Interaction of Hemorrhagic Metalloproteinases with Human α2-Macroglobulin†

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ABSTRACT: The interaction between four *Crotalus atrox* hemorrhagic metalloproteinases and human α2-macroglobulin was investigated. The proteolytic activity of the hemorrhagic toxins Ht-c, -d, and -e against the large molecular weight protein substrates, gelatin type I and collagen type IV, was completely inhibited by α2-macroglobulin. The proteolytic activity of Ht-a against the same substrates was not significantly inhibited. Each mole of α2-macroglobulin bound maximally 2 mol of Ht-e and 1.1 mol of Ht-c and Ht-d. These proteinases interacted with α2-macroglobulin rapidly at 22 °C. Rate constants based on intrinsic fluorescence measurements were 0.62×10^5 M^{-1} s^{-1} for interaction of α2-macroglobulin with Ht-c and -d and 2.3×10^5 M^{-1} s^{-1} for the interaction of α2-macroglobulin with Ht-e. Ht-a interacted with α2-macroglobulin very slowly at 22 °C. Increasing the temperature to 37 °C and prolonging the time of interaction with α2-macroglobulin resulted in the formation of M_r 90 000 fragments and high molecular weight complexes (M_r >180 000), in which Ht-a is covalently bound to the carboxy-terminal fragment of α2-M. The identification of the sites of specific proteolysis of α2-macroglobulin shows that the cleavage sites for the four metalloproteinases are within the bait region of α2-macroglobulin. Ht-c and -d cleave only at one site, the Arg_{696}–Leu_{697} peptide bond, which is also the site of cleavage for plasmin, thrombin, trypsin, and thermolysin. Ht-a cleaves α2-macroglobulin primarily at the same site, but a secondary cleavage site at the His_{694}–Ala_{695} peptide bond was also identified. Ht-e cleaves α2-macroglobulin at two sites which are different from those observed with Ht-a, -c, and -d. With Ht-e the primary cleavage site is the Val_{689}–Met_{690} peptide bond and the secondary site at Gly_{693}–His_{694}. Of the four toxins investigated, Ht-a is the most potent hemorrhagic toxin in vivo. We propose that reaction of Ht-a with the primary plasma proteinase inhibitor α2-M may partially explain the high hemorrhagic activity of this toxin.

Human α2-macroglobulin (α2-M) is a large plasma glycoprotein (M_r 720 000) that inhibits proteinases of all four classes (cysteine, serine, aspartate, and metallo) (Barrett & Starkey, 1973; Werb et al., 1974). The tetrameric molecule of α2-M is formed by the noncovalent association of two disulfide-bonded dimers (M_r 360 000) in which the monomers (M_r 180 000) are arranged in an antiparallel fashion (Harpel, 1973; Feldman et al., 1985; Jensen & Sottrup-Jensen, 1986; Sottrup-Jensen, 1989). The dimers constitute the functional proteinase binding units of α2-M (Pochon et al., 1978; Barrett et al., 1979; Swenson & Howard, 1979; Christensen & Sottrup-Jensen, 1984; Roche et al., 1988; Sottrup-Jensen, 1989). The mechanism of proteinase inhibition is explained by the "trap model" (Barrett & Starkey, 1973) according to which a proteinase first cleaves a peptide bond in the bait region sequence of α2-M, giving rise to the characteristic M_r 90 000 fragments (Harpel, 1973; Barrett et al., 1979). The α2-M then undergoes conformational changes resulting in the formation of tight, irreversible complexes with the proteinase (Barrett et al., 1979; Gonias et al., 1982; Gonias & Pizzo, 1983; Larsson et al., 1987). As a result of bait region cleavage, internal β-cysteinyl-γ-glutamyl thiol esters in α2-M become exposed and may react with nucleophilic groups (ε-amino groups of lysine) on the surface of the proteinase, thus forming covalently bound complexes (Sottrup-Jensen et al., 1980; Salvesen et al., 1981). The active site of the "trapped" proteinase is generally inhibited with respect to large substrates. The "trapped" proteinase is still accessible to small substrates and inhibitors (Barrett, 1981; Ganrot, 1966; Bieth et al., 1981).

A large variety of proteinases form complexes with α2-M (Mortensen et al., 1981; Sottrup-Jensen et al., 1981; Abe & Nagai, 1972). The rate of complex formation depends largely on the nature and the size of the proteinase. Some proteinases with restricted substrate specificity and large size react slowly with α2-M (Werb et al., 1974). It has been shown that some collagenases (metalloproteinases with limited substrate specificity) react slowly with α2-M, requiring many hours to go to completion (Werb et al., 1974). By contrast, Sottrup-Jensen and Hansen (1989) and Enghild et al. (1989) reported rapid reactions of collagenases with human α2-M.

