Design of a new protease inhibitor by the manipulation of the bait region of α_2 -macroglobulin: inhibition of the tobacco etch virus protease by mutant α_2 -macroglobulin

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Human α_2 -macroglobulin (α_2 M) inhibits a broad spectrum of proteases by changing its conformation and physically confining the enzyme. The inhibitory spectrum of α_2 M is defined by a stretch of 39 amino acids, the bait region, located near the middle of the α_2 M monomers. To investigate whether a new inhibitory specificity can be introduced by the manipulation of the bait region, recombinant α_2 M ($r\alpha_2$ M) was produced in which the primary cleavage site was replaced by a heptapeptide containing

the cleavage specificity of tobacco etch virus (TEV) protease. This protease is not inhibited by wild-type α_2 M. The $r\alpha_2$ M, produced in an episomal expression system, was fully functional and able to inhibit the tobacco etch virus protease according to its normal 'trap' mechanism. The manipulation of the bait region of α_2 M thus allows the design of new, specific protease inhibitors.

INTRODUCTION

α -Macroglobulins (α Ms) inhibit a broad range of proteases of the four different catalytic classes. Inhibition is achieved by steric shielding of the protease from its substrate [1], leaving the active site of the protease functional. Inhibition is initiated by cleavage of a peptide stretch located on the surface of each of the α M monomers, the bait region. This results in the hydrolysis of an internal thiol ester bond of the monomers which in turn triggers a conformational change responsible for the sequestration of the protease [2]. As a result of this unique inhibition mechanism, α Ms are broad-spectrum protease inhibitors, and the specific inhibitory spectrum of each α M is defined by its bait region.

One or more α Ms are found in all vertebrates, and similar proteins have been traced back as far as *Limulus* [3] and octopus [4]. α M sequences are strongly conserved except for the bait region, which varies both in length and composition, suggesting that the bait sequence selects the proteases that can be inhibited but does not influence the steric inhibition mechanism itself.

Human α_2 M has a bait region of 39 amino acids which forms a substrate for various proteases [5]. A naturally occurring mutant was recently detected (Arg681His) [6] but the functional implication of this mutation was not studied.

We investigated whether it was possible to change the inhibitory specificity of α_2 M by manipulating its bait region. To this aim an episomal expression system for α_2 M was used and tobacco etch virus (TEV) protease, which is not inhibited by α_2 M, was chosen as a model protease.

EXPERIMENTAL

Recombinant α_2 M ($r\alpha_2$ M)

Mutagenesis, production and purification of $r\alpha_2$ M were performed exactly as described in the preceding paper [7]. To mutate the α_2 M bait region, the full-length α_2 M cDNA was cloned in

pAlter1B-N, and single-stranded DNA was generated from this construct. The sequence coding for a seven-amino-acid stretch Arg⁶⁸¹ to Ser⁶⁸⁷ of the bait region was replaced by a sequence coding for the heptapeptide Glu-Asn-Leu-Tyr-Phe-Gln-Gly by using the 60-mer: 5'-GGC-CTC-TTC-CCA-TTA-CAT-CTC-CTT-GGA-AAT-ACA-GGT-TCT-CTA-GAC-CTT-CAG-GTC-CAT-GCA-3'. The cDNA was sequenced to check for the correct introduction of the mutation. The cDNA containing the mutation was then subcloned in the pMEP4 vector (pMEP4- α_2 M/TEV).

Analysis of α_2 M

Native PAGE and SDS/PAGE were as described in the preceding paper [7]. SDS/4–20% gradient polyacrylamide gels were obtained from Novex. The prestained protein molecular-mass standard was from Life Technologies. TEV protease was obtained from Life Technologies (10 units/ μ l; 0.04 μ g/unit of TEV protease) and stored at -70°C . All reactions were performed at 30°C in 50 mM Tris/HCl, pH 8.0, containing 0.5 mM EDTA and 1 mM dithiothreitol. A 30 kDa prokaryotic fusion protein in which a 34-amino acid peptide was fused to glutathione S-transferase through a TEV-cleavage sequence (GST-TEV-CS34) [8], kindly donated by D. Polayes and R. Vlietstra from Life Technologies, was used as a control substrate for TEV protease. To analyse the time course of the interaction between TEV protease and TEV- $r\alpha_2$ M, Coomassie Blue-stained polyacrylamide gels were scanned with a Personal Densitometer (Molecular Dynamics).

