>G37bA</td>
<td>85 ± 9</td>
<td>1.2 ± 0.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>G37cA</td>
<td>579 ± 44</td>
<td>8.2 ± 0.5</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>S37dA</td>
<td>225 ± 45</td>
<td>3.2 ± 0.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>P37eA</td>
<td>&gt;4000</td>
<td>&gt;50</td>
<td>2.1 ± 0.07</td>
</tr>
<tr>
<td>P38A</td>
<td>&gt;4000</td>
<td>&gt;50</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>F40A</td>
<td>10 ± 1</td>
<td>0.15 ± 0.01</td>
<td>5.1 ± 0.9</td>
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<tr>
<td>K41A</td>
<td>78 ± 4</td>
<td>1.1 ± 0.04</td>
<td>2.5 ± 0.2</td>
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<tr>
<td>S71A</td>
<td>&gt;4000</td>
<td>&gt;50</td>
<td>n.d.</td>
</tr>
<tr>
<td>K72A</td>
<td>68 ± 13</td>
<td>1.0 ± 0.2</td>
<td>2.5 ± 0.3</td>
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<tr>
<td>E73A</td>
<td>983 ± 196</td>
<td>14 ± 2.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>S74A</td>
<td>46 ± 7</td>
<td>0.7 ± 0.1</td>
<td>2.8 ± 0.03</td>
</tr>
<tr>
<td>Y76A</td>
<td>&gt;4000</td>
<td>&gt;50</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>N77A</td>
<td>112 ± 16</td>
<td>1.6 ± 0.2</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>P78A</td>
<td>&gt;4000</td>
<td>&gt;50</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>K82A</td>
<td>&gt;4000</td>
<td>&gt;50</td>
<td>n.d.</td>
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<tr>
<td>Y93A</td>
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<td>1.4 ± 0.5</td>
<td>n.d.</td>
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<tr>
<td>Y94A</td>
<td>107 ± 34</td>
<td>1.5 ± 0.5</td>
<td>n.d.</td>
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<tr>
<td>Y99A</td>
<td>116 ± 6</td>
<td>1.6 ± 0.1</td>
<td>3.6 ± 0.3</td>
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<tr>
<td>E96A</td>
<td>159 ± 8</td>
<td>2.2 ± 0.1</td>
<td>n.d.</td>
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<td>H100A</td>
<td>155 ± 39</td>
<td>2.2 ± 0.5</td>
<td>n.d.</td>
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<td>T110A</td>
<td>141 ± 28</td>
<td>2.0 ± 0.4</td>
<td>n.d.</td>
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<td>S110aA</td>
<td>84 ± 14</td>
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<td>n.d.</td>
</tr>
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<td>F141A</td>
<td>6 ± 1</td>
<td>0.09 ± 0.02</td>
<td>n.d.</td>
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<td>K143A</td>
<td>33 ± 1.1</td>
<td>0.46 ± 0.02</td>
<td>n.d.</td>
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<td>E144A</td>
<td>126 ± 48</td>
<td>1.8 ± 0.7</td>
<td>n.d.</td>
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<tr>
<td>E146A</td>
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<td>n.d.</td>
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<tr>
<td>Y149A</td>
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<td>3.6 ± 0.5</td>
<td>1.4 ± 0.3</td>
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<td>Y151A</td>
<td>105 ± 32</td>
<td>1.5 ± 0.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>K153A</td>
<td>58 ± 4</td>
<td>0.82 ± 0.06</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>N154A</td>
<td>387 ± 110</td>
<td>5.5 ± 1.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>E222A</td>
<td>235 ± 73</td>
<td>3.3 ± 1.0</td>
<td>n.d.</td>
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</table>
**Table S4.** Residue contact analysis between Nb7 and muPA. The contact residues were analysed using the online PISA server located at [www.ebi.ac.uk](http://www.ebi.ac.uk)

<table>
<thead>
<tr>
<th>Hydrogen bonds</th>
<th>Nb7</th>
<th>Murine uPA&lt;sub&gt;16-244&lt;/sub&gt;</th>
<th>Distance (Å)</th>
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<tr>
<td><strong>CDR-H3</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glu99 [OE1]</td>
<td>Lys82 [NZ]</td>
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<tr>
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<td>Tyr76 [OH]</td>
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</tr>
<tr>
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<td>Lys82 [NZ]</td>
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</tr>
<tr>
<td>Pro103 [O]</td>
<td>Gln70 [NE2]</td>
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<td>Ser71 [N]</td>
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<td></td>
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<tr>
<td>Gly104 [N]</td>
<td>Lys153 [O]</td>
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<td>Tyr32 [OH]</td>
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Figure S1. Crystal contact in the crystal structure of muPA in the absence of ligands. The asymmetric unit contains four muPA molecules. Crystal contact of surface-exposed loop (coloured red in chain D) stabilises the observed inactive conformation of muPA in the crystals.
Figure S2. Analysis of the ability of crystallised muPA to form a covalent complex with PAI-1. Crystals of ligand free muPA was washed extensively in mother liquid containing 100 mM HEPES, pH 7.4 and 1.8 M Li₂SO₄, before dissolved in a 10mM HEPES buffer with 140 mM NaCl. 2 µg of muPA from the dissolved crystals, or from the active muPA stock use for crystallisation, were reacted with 2-fold molar excess of PAI-1 for 1 hour at 22°C. The samples were analysed by non-reduced SDS-PAGE analysis.
Figure S3. Detailed interactions between muPA and Nb7. a. Nb7 inserts the CDR-H3 into a hydrophobic pocket created by the 37-, 70- and 140-loops. The binding epitope of Nb7 is placed distantly from the S1 specificity pocket which is affected upon Nb7 binding. muPA is shown in two orientations in surface electrostatic potential. Nb7 is represented in cartoon (red). The amino acids of Nb7 (Trp100, Pro102 and Pro103), which inserts into the hydrophobic pocket is shown as sticks. The electrostatic potential (-62 to +62 kilotesla/charge) was calculated using the APBS plugin for PyMOL. b. A detailed front and back view of the amino acids (sticks) involved in interface interactions between muPA (wheat) and Nb7 (red). Red dashed lines indicates hydrogen bond. The hydrophobic pocket into which Nb7 binds is created by a cluster of amino acids (Phe30, Pro37e, Pro38 Tyr34, Phe40, Tyr67, Leu68, Pro70 and Phe141) from the 37-, 70- and 140-loops. Water molecules that mediates hydrogen bond are shown as spheres (blue) and the 2mFo-DFc electron density map (blue mesh) is contoured at σ=1.
**Figure S4.** Changes in the surface-exposed loop upon binding of EGR-cmk or p-aminobenzamidine. 