In the present investigation we studied the interaction of α2-M with four zinc metalloproteinases (Ht-a, -c, -d, and -e) purified from *Crotalus atrox* crude venom (Bjarnason & Tu, 1978). These proteinases give rise to the typical hemorrhage observed with *C. atrox* envenomation. Microscopically, they appear to disrupt basement membrane structure surrounding capillaries, allowing escape of capillary contents into the surrounding matrix (Ownby et al., 1978). The molecular weights of Ht-a, Ht-c, Ht-d, and Ht-e are 68 000, 24 000, 24 000, and 25 000, respectively. Zinc ligands in the active site of these proteinases and plays an important role in catalysis as well as maintenance of protein structural integrity (Fox & Bjarnason, 1983; Bjarnason & Fox, 1986). We have previously demonstrated that these toxins are capable of releasing digestion peptides from basement membrane preparations (Bjarnason et al., 1988). We have also shown that Ht-a, -c, -d, and -e digest several components of extracellular matrix, namely, collagen type IV (Shannon et al., 1989), laminin, nidogen, and fibronectin (Baramova et al., 1989).

The results of the present study indicate substantial differences in the rates of reaction of α2-M with Ht-a, -c, -d, and -e. Finally, the identified cleavage sites are within the bait

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region of α2-M and correspond to the peptide bond substrate specificities of these metalloproteinases.

Materials and Methods

Hemorrhagic toxins a and c–e were isolated from the crude venom of the Western diamondback rattlesnack, *C. atrox* (Miami Serpentarium), according to a modification of the method of Bjarnason and Tu (1978). The toxins were determined to be homogeneous by SDS and native PAGE. α2-M was purified from human plasma according to the method of Imber and Pizzo (1981). Human gelatin type I was prepared by heating collagen type I (Sigma) at 70 °C for 15 min and then maintained at 37 °C to prevent renaturation (Welgus et al., 1982). Collagen type IV, isolated from EHS tumor grown in C57/Bl lathyritic mice, was from Collaborative Research Inc. Hide powder azure was from Calbiochem.

Gelatin Digestion. Ht-a, -c, -d, or -e (40 μM of each) was incubated with a 20-fold excess (w/w) of gelatin at 37 °C for 15 min in 20 μL of 50 mM Tris-HCl, 10 mM NaCl, and 2 mM $CaCl_2$, pH 7.4. To test inhibition by α2-M, the enzymes were preincubated with 21 μg of α2-M at 22 or 37 °C, and then gelatin was added. Reactions were stopped by adding electrophoresis sample buffer (Laemmli, 1970) and boiling for 3 min. SDS–PAGE was performed by using a 7% separating gel with a 3% stacking gel according to Laemmli (1970), using myosin (M_r 200 000), β-galactosidase (M_r 116 250), phosphorylase B (M_r 97 400), bovine serum albumin (M_r 66 200), and ovalbumin (M_r 45 000) as molecular weight standards.

Collagen Digestion. Collagen type IV was dialyzed against 20 mM sodium phosphate and 150 mM NaCl, pH 7.4 (PBS) for 6 h at 4 °C. Four micrograms of Ht-a, -c, -d, or -e was incubated with 40 μg of collagen for 24 h at 25 °C in 40 μL of PBS. To test inhibition by α2-M, Ht-a, Ht-c, and Ht-d were preincubated with an equimolar amount of α2-M and for Ht-e at a ratio of proteinase:α2-M of 2:1 at 25 °C. Collagen type IV was subsequently added, and electrophoresis was performed as described above.

α2-M Digestion. α2-M was dialyzed against PBS for 6 h at 4 °C. Aliquots of 7.4 μM α2-M were incubated with increasing concentrations of Ht-a, -c, -d, and -e (5.7–37 μM; I:E = 1:0.77 to 1:5 mol/mol) at 22 °C for 2 min in a total volume of 33 μL. The incubation contents were prepared for SDS–PAGE as described above. Quantitative data were obtained by scanning gels on a Visage 2000 instrument (Kodak Co.).

Hide Powder Assay of Proteinases. The proteolytic activity of Ht-a, -c, -d, and -e was assayed with hide powder azure as described by Barrett (1981) with some modifications. The buffer used was PBS, and the substrate was suspended in the same buffer. Proteinases were added to the substrate suspension and incubated for 60 min at 37 °C with continuous agitation.