Pancreatic porcine elastase was obtained from Calbiochem. Reactions between elastase and wild-type $r\alpha_2$ M (WT- $r\alpha_2$ M) or TEV- $r\alpha_2$ M were carried out as described by Sottrup-Jensen et al. [9]: 6 μ g of α_2 M was incubated with a twofold molar excess of elastase for 1 min at 37°C in 10 μ l of assay buffer (50 mM Tris/HCl, pH 8.0). SDS/PAGE loading buffer [2% (w/v) SDS and 1% (w/v) 2-mercaptoethanol final concns.] was added and samples were heated for 10 min at 95°C .

Abbreviations used: $r\alpha_2$ M, recombinant α_2 -macroglobulin; TEV, tobacco etch virus; α M, α -macroglobulin; GST-TEV-CS34, glutathione S-transferase/TEV cleavage sequence/34 amino acid polypeptide.

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TosPheCH₂Cl-treated trypsin was obtained from Sigma.

Protease inhibition was analysed using resorufin-labelled casein (Boehringer) as a substrate as previously described [7].

Amino acid sequence analysis

α_2 M treated with elastase was separated by SDS/PAGE (7.5% gel) and electroblotted on Problott Membranes (Applied Biosystems) using 192 mM glycine/10% methanol/25 mM Tris/HCl, pH 8.3, as the transfer buffer. The membrane was stained with Coomassie Blue R-250. Protein bands were cut out and subjected to automated pulsed liquid protein sequence analysis on an Applied Biosystems protein sequencer (model 477A/120A).

RESULTS

TEV protease is responsible for processing of the single polypeptide synthesized from the RNA of the potyvirus. Cleavage occurs between residue 6 and 7 of a strictly conserved heptapeptide sequence: Glu-Xaa-Xaa-Tyr-Xaa-Gln-Ser/Gly [10]. This sequence is absent from the α_2 M bait region, and consequently the protease is not inhibited by α_2 M. TEV protease was therefore selected as a model enzyme for the introduction of a novel inhibitory specificity in α_2 M.

The majority of proteases inhibited by α_2 M cleave the bait region at a particular stretch of seven amino acids: Arg⁶⁸¹ to Ser⁶⁸⁷ [5]. This 'primary cleavage region' was replaced by Glu⁶⁸¹-Asn-Leu-Tyr-Phe-Gln-Gly⁶⁸⁷ by mutagenesis of the α_2 M cDNA. The mutant protein (TEV- α_2 M) was expressed using the episomal expression vector pMEP4 in the erythroleukaemia cell line K-562 [7]. TEV- α_2 M and WT- α_2 M were purified as described in the Experimental section and characterized by PAGE. On SDS/PAGE under reducing conditions, the 180 kDa α_2 M monomer and its characteristic 120 and 60 kDa heat fragments were observed for both TEV- α_2 M and WT- α_2 M. No heat fragments were present after treatment of TEV- α_2 M with methylamine, indicating the presence of the internal thiol ester in TEV- α_2 M monomers (Figure 1). Incubation of WT- α_2 M with TEV protease did not change this pattern showing that TEV protease did not cleave the wild-type bait region. After incubation of TEV- α_2 M with TEV protease, however, a completely different pattern was generated: the 180 kDa monomer disappeared and a major

Figure 1 Characterization of TEV- α_2 M by SDS/PAGE

TEV- α_2 M (T) and WT- α_2 M (W) were untreated (—) or treated with methylamine (MA) and TEV protease (TEV). The last lane contains molecular-mass markers.

Figure 2 Characterization of TEV- α_2 M by native PAGE

(a) TEV- α_2 M (T) and WT- α_2 M (W) were untreated (—) or treated with TEV protease (TEV), methylamine (MA) or 1% SDS (SDS). (b) TEV- α_2 M (T) and WT- α_2 M (W) were treated with TEV protease (TEV) or trypsin followed by incubation with the monoclonal antibody F2B2 as indicated. Both gels were silver stained.

band with a mobility of 90 kDa and some minor species with a higher molecular mass were generated. This strongly suggests the cleavage of the mutant bait region of TEV- α_2 M by TEV protease. The additional bands of molecular mass higher than 200 kDa (Figure 1) probably resulted from covalent linkage of the protease through its lysine side chains to the activated Glx residue generated by the hydrolysis of the internal thiol ester of α_2 M. The mutant bait region of TEV- α_2 M was thus a good substrate for TEV protease.