**a.** Cartoon representation of the structures of complexes between muPA:Nb7 (wheat), muPA:Nb7:EGR-cmk (teal) and muPA:Nb7:p-aminobenzamidine (magenta). EGR-cmk (yellow) and p-aminobenzamidine (orange) is shown in sticks. Residues that interconnects the N-terminal activation loop, the 140-loop, the 180-loop and the 220-loop is shown as sticks. In **b. c. and d.** is shown the electron density of the area in the three structures. Note that the surface-exposed loop especially the 140-loop and the area around the Cys191-Cys220 disulphide bond becomes more ordered as EGR-cmk or p-aminobenzamidine binds at the active site. Hydrogen bonds are indicated as red dashed lines, and the 2mFo-DFc electron density map is contoured at $\sigma=1$. 
**Figure S5.** Cartoon representation of the crystal packing effects of Nb7. Two Nb7 molecules from adjacent asymmetric units (grey) form crystal contacts with the 60-, 90-, 130-, 170- and 220-loops of muPA (wheat and blue). The 37- and 70-loop binding Nb7 molecule is shown in red.
APPENDIX 6

Manuscript VI

A Camelid-Derived Antibody Fragment Targeting the Active Site of a Trypsin-like Serine Protease Balances Between Inhibitor and Substrate Behaviour

Tobias Kromann-Hansen, Emil Oldenburg, Kristen Wing Yu Yung, Gholamreza H. Ghassabeh, Serge Muyldermans, Paul J. DeClerck, Mingdong Huang, Peter A. Andreasen and Jacky Chi Ki Ngo

(Manuscript in preparation)
A Camelid-derived Antibody Fragment Targeting the Active Site of a Trypsin-like Serine Protease Balances Between Inhibitor and Substrate Behaviour

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Abbreviations. uPA, urokinase-type plasminogen activator; EGR-cmk, H-Glu-Gly-Arg-chloromethylketone; Nb4, nanobody-4; r.m.s.d, root mean square deviation; PAI-1, plasminogen activator inhibitor-1; HEK293, human embryonic kidney 293 cells; PBS, phosphate buffered saline; HBS, HEPES buffered saline; BSA, bovine serum albumin; CS-61(44), pyro-Glu-Gly-Arg-para-nitroaniline-HCl; uPAR, urokinase-type plasminogen activator receptor; CDR, complimentary determining region; BPTI, bovine pancreatic trypsin inhibitor. RCL, reactive centre loop.
Summary

The conserved architecture of the active site of trypsin-like serine protease active sites poses a challenge for the development of specific active site binding inhibitors. In this report, we demonstrate how specific active site inhibition of trypsin-like serine proteases can be achieved by a Camelid derived antibody fragment. X-ray crystal structure analysis of the complex between an antibody fragment and human urokinase-type plasminogen activator (uPA), demonstrates that the antibody fragment combines in a substrate-like manner with the active site and with exosite binding ensuring specific and potent inhibition of uPA. The inhibitor has a unique mechanism of inhibition by inserting a CDR-H3 loop into the active site region of uPA. We demonstrate that the potency of the inhibitor relies on a complex hydrogen bond network in the CDR-H3 loop in a manner similar to that of naturally occurring standard mechanism trypsin-like serine protease inhibitors. Mutagenesis of residues involved in the hydrogen-bonding network converted the inhibitory antibody to a uPA-substrate. Our studies reveal new information about the differences between protease inhibitors and protease substrates.
Introduction

The catalytic machinery of trypsin-like serine proteases (Clan PA, family S1) consists of the catalytic triad (His57, Asp102 and Ser195, chymotrypsin numbering) and the oxyanion hole (backbone NHs of Gly193 and Ser195). The hydrolysis of a peptide bond proceeds through a well-established reaction mechanism involving two tetrahedral intermediates and formation of an acyl-enzyme complex. The oxyanion hole stabilises the tetrahedral intermediates whereas the catalytic triad acts as a charge relay system to generate the required nucleophiles for formation and breakdown of the acylenzyme. The important functions of the catalytic triad and the oxyanion hole are assisted by the substrate specificity pockets (denoted S1-Sn and S1’-Sn’ on the acyl and leaving group side of the scissile bond, respectively). Especially residue 189 in the bottom of the S1 pocket, and residues 214-217, which is part of the S2-S4 pockets, determines the primary specificity of the protease by providing specific recognition sites for the substrate (1).

The activity of trypsin-like serine proteases is regulated at the post-transcriptional level by zymogen activation, cofactor binding or by inhibition by naturally occurring inhibitors. 18 out of 22 families of natural trypsin-like serine protease inhibitors inhibit their target protease by a highly conserved mechanism termed the ‘standard mechanism’ or ‘Laskowski mechanism’ (2, 3). Standard mechanism inhibitors exert their inhibitory effect through high-affinity interactions with the active site of the protease by inserting a reactive-loop in a substrate-like manner. The reactive-loop has a single reactive-peptide bond, which is hydrolysed slowly by the protease with half-lives of many hours. Once cleaved, the inhibitor remains associated with the protease and the reactive-peptide bond is believed to be resynthesized by the protease (4, 5). However, the information about factors determining inhibitor or substrate behaviour is sparse.

Intriguingly, antibody fragment that binds to the active site of trypsin-like serine proteases by insertion of the CDR-H3 has recently been demonstrated. Structural data demonstrated, that the antibody fragments were inhibitors and not substrates as the CDR-H3 adopt a non-substrate like conformation in the active site (6-9). However, the current understanding of targeting the active site
of trypsin-like serine proteases with antibody fragments is limited to these very few studies. We have now isolated an antibody fragment against a trypsin-like serine protease, which throw new light on the balance between inhibitor and substrate behaviour.

_Camelid_ derived single-domain antibody fragments, so-called nanobodies, seem ideally shaped for interacting with concave clefts such as the active site of an enzyme (10, 11). Until now, nanobodies has been demonstrated to possess the ability to insert a long protruding complementary determining H3 loop (CDR-H3) into the substrate binding cleft of non-proteolytic enzymes such as lysozyme (12). In this report, we describe a nanobody that specifically targets the active site of human uPA. X-ray crystal structure analysis reveals that the CDR-H3 inserts into the active site in a canonical substrate-like manner and that specificity is achieved by interaction of the nanobody with the surface-exposed 37-loop of uPA. We further demonstrate that the inhibitory mechanism of the nanobody relies on a stable fold of the CDR-H3, which resembles the reactive-loop conformation of the standard mechanism inhibitors. A weakening of the affinities by site-directed mutagenesis converted the nanobody from an inhibitor to a substrate.
Results

**Inhibition of human uPA by an anti-uPA nanobody.** A nanobody library was generated by immunising an Alpaca (*Vicugna pacos*) with active human uPA. Screening the library resulted in identification of a nanobody (Nb4), which potently inhibited proteolytic activity of uPA towards the macromolecular substrate plasminogen and its amidolytic activity towards the small chromogenic substrate CS-61(44) (figure 1a and 1b). Testing the specificity of Nb4 towards a panel of human trypsin-like serine proteases and uPA from other species, we showed that Nb4 is highly specific towards uPA (figure 1c), as it potently inhibited the catalytic activity of uPA from human, dogs and rabbits, and less potently uPA from mouse, while it did not inhibit rat uPA measurably.

Sequencing of Nb4 revealed an arginine rich motif in the CDR-H3 region (figure 2). Since uPA is highly specific towards substrates with arginine in the P1 position we hypothesised that Nb4 inserts the CDR-H3 into the active site of uPA. To test this hypothesis, we immobilised Nb4 on a CM5 surface sensor chip, and measured binding affinities towards different uPA variants using surface plasmon resonance (SPR). The data shows that Nb4 binds tightly to active human uPA with a $K_D$ around 50 pM (table S1). Interestingly, we were not able to measure any affinity towards uPA in which the active site had been blocked with the covalent inhibitor Glu-Gly-Arg-chloromethylketone (EGR-cmk). Furthermore, Nb4 is highly specific towards active uPA, as we did not detect any binding to the zymogen form pro-uPA.