Rate of Interaction of α2-M with Ht-a, -c, -d, and -e. The kinetics of reaction of α2-M with Ht-a, -c, -d, and -e were studied by monitoring α2-M intrinsic fluorescence as described by Christensen and Sottrup-Jensen (1984). Excitation was at 280 nm with 3-nm slit, and fluorescence emission was at 340 nm with 20-nm slit, in a Perkin-Elmer LS-5 fluorometer. The protein concentration of Ht-a, -c, -d, and -e was 0.1 μM. Incubation was carried out with equimolar concentrations of α2-M and proteinase. Previous studies suggest that the reaction of α2-M with proteinases involves a number of distinct steps including reversible complex formation, bait region cleavage, conformational change, and thiol ester bond cleavage (Christensen & Sottrup-Jensen, 1984; Gonias & Figler, 1988). The fluorescence technique detects conformational change (a later step in the reaction mechanism). In these experiments, by assuming formation of only 1:1 complexes in a simple bimolecular reaction (the concentration of α2-M was equal to the concentration of proteinase), the second-order rate constants (k_2) may be approximated by using the equation:

$$a_0/a_t = k_2 t a_0 + 1$$

where a_0 = the concentration of enzyme at time = 0 and a_t equals the concentration of unreacted enzyme at time = t. In the intrinsic fluorescence experiments

$$a_0/a_t = 1/(1 - \Delta F_t/\Delta F_{max})$$

where ΔF_t is the fluorescence change at time t and ΔF_{max} is the fluorescence at the completion of the reaction.

Amino Acid Sequencing. The sites of α2-M cleavage by the metalloproteinases were determined by sequence analysis. The digestion reactions were carried out by incubating a 2-fold excess of Ht-c, -d, and -e with α2-M for 2 min at 22 °C while Ht-a was incubated with α2-M for 30 min at 37 °C, after which SDS–PAGE was carried out, electroblotted onto poly(vinylidene difluoride) membrane according to the method of Matsudaira (1987). The visualized protein bands were excised from the membrane and stored at −20 °C prior to sequence analysis. The excised band was cut into 2-mm^2 fragments and loaded onto a Polybrene-treated glass fiber disk in an Applied Biosystems Model 470A sequenator interfaced with an Applied BioSystems Model 120A on-line PTH analyzer. The Edman degradations were carried out with the standard 03RPTH program.

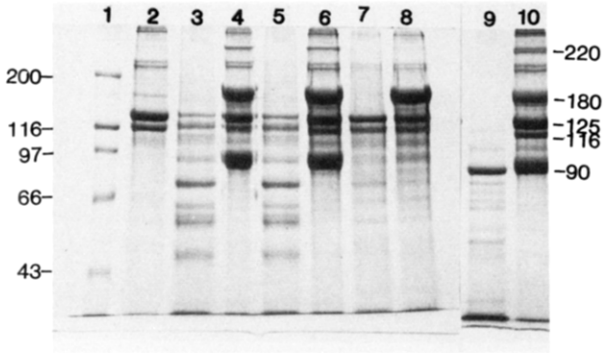

FIGURE 1: Digestion of gelatin type I by Ht-a, -c, -d, and -e in the absence and presence of α2-M. Ht-a, -c, -d, and -e (1 μg) were incubated with gelatin type I (20 μg) at 37 °C for 15 min. To test inhibition by α2-M, the enzymes were preincubated with an equimolar amount of the inhibitor at 22 °C for 2 min prior to addition of substrate. Lane 1, molecular weight markers; lane 2, gelatin type I, control; lanes 3, 5, 7, and 9, gelatin type I incubated with Ht-c, -d, -a, and -e, respectively; lanes 4, 6, 8, and 10, Ht-c, -d, -a, and -e, respectively, preincubated with α2-M.

Results

Digestion of Gelatin Type I and Collagen Type IV by Ht-a, -c, -d, and -e in the Presence of α2-M. Gelatin type I is rapidly digested by Ht-a, -c, -d, and -e, leading to the formation of fragments of M_r 90 000 or less (Figure 1). When Ht-c, -d, and -e were preincubated with α2-M for 5 min at 22 °C, the proteolytic activities against gelatin type I were nearly completed inhibited. The three enzymes formed high molecular weight covalent complexes with α2-M (M_r 220 000 in reducing SDS gels). In addition, the α2-M subunits were cleaved, giving rise to the characteristic M_r 90 000 protein fragments (Figure 1) (Barrett et al., 1979). The proteolytic activity of Ht-a against gelatin type I was not inhibited when the enzyme was preincubated with α2-M, and only minimal formation of the M_r 90 000 fragments was observed (Figure 1).