The interaction of TEV protease with TEV- α_2 M was further analysed by native PAGE (5% gel). In this system the conformational change in α_2 M induced by interaction with a protease is detectable as an increase in mobility from 'slow' α_2 M to 'fast' α_2 M [11]. The majority of the TEV- α_2 M (> 90%) migrated with the same mobility as WT- α_2 M or 'slow' α_2 M in this gel system (Figure 2a). Some TEV- α_2 M with the mobility of 'fast' α_2 M and three intermediate bands were also present. This indicated the absence of the internal thiol ester from part of the TEV- α_2 M preparation. The methylamine-treated TEV- α_2 M migrated with a higher mobility identical with that of methylamine-treated WT- α_2 M ('fast' α_2 M). Treatment of WT- α_2 M with TEV protease did not affect its mobility (Figure 2a). Treatment of TEV- α_2 M with TEV protease in contrast increased its mobility similarly to that of 'fast' α_2 M. This showed that cleavage of TEV- α_2 M by TEV protease resulted in a conformational change similar to that observed after hydrolysis of the internal thiol esters of α_2 M. The conformational change induced by cleavage of the bait region of α_2 M by a protease also results in the exposure of a receptor-binding site which can mediate the binding of the 'fast' α_2 M to the α_2 M/LRP (low-density-lipoprotein receptor-related protein) receptor. This receptor-recognition site is also recognized by the monoclonal antibody F2B2 [12]. Incubation of TEV-protease-treated TEV- α_2 M with the monoclonal antibody F2B2 decreased the mobility of TEV- α_2 M

Figure 3 Inhibition of TEV protease by TEV- α_2 M

SDS/PAGE analysis of TEV- α_2 M treated with TEV protease, followed by incubation with control substrate. (a) 0, 5, 7.5, 10, 15 and 30 μ g of TEV- α_2 M (lanes 3, 4, 5, 6, 7 and 8 respectively) were incubated with 10 units of TEV protease for 5 min at 30 °C in a volume of 25 μ l; 4 μ g of control substrate was then added to the reaction mixtures and incubation was continued for 30 min at 30 °C. The reaction was stopped by adding 5 $\times$ SDS/PAGE loading buffer. Lane 1, 10 units of TEV protease; lane 2, 4 μ g of control substrate. (b) 30 μ g of WT- α_2 M (lane 4) and TEV- α_2 M (lane 5) were incubated with 10 units of TEV protease in a volume of 25 μ l for 5 min at 30 °C, followed by the addition of 4 μ g of control substrate and a 30 min incubation at 30 °C. The reaction was stopped as in (a). Lane 1, 10 units of TEV protease; lane 2, 4 μ g of control substrate; lane 3, 4 μ g of control substrate incubation with 10 units of TEV protease for 30 min. Lane M, molecular-mass markers.

on native PAGE (Figure 2b), indicating binding of the antibody to the inhibitor. This showed that the conformational change induced by the TEV protease also resulted in expression of the receptor-recognition sites.

To evaluate whether the TEV protease was inhibited by TEV- α_2 M, a recombinant protein with a TEV cleavage sequence between a 34-amino acid polypeptide and glutathione S-transferase (GST-TEV-CS34) was used as substrate. Incubation of 4 μ g of TST-TEV-CS34 with 10 units of TEV protease at 30 °C for 30 min resulted in complete cleavage of the substrate (Figure 3a, lane 3). Next, 10 units of the TEV protease was incubated for 5 min at 30 °C with an increasing amount of TEV- α_2 M (5, 7.5, 10, 15 and 30 μ g). Then 4 μ g of GST-TEV-CS34 was added and the mixture incubated for an additional 30 min. Figure 3(a) shows that when increasing amounts of TEV- α_2 M were added, an increasing amount of uncleaved substrate was still present after the 30 min incubation period. The TEV protease was completely inhibited when 30 μ g of TEV- α_2 M was used. When 30 μ g of WT- α_2 M was used in an identical experiment, the control substrate was completely cleaved (Figure 3b).