**Crystal structure of Nb4.** We determined the X-ray crystal structure of the free form of Nb4 (Nb4$_{\text{free}}$) to a resolution of 2.16Å ($R_{\text{free}}= 0.2177$ and $R_{\text{work}}= 0.1766$) by molecular replacement with four molecules in the asymmetric unit (table S2). Only molecule B is described, as all residues are visible in the electron density of this molecule. The structure reveals an unusual fold of the CDR-H3 (figure 3). The CDR-H3 of Nb4 is 18 amino acids long and can be divided into a N-terminal (Asp95 to Leu99) and C-terminal (Thr100a- Val100l) portion by Cys100, which is centrally positioned in the CDR-H3 forming a disulphide bond to Cys50 in the CDR-H2. In the anti-lysozyme nanobody cAb-Lys3, the N-terminal portion of the
CDR-H3 extents away from the scaffold of the nanobody forming a protruding loop, and the C-terminal portion folds back and covers the hydrophobic interface (Phe37 and Trp103) that in conventional antibodies forms an interaction surface with the variable light chain. This structural motif is a typical characteristic of nanobodies and increases the solubility of the single domain (12). In Nb4 free the hydrophobic patch is only partly covered by the CDR-H3, and in contrast to cAb-Lys3, it is the N-terminal portion that folds back to partly cover this area. The C-terminal part of the CDR-H3 forms the protruding loop. This arrangement of the CDR-H3 is unique to Nb4 free and is most likely due to the unusual disulphide bond between the CDR-H3 and CDR-H2, which is often observed in heavy chain antibodies from alpacas (13).

**Crystal structure of human uPA complexed with Nb4.** The crystal structure of the catalytic domain of human uPA in complex with Nb4 was determined to a resolution of 1.97Å (Rfree = 0.2236 and Rwork = 0.1754) with two complexes in the asymmetric unit. Nb4 covers 885.3Å² of accessible surface area on uPA and the interface is characterised by a high degree of shape complementarity (figure S1). In agreement with the biochemical data Nb4 occludes the active site of uPA by inserting the CDR-H3. The CDR-H1 of Nb4 is also involved in binding to uPA by forming exosite interactions with the surface-exposed 37-loop of uPA (figure 4). A contact list is given in table S3.

**Interaction of Nb4 with the active site of uPA.** From the X-ray crystal structure it is evident that the CDR-H3 of Nb4 inserts into the active site of uPA in a substrate-like manner similar to that of irreversible active site inhibitor EGR-cmk in complex with human uPA (figure 5a). Interactions of Nb4 with the active site of uPA involve residues from Ser100b to Glu101 of the CDR-H3. The residues from Glu100c to Tyr100i form a tight loop that interacts extensively with the catalytic machinery of uPA. The P1 Arg100f inserts into the S1 specificity pocket to form extensive interactions with residues in uPA (figure 5b). The carbonyl group of Arg100f forms a hydrogen bond with the backbone amide of Ser195 and occupies the oxyanion hole by contacting the backbone of Gly193, Ser195 and Asp194. The backbone amide of Arg100f hydrogen bonds with the
side chains of His57 and Ser214. The side chain of Arg100f occupies the S1 specificity pocket and forms a salt-bridge to Asp189 and the guanidinium group mediates three additional hydrogen bonds with Ser190 and Gly218. The backbone carbonyl of P2 Gly100e forms a hydrogen bond to the side chain of Gln192 and occupies the S2 pocket, which is formed by His57 and His99 in uPA. Ser100d of Nb4 occupies the S4 pocket and its side chain form hydrogen bonds to His99 and Leu97b in the 90-loop. Ser100d can also interact with Gly216 in the 220-loop of uPA as its side chain adopts two alternate rotamers in one of the complexes within the asymmetric unit. The interaction between Nb4 and uPA is further strengthened by direct interactions between Glu100e and Ser100b of Nb4 and Arg217 and His99 of uPA respectively. On the C-terminal side of the P1 residue, the backbone amide of P1’ Arg100g interacts with His57 and Gly193 (figure 5c). The side chain of Arg100g is positioned away from the active site to interact with Asp60a. In addition, Arg100h interacts with Tyr40 and Val41 in the S1’ pocket, and Tyr100i and Glu101 make further contacts with Lys143 and Tyr151 in the S2’ pocket via either side chain-side chain or side chain-backbone interactions. The extensive network of interaction observed partly explains the high affinity binding of Nb4 to uPA and the X-ray crystal structure is in good agreement with the biochemical data that the positioning of CDR-H3 in the active site region interferes with the binding of EGR-cmk.

**Inhibitory mechanism of Nb4.** The X-ray crystal structure analysis revealed that the backbone carbonyl of Arg100f is aligned in the oxyanion hole and that Ser195 is perfectly positioned 2.9 Å away for the nucleophilic attack. To evaluate why Nb4 is not acting as a substrate of uPA we generated a panel of alanine mutants of uPA and Nb4. Alanine residue substitutions were made at key positions identified from the crystal structure to determine the difference between the mutant and wild-type $K_i$ values for Nb4 inhibition of the chromogenic substrate CS-61(44). Overall, the analysis validated the interactions observed in the crystal structure of the complex, as substitutions of the interface residues by alanines resulted in increased $K_i$ values (table S4). As expected, the mutation of Arg100f completely abolished the inhibitory activity of Nb4. Interestingly, mutations of Arg100g and Tyr100i in Nb4 have larger effects on
the \( K_i \) value than mutating their interacting counter residues in uPA (Asp60a and Tyr151). From the crystal structure it is evident that the side chains of Asp 95, Arg100g and Tyr100i are important for the conformation and stability of the CDR-H3 as they form several intra-loop hydrogen bonds (figure S2). We speculate that the complex hydrogen bond network of the CDR-H3 is key to the inhibitory mechanism of Nb4. To investigate this, we performed SDS-PAGE analysis with the Nb4 R100gA, Y100iA and D95A mutants and wild-type Nb4 after different times of incubation with uPA. The analysis revealed that wild-type Nb4 is slowly hydrolysed by uPA resulting in two fragments at 12kDa and 5kDa respectively (figure 6a). After 4 hours the reaction reaches steady-state, as the same fraction of Nb4 remains intact even after 96 hours of incubation. For the R100gA and D95A mutants, the cleavage proceeds much faster, as all nanobody is cleaved within 0.5 hours (figure 6b and 6c). For the Y100iA mutant the cleavage also proceeds faster than for the wild-type Nb4 but slower than for the R100gA and D95A mutant (figure 6d). N-terminal sequencing revealed that Nb4 is cleaved at the putative scissile bond (Arg100f-Arg100g) in the CDR-H3 as the 5kDa fragment has a N-terminus starting at the P1’ residue \( R_{100g}^{100g} \) RYLEV-GQ for wild-type Nb4 and the D95A mutant, \( A_{100g}^{100g} \) RYLEV-GQ for the Nb4 R100gA mutant and \( R_{100g}^{100g} \) RALEV-GQ for the Y100iA mutant (the blank read is a Trp residue). The size of the 5kDa band indicates that the fragment extends to the C-terminus including the HA-tag and the His-tag (36 amino acids, 3960Da). On the other hand, the 12kDa band begins at the N-terminus (Q1VQLES) and ends at the scissile bond (110 amino acids 11565Da). These data demonstrates that the inhibitory potential of Nb4 strongly depends on the intra-loop hydrogen bond network in the CDR-H3, as removal of the hydrogen bond lead to substrate behaviour.