To evaluate the kinetics of the interaction between TEV- α_2 M and TEV protease, the TEV protease was allowed to react with TEV- α_2 M. After 10 s, 1, 5, 15, 30 and 60 min the reaction was stopped by the addition of TosLysCH₂Cl (1 mM final concn.) or SDS/PAGE protein buffer. The final SDS concentration of the samples was 2 %, whereas the TEV protease is inhibited by SDS concentrations as low as 0.01 % [13]. The samples were separated by SDS/PAGE, and the generation of 90 kDa fragments was analysed by densitometric scanning. This time course was then also compared with that of the interaction between TEV- α_2 M and trypsin and between WT- α_2 M and TEV protease or trypsin. Analysis of the reaction between TEV- α_2 M and TEV protease showed that, after 15 min, no increase in 90 kDa bands occurred (Figure 4). Some 78 % of the amount of TEV- α_2 M cleaved after 15 min was already cleaved after 10 s, showing that the mutated bait region was an excellent substrate for the TEV protease. Some 180 kDa fragments remained after the 15 min incubation. For the reaction between WT- α_2 M and TEV protease on the other hand, no cleavage was detected. When TEV- α_2 M and

Figure 4 Time course of the interaction of TEV protease and TEV- α_2 M

WT- α_2 M or TEV- α_2 M (6.5 μ g each) was treated with 13 units of TEV protease or a 3-fold molar excess of trypsin for 10 s, 1, 5, 15, 30 and 60 min at 30 °C in 25 μ l. The reaction was stopped by the addition of 5 $\times$ SDS/PAGE protein buffer or TosLysCH₂Cl (1 mM final concn.) and the samples were heated for 15 min at 95 °C. Samples were loaded on an SDS/7.5 % polyacrylamide gel and stained with Coomassie Blue R-250. The amounts of cleaved and uncleaved WT- α_2 M and TEV- α_2 M were determined by densitometric scanning. Data on the x-axis are depicted on a logarithmic scale. $\blacktriangledown$, WT- α_2 M + trypsin; $\blacksquare$, TEV- α_2 M + trypsin; $\blacktriangle$, TEV- α_2 M + TEV protease; $\blacklozenge$, WT- α_2 M + TEV protease.

WT- α_2 M were allowed to react with trypsin, the reactions showed an identical time course (Figure 4). More than 95 % of the monomers were cleaved within 10 s of the addition of trypsin. Thus the absence of the primary cleavage site as well as substitution of part of the bait region did not result in a drastic decrease in bait-region cleavage by trypsin. This was not unexpected as a second function trypsin-cleavage site was located 15 amino acids downstream of the primary one [14].

Further analysis of the interaction between TEV- α_2 M and trypsin showed the conversion of 'slow' form TEV- α_2 M to 'fast' form TEV- α_2 M which was recognized by the monoclonal antibody F2B2 (Figure 2b). The two elastase-cleavage sites of p- α_2 M are both located in the seven-amino-acid sequence replaced in the TEV- α_2 M [9]. We therefore compared the interaction of elastase with WT- α_2 M and TEV- α_2 M. Incubation of TEV- α_2 M with porcine elastase also induced the conformational change, as detected by non-denaturing PAGE (results not shown). SDS/PAGE of TEV- α_2 M incubated with porcine elastase showed essentially only a 90 kDa band identical with that observed with WT- α_2 M, indicating cleavage of the mutant bait region (Figure 5). In order to determine the site of cleavage, N-terminal sequencing was performed on the electroblotted 90 kDa fragments. Sequencing the 90 kDa fragments generated after elastase cleavage in the WT- α_2 M bait revealed two equimolar sequences. One sequence could be attributed to the N-terminus of the 180 kDa monomer. The other sequence was the result of cleavage of the α_2 M monomer at the described primary cleavage site of elastase between Val⁶⁸² and Gly⁶⁸³. Sequencing of the 90 kDa fragments generated after incubation of TEV- α_2 M with elastase also revealed two sequences. One sequence was the N-terminus of the 180 kDa monomer: Ser-Val-Ser-Gly-Lys. The second sequence was Tyr-Phe-Gln-Gly. Thus cleavage occurred between Leu⁶⁸³ and Tyr⁶⁸⁴ introduced in the bait region of TEV- α_2 M.

These experiments show that both trypsin and elastase cleave the modified bait region, thereby inducing the 'slow' into 'fast' conversion. To investigate whether the cleavage of the bait region also resulted in protease inhibition, hydrolysis of casein-labelled resorufin by both proteases was measured in the absence

Figure 5 SDS/PAGE of elastase-treated WT- α_2 M and TEV- α_2 M

WT- α_2 M (lane 1) and TEV- α_2 M (lane 2) were incubated for 1 min with porcine pancreatic elastase as described in the Experimental section.

Table 1 Assay of functional inhibition of trypsin and elastase by WT- and TEV- α_2 M

The assay was performed as described in the preceding paper [7].