**Para-aminobenzamidine displacement assays.** To further verify our structural and biochemical observations we performed fluorescent spectroscopy to measure binding of the reversible ligand \( p \)-aminobenzamidine in the S1 specificity pocket of uPA in the presence or absence of Nb4 wild-type or alanine mutants. Binding of \( p \)-aminobenzamidine results in a high intensity fluorescent signal with \( \lambda_{\text{max}} \) at 362nm, whereas the low intensity signal of \( p\-
aminobenzamidine in solution has $\lambda_{\text{max}}$ at 375nm (figure S). Adding Nb4 displaces $p$-aminobenzamidine from the S1 specificity pocket as the intensity of the signal decreases and a red shift has occurred as observed for free $p$-aminobenzamidine in solution. Mutating Arg100f and Arg100g in Nb4 to alanine affected the ability of Nb4 to displace $p$-aminobenzamidine whereas mutation of Arg100h to alanine only had a minor effect. This result suggests, in reasonable agreement with the proteolysis experiments, that the integrity of the P1’ site in Nb4 is important to its inhibitor behaviour.
Discussion

In this report, we describe a completely new mechanism of inhibition of a trypsin-like serine peptidase, by development of a Camelid derived antibody fragment, which binds in a substrate-like manner to the active site of human uPA. The availability of structural information about the binding mechanism of the antibody fragment enabled us to ask the question: Which factors determine whether an agent binding in the active site of an endopeptidase in a substrate-like manner is a substrate or an inhibitor?

As evaluated by our findings, Nb4 exerts its inhibitory effect by inserting a protruding CDR-H3 loop into the active site of uPA in a canonical substrate-like manner thereby occluding the substrate binding pockets. The fast association ($k_{on}$) between Nb4 and uPA suggest a lock and key interaction in which neither Nb4 or uPA undergoes large conformational changes. This is in agreement with the X-ray crystal structure information, which demonstrates that the conformation of Nb4 before and after binding to uPA is highly similar (RMS=0.489Å) (figure S2). Also uPA do not undergo large conformational changes upon binding of Nb4 (RMS=0.268Å), when compared to the ligand free form of human uPA (PDB:4dva). Only minor changes in the 37- and 90-loops, and the side chains of His57, Ser195 and Gln192 occur to accommodate the CDR-H3 of Nb4 (figure S2). The lack of conformational change in the long CDR-H3 loop of Nb4 is most likely a result of the interloop disulphide bond (Cys50-Cys100), which restricts the conformational flexibility of the CDR-H3, thereby minimising the entropic penalty associated with complex formation. Once bound, Nb4 remains tightly associated with uPA as determined by a slow dissociation rate constant ($k_{off}$). Hence, uPA competitively inhibits the interaction of uPA with its physiological substrate plasminogen, as well as hydrolysis of small chromogenic substrates.

The X-ray crystal structure of Nb4 in complex with uPA also provides a rationale for the exquisite selectivity of Nb4 towards uPA. It is evident from the crystal structure that the CDR-H1 loop of Nb4 interacts with the 37-loop of uPA. The 37-loop is a non-conserved region that is variable in sequence and length in trypsin-like serine proteases. The specific interactions observed between Nb4
and this region thus explain the selectivity of Nb4 towards uPA. In agreement with this observation, Nb4 efficiently inhibits dog and rabbit uPA in which the 37-loop and the residues that interact with Nb4, namely Arg35, His37, Arg37a and Ser37d, are completely conserved (figure S4).

Structural insight into how active site binding antibodies inhibit the activity of trypsin-like serine proteases arises from a series of antibody fragments that target matriptase, hepatocyte growth factor activator (HGFA) and plasma kallikrein (6-9). Common to all the antibodies is that they inserts one or two of their CDR loops into the active site of the protease in a non-substrate like manner. In the case of the anti-matriptase antibodies E2, S4 and A11 they inserts the CDR-H3 loop in a reverse non-substrate like C- to N-terminal orientation, which results in a suboptimal interaction of the P1 arginine with Asp189 in the bottom of the S1 pocket through a water-mediated hydrogen bond. The reverse orientation, however, places the putative scissile bond out of reach for a nucleophilic attack by Ser195. Contrary, the CDR-H3 of the anti-plasma kallikrein antibody DX-2930 binds in the normal N- to C-terminal orientation but the orientation of the loop prevents its interaction with the S1'–S3' pockets and thereby prevents cleavage of the putative scissile bond. The anti-HGFA Fab58 also binds into the active site region of HGFA occluding the S2 and S3 but not the S1 specificity pocket. In contrast, Nb4 binds in a substrate-like manner utilising the S3-S3' specificity pockets. This difference emphasises the unique mechanism of Nb4, of which a tight and optimal substrate-like interaction with uPA is an important part of the inhibitory mechanism.

A structural alignment of the CDR-H3 of Nb4 with the reactive center loop (RCL) of the serpin plasminogen activator inhibitor 1 (PAI-1) revealed that their reactive loops adopt a nearly identical conformation (figure 7a). In contrast to Nb4, the covalent and irreversible inhibitory mechanism of serpins relies on an unusually high flexibility of the RCL as the cleavage of the scissile bond and consequently the formation of the acyl-enzyme traps and translocate the protease to the distal side of the serpin via a large conformation change (14). As a result, only a few intra-loop hydrogen bonds connecting the P and P' site of the scissile bond is found in the RCL of PAI-1. In contrast, Nb4 displays all characteristics of a standard mechanism trypsin-like serine protease inhibitor.
Superposition of the CDR-H3 loop to the reactive-loop of the bovine pancreatic trypsin inhibitor (BPTI) in complex with trypsin reveals a similar conformation and architecture of the two loops (figure 7b). In BPTI, a disulphide bridge (Cys14-Cys38) adjacent to the P1 residue (Lys15) connects the P- and P′-site of the scissile bond and stabilises the reactive-loop at the active site of trypsin (15). Thus, BPTI inhibits trypsin reversibly by forming a tight interaction in which the reactive loop is maintained in a highly stable conformation. In Nb4, the P- and P′-site of the scissile bond is only connected through non-covalent interaction involving the Arg100g-Asp95 salt-bridge and the hydrogen bonds between Tyr110i and the Thr100a-Gly100e segment. Our results demonstrate that weakening of this hydrogen bond network, through mutations, converts Nb4 from an inhibitor to a substrate. Thus, similar to other standard mechanism inhibitors, the inhibitory potential of Nb4 relies heavily on the stability of the CDR-H3, as a tight and highly complementary interaction with uPA is key to the inhibitory mechanism. However, in contrast to standard mechanism inhibitors, which often display broad protease specificity, Nb4 is highly specific towards uPA. This emphasises the importance of exosite interaction to achieve specific inhibition of trypsin-like serine proteases.