	Enzyme activity ($\Delta A_{574}/h$ per 100 μ mol of protease)		
	Trypsin	Elastase	No protease
WT- α_2 M	0.134	0.204	0
TEV- α_2 M	0.135	0.198	0
No α_2 M	0.524	0.475	0

or presence of WT- α_2 M or TEV- α_2 M. Table 1 shows that both WT- α_2 M and TEV- α_2 M reduce the proteolytic activity towards casein significantly.

DISCUSSION

The bait region sequences of α M of different species show no similarity [5,15]. The exchange of the bait region of α_2 M for the bait region derived from pregnancy zone protein [16] results in an active protease inhibitor with the substrate specificity of pregnancy zone protein. It is thus unlikely that there are restrictive structural constraints for a functional bait region in an α M. Indeed the bait region appears as a flexible loop at the surface of α_2 M [17,18]. We show here that manipulation of the bait region, specifically the exchange of seven amino acids for the cleavage site of TEV protease, results in a structurally normal inhibitor which now gains inhibitory activity against TEV protease. A semiquantitative kinetic analysis indicates that inhibition of TEV protease by TEV- α_2 M occurs within seconds, although the

interaction appears to be somewhat slower than the interaction between trypsin and WT- α_2 M.

The available evidence shows that TEV protease is inhibited by TEV- α_2 M according to the classical 'trap' mechanism [1]: (i) incubation of TEV- α_2 M with TEV protease induces a conformational change which is detected as an increase in mobility in native PAGE; (ii) the 'fast' TEV- α_2 M is recognized by the conformation-specific monoclonal antibody F2B2, indicating expression of the receptor-recognition site; (iii) cleavage of the 30 kDa control substrate by TEV protease is inhibited after interaction with TEV- α_2 M; (iv) the high-molecular-mass bands observed on SDS/PAGE of the protease-treated TEV- α_2 M suggest covalent linkage of lysine side chains of the protease to the activated Glx residue of the hydrolysed thiol ester from the TEV- α_2 M monomers as is shown for the interaction between trypsin and α_2 M [19]. Fluorescence energy-transfer experiments [20] and electron microscopy [21] show that the internal thiol ester residues are located at the bottom of the deep cavities that constitute the protease traps.

The manipulation of the bait region performed here seems to have few consequences on the interaction of proteases known to be inhibited by α_2 M. Trypsin treatment of TEV- α_2 M also results in the conformational change and cleavage of the TEV- α_2 M monomers with kinetics that are very similar to those of the interaction between WT- α_2 M and trypsin. This could be easily explained by the presence of a second trypsin-cleavage site downstream of the modified sequence in the TEV- α_2 M bait region [14]. Moreover, the modified α_2 M retained the same inhibiting activity as WT- α_2 M towards trypsin as it protected a high-molecular-mass substrate (casein: 23.6 kDa) from proteolysis. The two cleavage sites of porcine pancreatic elastase are located in the heptapeptide sequence replaced by the TEV protease-recognition sequence. TEV- α_2 M is, however, still able to trap and inhibit elastase from cleaving casein. Protein sequencing indicates that elastase, which normally cleaves its substrates at the carboxyl side of unchanged or aromatic residues, cleaves the modified bait sequence in accordance with its specificity. This further supports the hypothesis that the cleavage of the modified bait region reflects the specificity of the protease rather than being an aspecific cleavage.

We show here that by manipulation of the α_2 M bait region a protease inhibitor with a new specificity can be designed. The length of the α_2 M bait region (39 amino acids) therefore could allow the introduction of complex cleavage sites. The trap mechanism thus also permits the use of α_2 M as an affinity reagent for a protease where the inclusion of primary amines during interaction with the protease [22] would prevent its covalent linkage to the α_2 M. Inhibition of trypsin bound to agarose beads has been described [23]. This would suggest that the approach described above could be extended to membrane-bound proteases. A limitation peculiar to the trap mechanism is the requirement that the protease fits within α_2 M cavities. The TEV protease used here has a molecular mass of 29 kDa. It is not clear what the upper limit would be, but it should be noted that α_2 M is able to interact with plasmin, which has a molecular mass of 85 kDa, although with relatively slow kinetics [24].

The study of the requirements for a functional bait region will be the subject of future experiments. Questions of what constitutes the minimal size of the bait region and, related to this, whether the specificity of the α_2 M could be narrowed to a single protease or a single specificity will be addressed.

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