In standard mechanism inhibitors, the scissile bond in the reactive loop is hydrolysed slowly by the protease at neutral pH (16). However, the scissile bond can be religated due to a tight association between the protease and the inhibitor. Although poorly understood, it has been proposed that the position of the P1′ residue, which is stabilised by interactions with both the protease and the inhibitor, excludes the hydrolytic water required for the second step of the hydrolysis reaction to proceed (5). Thus, the rigid position of the P1′ residue favours the reformation of the peptide bond and disfavours deacylation of the acyl-enzyme intermediate (17). Our data suggests a rationale behind this model. From our SDS-PAGE analysis, it is evident that Arg100g and Asp95 mutants are cleaved much faster than the Y100i mutant. This indicates that the Arg100g-Asp95 salt-bridge is essential to prevent hydrolysis of Nb4. Although Arg100g forms a hydrogen bond to Asp60a of uPA, it is not enough to prevent hydrolysis when the side chain of Asp95 is missing. This strongly suggests that the position of P1′ Arg100g is detrimental for the hydrolysis reaction to proceed. The X-ray
crystal structure of a cleaved variant of BPTI in complex with inactive trypsin S195A suggests that the P1’ residue (Ala16) does not change in position after cleavage (5). Thus, it is reasonable to speculate that a locked position of Ala16 in BPTI and Arg100g in Nb4 prevents access of the hydrolytic water even in their cleaved forms (figure S5). Alternatively, the R100gA mutation may provide enough flexibility of the CDR-H3 loop to allow proper alignment into the active site of uPA for a more efficient hydrolysis and dissociation of the leaving group. This suggests that the rigidity of the acyl-enzyme intermediate is more important for the inhibition by Nb4 that the rigidity of the enzyme-inhibitor complex.

We therefore propose that Nb4 mimics the inhibitory mechanism of naturally occurring standard mechanism inhibitors, in which the leaving group is retained in the active site of the protease to act as a nucleophile for the reformation the scissile bond. The availability of an antibody fragment that inserts a CDR loop into the active site in a substrate-like manner further allowed us to address mechanisms by which active site agents acts as inhibitors and not substrates. As such, the unique inhibitory mechanism of Nb4 would provide an alternative way of studying the interaction between standard mechanism inhibitors and their target protease. Moreover, the X-ray crystal structure presented here may allow design of a framework for development of active site binding agents for molecular intervention with the activity of serine proteases.
Materials and Methods

**Generation of anti-uPA nanobodies.** The immunization and construction of the nanobody phage library was conducted as described previously (18). Selection of anti-murine nanobodies was performed by immobilizing human uPA (ProSpec-Tany TechnoGene) (100 µg/mL) in 96-well MaxiSorp immunoplates (Nunc). After adding the nanobody phage library bound phages were eluted with triethyleamine (100 mM) and neutralized with 1 M Tris pH 8.2. Recovered phages were amplified in *E.coli* TG1 cells. During three subsequent selection rounds, the stringency of plate washing was gradually increased. uPA binding nanobodies were identified by a polyclonal phage ELISA by randomly picking single colonies. Positive clones were sequenced and unique clones were transformed into *E.coli* WK6 (su⁻) cells and produced as described previously (19). Alanine mutants of Nb4 was prepared by standard QuickChange site-directed mutagenesis and expressed in *E.coli* WK6 (su⁻) cells.

**Generation of the catalytic domain of uPA and alanine mutants.**

Full-length human uPA and uPA site-directed mutants were cloned into the pCNDA 3.1 vector. cDNAs were transfected into HEK293 6E suspension cells and cultured in a humidified 5% CO₂ incubator at 37 °C. The media used was Freestyle F17 (Gibco) containing 4 mM L-glutamine, 0.1% Pluronic F68 (Gibco), 1% penicillin/streptomycin (Invitrogen), 25 µg/mL G418 (Geneticin, Thermo Fisher Scientific). Linear polyethyleneimine (PEI) (2.2mg) and cDNA (1.1 mg) was mixed in PBS for 15 min and added to 1 L of cell cultures at a density of 1x10⁶ cells/mL. Tryptone N1 (0.5 %) was added to the culture 24 hours post-transfection. The cultures were continued for 6 days before harvesting the conditioned media. The catalytic domain of uPA was recombinant expressed in *Pichia pastoris* as described previously (20).

**Enzymatic assays.** All enzymatic assays were performed in HEPES buffered saline (HBS; 10 mM HEPES pH 7.4; 140 mM NaCl) with 0.1% bovine serum albumin (BSA) and the initial velocities was monitored at an absorbance of 405 nm for 1 hour at 37 °C in a kinetic microplate reader (Multiscan Go, Thermo Scientific).
For the plasminogen activation assay various concentrations of Nb4 (120-0 nM) was preincubated with uPA (0.25 nM) for 15 min at 37 °C. Next, the reaction was initiated by adding human plasminogen (100 nM), purified from plasma, and plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide (0.5 mM) (S-2251, chromogenix, Sweden). To determine the velocity of plasminogen activation, the data were transformed to plot ΔA405/Δtime on the ordinate and time on the abscissa (21). Velocities were calculated from the time interval 5-20 min of these plots and used for calculation of IC50 values.

For inhibition of Pyro-Glu-Gly-Arg-pNa-HCL (CS-61(44), Hyphen Biomed), various concentration of Nb4 (250-0 nM) were preincubated with uPA (2 nM) for 15 min at 37 °C before adding CS-61(44) (47 µM) to initiate the reaction. The apparent inhibitory constant $K_D^{app}$ was calculated as described in (22), and $K_i$ values were determined by $K_i = \frac{K_D^{app}}{(1+[S])}$. $K_M$ values were determined by measuring initial velocities of hydrolysis of CS-61(44) (2-0 µM) by wild-type uPA (2 nM) or by various uPA alanine mutants in conditioned media. The $K_M$ values were calculated by fitting the data to standard standard Michaelis Menten kinetic.

To evaluate the specificity of Nb4 we incubated Nb4 (4 µM) with human plasma kallikrein (2 nM, 300 µM S-2302), human coagulation factor VIIa (20 nM, 1000 µM S-2288), human plasmin (5 nM, 750 µM S-2288), matriptase (2 nM, 100 µM S-2288), human activated protein C (8 nM, 375 µM S-2366), human hepsin (4 nM, 170 µM S-2366), human tissue-type plasminogen activator (2 nM, 500 µM S-2288), murine uPA (2 nM, 750 µM CS-61(44)), dog uPA (4 nM, 750 µM CS-61(44)), rat uPA (4 nM, 750 µM CS-61(44)), rabbit uPA (4 nM, 750 µM CS-61(44)) or human uPA (2 nM, 47 µM CS-61(44)) for 15 min at 37 degrees before adding the chromogenic substrates.

**Surface Plasmon Resonance.** The equilibrium dissociation constant $K_D$, the association rate $k_{on}$, and the dissociation rate $k_{off}$ of Nb4 binding to active human uPA, pro-uPA (Abbott Laboratories) or the active site H-Glu-Gly-Arg-chloromehylkentone (Bachem Switzerland) blocked variant uPA:Egr-cmk were determined by surface plasmon resonance on a BiaCore T200 (Ge Healthcare). In
all experiments the uPA variant were diluted in running buffer HBS+0.1% BSA. Nb4 were diluted to 0.5µg/mL in immobilization buffer (10 mM Sodium Acetate pH 5), and immobilised on a CM5 sensor chip (GE Healthcare) by amine coupling to approximately 200 response units (RU). Active uPA (1-0 nM), pro-uPA (50-0 nM) or uPA:Egr-cmk (25-0 nM) were injected for 480s with a flow rate of 30 µL/min and the dissociation was monitored for 3600s before regenerating the surface with 10 mM Glycine, 0.5 M NaCl, pH 2.5. The kinetic constants were determined at 25 °C, and the experimental curves were fitted to a 1:1 binding model using the BiaCore evaluation software.

**Fluorescent assay.** uPA (0.4 µM) alone or mixed with Nb4 (500 nM), Nb4 R100fA (500 nM), Nb4 R100gA (500 nM) or Nb4 R100hA (500 nM) were incubated for 15 min at 22 °C before adding p-aminobenzamidine (20 µM). Fluorescence emission spectrums were recorded at 25 °C on a PTI quantamaster spectrofluorometer in a 2 mm x 10 mm semi-micro quarts cuvette. An emission scan of 340 – 400 nm using an excitation wavelength of 335 nm and an integration of 1-2 s over a 1.0 nm step resolution was used. The buffer used was HBS supplemented with 0.1 % polyethylene glycol 8000.

**Crystallography.** Crystals of the free form of Nb4 were grown by sitting drop vapour diffusion by adding 2 µL of Nb4 (20 mg/ml) to 2 µL mother liquid (0.1 M MES, 0.2 M ammonium sulphate, 30 % w/v PEG5000, pH 6.5). Crystals grew within 3 days and were cryoprotected in mother liquid supplemented with 5 % ethylene glycol and vitrified in liquid nitrogen. A native data set to 2.16 Å resolution was collected at 100 K at a wavelength of 0.98 Å on the BESSY beamline at the Berlin Electron Storage Ring Society for Synchroton Radiation, Berlin, Germany. The diffraction data was indexed, integrated and scaled in space group P43212 using the XDS program package (23). The initial phases were obtained by molecular replacement with PhenixMR (24) with PDB ID 4JVP as search model (25). Model building was performed in Coot (26); refinement and structure validation were performed using PHENIX (24). Molecular graphics were prepared in PyMOL (27).
Crystals of the catalytic domain of human uPA in complex with Nb4 were grown using hanging drop vapour diffusion by adding 1 µL of uPA:Nb4 (3 mg/ml) to 1 µL mother liquid (1.4 M of ammonium sulfate, 0.1 M MES, pH 6.25, 0.1 M disodium phosphate dibasic and 0.5% (v/v) Tween 20) at 16°C. Crystals were cryoprotected in mother liquid supplemented with 30% (v/v) glycerol and frozen in liquid nitrogen. Crystals were tested and screened at 100K using the Rigaku FRE+ X-ray source at the Centre for Protein Science and Crystallography, The Chinese University of Hong Kong. The highest resolution data set at 1.97 Å was collected on the 13B1 beamline at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. The diffraction data was indexed, integrated and scaled in space group C121 using the HKL2000 program package (23). The initial phases were obtained by molecular replacement with PHASER of the CCP4 program suite (28) with the structures of uPA (PDB ID= 4DVA) and Nb4 as search model. Model building was performed in Coot (26); refinement was performed using REFMAC (29); structure validation and conformational quality were assessed by Procheck (30).

**SDS-PAGE analysis.** Nb4 (3µg), Nb4 R100gA (3 µg), Nb4 Y100iA (3 µg) or Nb4 D95A (3 µg) were incubated with active uPA (3 µg) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and the reactions were quenched by adding pre-warmed (96 °C) non-reducing sample buffer and the protease inhibitor Phenylmethylsulfonylfluoride (PMSF, 1 mM) at the indicated time points. The samples were separated by non-reducing 18% SDS-PAGE and the digestion products were N-terminal sequenced at the Department of Molecular Biology and Genetics, Aarhus, Denmark to determine the cleavage site.
References.


Figure 1. Inhibition of uPA-catalysed plasminogen activation and hydrolysis of a small chromogenic substrate. a. uPA (0.25 nM) was preincubated with Nb4 (120-0 N) before addition of plasminogen (100 nM) and the plasmin substrate S-2251 (0.5 mM) to start the reactions. The experimental data were fitted to zymogen activation kinetic to determine the IC50-values by non-linear regression. Nb4 was found to inhibit plasminogen activation with an IC50 value of 1.8±0.2 b. Nb4 (250-0 nM) inhibits hydrolysis of the small chromogenic substrate CS-61(44) (47 µM) by uPA (2 nM) with an IC50 values of 3.7±0.3 nM as determined by non-linear regression. c. Nb4 do not significantly inhibit any human trypsin-like serine proteases related to uPA, but the amidolytic activity of mouse-, dog- and rabbit-uPA is inhibited by Nb4. Error bars represent the standard deviation for three individual determinations.
Figure 2. Alignment of the amino acid sequence of the anti-lysozyme nanobody cAb-Lys3 (1) and the anti-uPA nanobody Nb4. The Kabat (2) numbering and the sequential crystallographic numbering are indicated below in bold and italic respectively. Cysteine forming disulphide bonds are indicated in yellow, and the arginine rich motif in the CDR-H3 is indicated in green.
Figure 3. X-ray crystal structure of Nb4 and a structural comparison between to the anti-lysozyme nanobody cAb-Lys3 (1mel) (grey). In the crystal structure of Nb4, the N-terminal portion of the CDR-H3 is coloured red, and the C-terminal portion is coloured brown. The residues (W103 and F37), which are part of the contact interface with the variable light chain in conventional antibodies, are shown as sticks. In Nb4free the CDR-H1 is coloured blue and the CDR-H2 is coloured green. Disulphides are shown as sticks.
Figure 4. X-ray crystal structure of the catalytic domain of uPA in complex with Nb4. The catalytic domain of uPA is shown as a cartoon in the standard orientation (wheat). The catalytic triad (His57, Asp102 and Ser195) and the S1 specificity pocket (Asp189) of uPA are shown in sticks. Nb4 is shown as a cartoon (blue). Arg100f in the CDR-H3, which inserts into the S1 specificity pocket, and Gly26, Ser31 and Tyr32 of the CDR-H1 is shown as sticks. Red dashed lines indicate potential hydrogen bonds.
Figure 5. Nb4 inserts the CDR-H3 in the active site of uPA in a substrate-like manner. a. A surface representation of the active site of uPA (wheat). The S4-S3’ substrate specificity pocket are indicated in blue with the corresponding P4-S4’ resides of the CDR-H3 of Nb4 is shown as sticks (blue). The position of specificity determining residues in uPA is indicated, and the catalytic Ser195 is coloured red. The position of EGR-cmk (yellow sticks) is indicated by superimposing the complex of uPA:Nb4 onto that of uPA:EGR-cmk (PDB:1lmw). Note the salt-bridge between Arg100g and Asp95 in the CDR-H3 of Nb4. b and c. A detailed overview of the hydrogen bond pattern on the b. P-site or the c. P’ site of the putative scissile bond. The distance from the oxygen atom of Ser195 to the carbonyl group of Arg100f is indicated with a black dashed line. Red dashed lines indicated potential hydrogen bonds.
Figure 6. Balance between inhibitor or substrate behaviour of Nb4. a. Nb4 (3 μg) is slowly hydrolysed at the putative Arg100f-Arg100g scissile bond at neutral pH 7.4 by uPA (3 μg) resulting in two cleavage products at approximately 12kDa and 5kDa respectively. The N-terminal sequence of the cleavage products is indicated to the right. The reaction seems to reach a steady state as the fraction of cleaved Nb4 remains the same after 3h of incubation. b. and c. Mutation of Arg100g and Asp95 to an Ala significantly accelerates the cleavage of Nb4 by uPA. d. Mutation of Y100i to Ala results in accelerated cleavage of Nb4 by uPA. However, the cleavage is slower than for the Arg100g and Asp95 mutants.
Figure 7. Structural comparison of the conformation of the CDR-H3 in Nb4 with the reactive center loop (RCL) of the serpin PAI-1 and the reactive loop of BPTI. a. The crystal structure of the Michaëlis complex between uPA and PAI-1 (3pb1) (red) was aligned to the uPA:Nb4 complex (wheat and blue). In Nb4 the P and P’ site of the putative sessile bond is connected by intra-loop hydrogen bonds (red dashed lines) between Arg100g to Asp95, and more complex water-mediated hydrogen bond network between Tyr100i and the Thr100a-G100e segment. b. The crystal structure of BPTI in complex with trypsin (PDB: 3y0y) (green) was superimposed to uPA:Nb4. In BPTI the P and P’ site of the sessile bond is connected primarily through the Cys14-Cys38 disulphide bond.
References


Supporting information

A Camelid-derived Antibody Fragment Targeting the Active Site of a Trypsin-like Serine Protease Balances Between Inhibitor and Substrate Behaviour

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Table S1. Kinetic analysis of the binding of Nb4 to active uPA, Pro-uPA or EGR-cmk inhibited uPA (uPA:EGR-cmk). The equilibrium constant \((K_D)\) were determined by fitting the SPR data to a 1:1 binding model. The data represent the mean ± standard deviations of three independent experiments. There was no measurable binding (n.b.) of Nb4 to pro-uPA or uPA:EGR-cmk when using 50 nM or 25 nM of the protease respectively.

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<th>(k_{off} \text{ (1/s) x 10^-5})</th>
<th>(K_D \text{ (pM)})</th>
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<td>54.4 ± 14.5</td>
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<td>-</td>
<td>n.b.</td>
</tr>
<tr>
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Table S2. X-ray data collection and model refinement statistics.

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*a* Numbers in parentheses refer to the highest resolution shells.
**Table S3.** Contact list between Nb4 and uPA from PISA server located at http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver.

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Table S4. $K_M$ determinations for wild-type uPA and variants, and $K_i$ for inhibition of Nb4 mutants and uPA mutants. The data represent the mean ± standard deviations of three independent experiments.

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<td>$2.7±0.2$</td>
</tr>
<tr>
<td>R35A</td>
<td>$0.11±0.01$</td>
<td>$44±4$</td>
</tr>
<tr>
<td>D60aA</td>
<td>$0.09±0.01$</td>
<td>$52±7$</td>
</tr>
<tr>
<td>Y60bA</td>
<td>$0.09±0.01$</td>
<td>$2.4±0.2$</td>
</tr>
<tr>
<td>H99A</td>
<td>$0.46±0.13$</td>
<td>$205±39$</td>
</tr>
<tr>
<td>Q192A</td>
<td>$0.21±0.03$</td>
<td>$16±1$</td>
</tr>
<tr>
<td><strong>Nb4 variant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y59A</td>
<td>-</td>
<td>$8±2$</td>
</tr>
<tr>
<td>L99A</td>
<td>-</td>
<td>$12±1$</td>
</tr>
<tr>
<td>S100bA</td>
<td>-</td>
<td>$25±3$</td>
</tr>
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<td>Y100iA</td>
<td>-</td>
<td>$934±49$</td>
</tr>
<tr>
<td>R100fA</td>
<td>-</td>
<td>$&gt;4000$</td>
</tr>
<tr>
<td>R100gA</td>
<td>-</td>
<td>$&gt;4000$</td>
</tr>
</tbody>
</table>
**Figure S1.** The interface between uPA and Nb4 display a high degree of shape complementarity. In panel a. is shown the electrostatic potential of the crystal complex between uPA and Nb4. The interaction of Nb4 with the 37-loop, the 60-loop, the 90-loop, the 140-loop and the 220-loop is highlighted with green circles. In panel b. is shown the exosite interaction between the CDR-H1 and the 37-loop in uPA, and the interaction between the CDR-H3 (E100c-E101) and uPA (right). The electron density of the CDR-H3 and CDR-H1 (magenta mesh) is contoured at $\sigma=1$. Residues of the loops highlighted in a. are indicated along with the primary specificity pockets (S5-S2'). Panel c. displays the corresponding electron density (magenta mesh, $\sigma=1$) of residues in uPA involved in the interaction with Nb4. The view is equivalent to that found in figure 4 in the main text. Note the possibility of a alternative conformation of the Arg217 side chain. Red dashed lines indicate hydrogen bonds.
**Figure S2.** Structural transition of uPA and Nb4 upon complex formation. In **a.** is shown an alignment of Nb4\textsubscript{free} (orange) and Nb4\textsubscript{bound} (blue). In **b.** is shown uPA\textsubscript{free} (green) and uPA\textsubscript{bound} (wheat). The 37- and 90-loops that changes conformation upon binding to Nb4 is highlighted in red, and the catalytic triad (His57, Asp102 and Ser195) along with Gln192 is shown as sticks. Panel **c.** displays the electron density of the CDR-H3 (magenta mesh, σ=1) of Nb4\textsubscript{bound} in a front and back view. The conformation of the CDR-H3 is stabilised by hydrogen bonds between Arg100g and Asp95 and by water-mediated hydrogen bond between Y110i and the Thr100a-Gly100e segment. Note a possible double conformation of the Ser100d side chain. In panel **d.** is shown the electron density of the CDR-H3 (magenta mesh, σ=1) of Nb4\textsubscript{free} in a front and back view. Similar to Nb4\textsubscript{bound} a hydrogen bond network involving Arg100g, Asp95, Y110i and the Thr100a-Gly100e segment can be observed. Red dashed lines indicate potential hydrogen bonds. Waters are shown as yellow spheres with blue mesh contoured at σ=1.
**Figure S3.** Fluorescent spectroscopy of p-aminobenzamidine (20 µM) in solution (cyan) or when bound in the S1 specificity pocket of uPA (0.4 µM) (black). Nb4 (500 nM) (red) and the Nb4 R100hA (500 nM) mutant competitively displace p-aminobenzamidine from the S1 specificity pocket whereas the Nb4 R100fA (500 nM) (green) and R100hA (500 nM) (orange) mutants are unable to displace p-aminobenzamidine.
Figure S4. Sequence alignment of the 37-loop of uPA related human trypsin-like serine proteases and uPA from mouse, rat, dog and rabbit. Residues identical to the Nb4 interacting residues in human uPA are highlighted in red.

<table>
<thead>
<tr>
<th>Human uPA</th>
<th>WFAAIYRRHGGSVTVYVC</th>
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<tbody>
<tr>
<td>Plasma Kallikrein</td>
<td>WQVSLQVLTATQRHLC</td>
</tr>
<tr>
<td>Coagulation Factor VIIa</td>
<td>WQVLLVNGAQLC</td>
</tr>
<tr>
<td>Plasmin</td>
<td>WQVSLRTREGMHFC</td>
</tr>
<tr>
<td>Tissue-Type Plasminogen Activator</td>
<td>WQAIFKRRSPGERFLC</td>
</tr>
<tr>
<td>Matriptase</td>
<td>WQVSLHALGCBIHLC</td>
</tr>
<tr>
<td>Activated Protein C</td>
<td>WQVVLDSLKKLAC</td>
</tr>
<tr>
<td>Hepsin</td>
<td>WQVSLRYDGRHLC</td>
</tr>
<tr>
<td>Murine uPA</td>
<td>WFAAIYOKKPPSFKC</td>
</tr>
<tr>
<td>Dog uPA</td>
<td>WFAAIYRPGVTVYQC</td>
</tr>
<tr>
<td>Rat uPA</td>
<td>WFAAIYLNKPPSFKC</td>
</tr>
<tr>
<td>Rabbit uPA</td>
<td>WFAAIYRPGVTVYVC</td>
</tr>
</tbody>
</table>
**Figure S5.** Arg100g blocks entry of the hydrolytic water. The P1-P1’ bond and the catalytic His57, Asp189 and Ser195 (black arrow) is shown as stick in the BPTI:Trypsin complex (green, PDB:4y0y), BPTI<sub>cleaved</sub>:Trypsin<sub>S195A</sub> complex (1) (yellow, PDB:3fp7), and for the acyl-enzyme intermediate Succinyl-AAPR:Trypsin (2) (orange, PDB:2age). The Nb4:uPA P1-P1’ bond, the Asp95 and the catalytic residues H57, Asp189 and Ser195 is shown as sticks (blue). Red dashed line indicates hydrogen bonds, and the black dashed lines indicate distances from His57 to the hydrolytic water and from the hydrolytic water to the carbonyl group of Arg5 in succinyl-AAPR. The potential hydrolytic water (HOH26) from the acyl-enzyme intermediate is shown as a red sphere. The position of the P1’ Ala16 only changes slightly in cleaved BPTI (Yellow and Green). In the cleaved form the amide of Ala16 clashes with the hydrolytic water to prevent hydrolysis. A similar mechanism might apply to Nb4 where the P1’ Arg100g is lock in its position by the salt-bridge to Asp95.
References


APPENDIX 7

The Binding Epitope of Nb22 Mapped by Alanine-Scanning Mutagenesis
Appendix 7. IC_{50} values for inhibition by Nb22 of hydrolysis of the chromogenic substrate CS-61(44) (750 µM) by muPA site-directed mutants (1 nM). The data is reported as mean ± standard deviation of three independent experiments. *Significantly different from the value at 0 nM Nb22 (p< 0.01). All p-values were calculated with Student’s t-test. The epitope is shown on a surface representation of the catalytic domain of muPA.

<table>
<thead>
<tr>
<th>muPA variant</th>
<th>IC_{50} (nM)</th>
<th>Ratio (mutant/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>muPA (wt)</td>
<td>0.49 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>V17A</td>
<td>0.36 ± 0.03</td>
<td>0.73</td>
</tr>
<tr>
<td>G19A</td>
<td>0.51 ± 0.05</td>
<td>1.04</td>
</tr>
<tr>
<td>E23A</td>
<td>0.33 ± 0.05</td>
<td>0.67</td>
</tr>
<tr>
<td>F30A</td>
<td>0.97 ± 0.22</td>
<td>1.98</td>
</tr>
<tr>
<td>Q35A</td>
<td>0.43 ± 0.03</td>
<td>0.88</td>
</tr>
<tr>
<td>K37aA</td>
<td>0.25 ± 0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>G37bA</td>
<td>0.67 ± 0.12</td>
<td>1.37</td>
</tr>
<tr>
<td>S37dA</td>
<td>0.51 ± 0.04</td>
<td>1.04</td>
</tr>
<tr>
<td>P37eA</td>
<td>0.53 ± 0.07</td>
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</tr>
<tr>
<td>P38A</td>
<td>0.44 ± 0.12</td>
<td>0.90</td>
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<tr>
<td>F40A</td>
<td>0.52 ± 0.05</td>
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</tr>
<tr>
<td>K41A</td>
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<td>0.76</td>
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<td>Q60aA</td>
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<tr>
<td>K72A</td>
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<td>0.82</td>
</tr>
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<td>E73A</td>
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<tr>
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<td>S75A</td>
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</tr>
<tr>
<td>Y76A</td>
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<td>N77A</td>
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<td>P78A</td>
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<td>Y99A</td>
<td>0.57 ± 0.02</td>
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<td>T110A</td>
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<td>S110aA</td>
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<td>F141A</td>
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<td>K143A</td>
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<td>E146A</td>
<td>11.9 ± 0.40*</td>
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<tr>
<td>S147A</td>
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<td>Y149A</td>
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<td>V176A</td>
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<td>S190A</td>
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<td>K192Q</td>
<td>446 ± 162*</td>
<td>910</td>
</tr>
</tbody>
</table>
The binding epitope of Nb22. Surface representation of the catalytic domain of muPA in a front and side view orientation. Blue residues could be mutated to Ala without affecting the inhibitory activity of Nb22 as determined by an increase in IC₅₀-value for inhibition of CS-61(44) by Nb22 for the various mutants. Mutation of the red residues to Ala (except in the case of Lys192 which was mutated to a Gln residue) affected the IC₅₀-value for inhibition by Nb22 more than 5-fold. His57 and Ser195 of the catalytic triad is coloured yellow.
APPENDIX 8

Crystallographic Data and Refinement Statistics for the X-ray Crystal Structure of muPA in Complex with Nb22
Appendix 8. Crystallographic data and refinement statistics for the X-ray crystal structure of muPA in complex with Nb22.

<table>
<thead>
<tr>
<th>muPA:Nb22</th>
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<tbody>
<tr>
<td><strong>Data collection</strong></td>
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<tr>
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<tr>
<td>Cell dimensions</td>
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</tr>
<tr>
<td>a, b, c (Å)</td>
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<tr>
<td>a, b, g (°)</td>
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<td>Resolution (Å)</td>
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<tr>
<td>R_sym or R_merge</td>
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<tr>
<td>I / σI</td>
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</tr>
<tr>
<td>Completeness (%)</td>
<td>94.19 (92.88)</td>
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<tr>
<td>Redundancy</td>
<td>4.0 (3.4)</td>
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| **Refinement** |  |
| Resolution (Å) | 32.47-2.3 |
| No. reflections | 15135 (1474) |
| R_work / R_free | 0.2809/0.3370 |
| No. atoms | 3093 |
| Protein | 2853 |
| Ligand/ion | - |
| Water | 240 |
| B-factors |  |
| Protein | 23.9 |
| Ligand/ion | - |
| Water | 23.2 |
| R.m.s. deviations |  |
| Bond lengths (Å) | 0.005 |
| Bond angles (°) | 1.12 |
| Ramachandran favoured (%) | 85 |
| Ramachandran outliers (%) | 4.4 |

a Numbers in parentheses refer to the highest resolution shells.
Residue Contact Analysis Between muPA and Nb22
Appendix 9. Residue contact analysis between muPA and Nb22. The polar contacts were analysed using the online PISA server located at [www.ebi.ac.uk](http://www.ebi.ac.uk)

<table>
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<tr>
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<td>Ser147 [OG]</td>
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<td></td>
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<td>Asn58 [N]</td>
<td>Ser147 [OG]</td>
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<td>Lys192 [NZ]</td>
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<td>His47 [NE2]</td>
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<td>Asp100a [OD2]</td>
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Alignment of the Amino Acid Sequence of the Anti-lysozyme Nanobody cAb-Lys3 and the Anti-uPA Nanobody Nb22.
Appendix 10. Alignment of the amino acid sequence of the anti-lysozyme nanobody cAb-Lys3 (1) and the anti-uPA nanobody Nb22. The Kabat numbering (2) and the sequential crystallographic numbering is indicated below in bold and italic respectively. Cysteine forming disulphide bonds are indicated in yellow, and the P1 Arg100 in Nb22 is indicated in green.