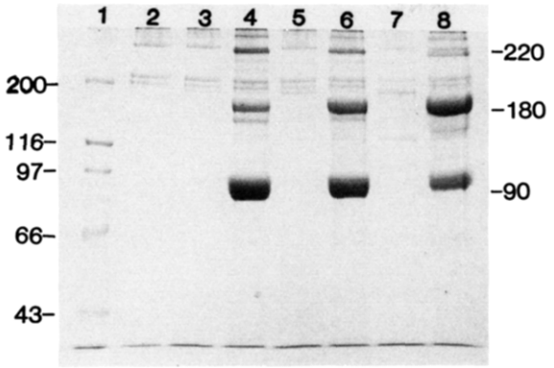

FIGURE 2: Digestion of collagen type IV by Ht-c, -d, and -e and inhibition by α2-M. Aliquots of collagen type IV (20 μg) were incubated with Ht-c, -d, and -e (2 μg) at 25 °C for 24 h. To test inhibition by α2-M, the enzymes were preincubated with α2-M for 5 min at 22 °C. Lane 1, molecular weight markers; lane 2, collagen type IV, control; lanes 3, 5, and 7, collagen type IV digested by Ht-c, -d, and -e, respectively; lanes 4, 6, and 8, Ht-c, -d, and -e, respectively, preincubated with α2-M and then incubated with collagen type IV.

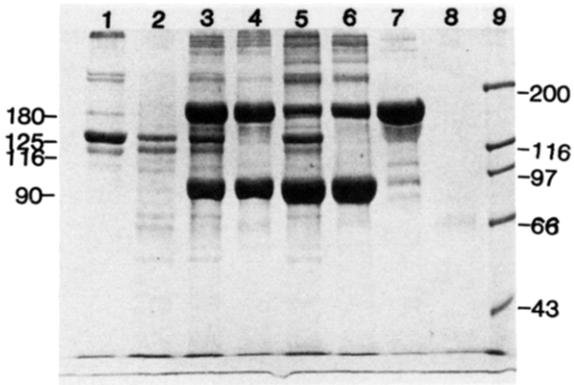

FIGURE 3: Digestion of gelatin type I by Ht-a in the presence and absence of α2-M. Gelatin type I (20 μg) was incubated with Ht-a (2 μg) at 37 °C for 15 min to measure proteolytic activity. Samples containing α2-M were preincubated with the inhibitor for 1 or 24 h at 37 °C. Lane 1, gelatin type I control; lane 2, gelatin type I digested by Ht-a; lanes 3 and 5, gelatin type I digested by Ht-a after preincubation with α2-M for 1 and 24 h, respectively; lanes 4 and 6, α2-M preincubated with Ht-a for 1 and 24 h; lane 7, α2-M, control; lane 8, Ht-a; lane 9, molecular weight markers.

α2-M inhibited the proteolytic activities of Ht-c, -d, and -e against another large molecular weight natural substrate, collagen type IV, over the course of 24-h incubation (24-h data not shown), as observed by the absence of the M_r 190 000 product, just below the α1(IV) and α2(IV) chains (Figure 2). The proteolytic activity of Ht-a against collagen type IV was not inhibited (results not shown).

In an attempt to increase the interaction of α2-M and Ht-a, the temperature of incubation was increased to 37 °C and the time of interaction extended up to 24 h. The SDS gel presented in Figure 3 shows that this more rigorous incubation caused partial inhibition of the proteolytic activity of Ht-a against gelatin type I as shown by the absence of the digestion products with M_r of approximately 66 000 and 100 000 and decreased degradation of the α1(I) chain. This incubation also lead to the formation of the M_r 90 000 α2-M fragments and high molecular weight complexes (Figure 3).

Cleavage Pattern of α2-M–Proteinase Complexes. In order to investigate the cleavage pattern of the complexes formed by α2-M with Ht-a, -c, -d, and -e, a constant concentration (7.4 μM) of the inhibitor was incubated with increasing concentrations of the enzymes (5.7–37 μM; proteinase:α2-M = 0.77–5 molar ratio). The samples were subjected to electrophoresis, and densitometry was performed. Ht-c, -d, and -e cleaved the M_r 180 000 subunit of α2-M, yielding the M_r 90 000 fragments. In each case, the extent of α2-M cleavage was related to the amount of the proteinase present (Table I). When the digestion pattern intensities of α2-M after incubation with the metalloproteinases are compared (Table I), it is clearly seen that Ht-e and Ht-c cleave the inhibitor to a similar extent. Ht-d produced the same digestion pattern as Ht-c (results not shown). Ht-a reacted very slowly with α2-M, and very little cleavage of the M_r 180 000 subunit was observed under these conditions.

Table I: Digestion of α2-M as a Function of Proteinase Concentration[a]

ratio E:α2-M	residual α2-M (%) after Ht-e digestion	residual α2-M (%) after Ht-c digestion	residual α2-M (%) after Ht-a digestion
0.77	46.4	48.2	84.9
1.0	39.6	39.8	93
1.25	24.2	29.2	97.1
1.66	21.9	22.3	110
2.50	10.7	18.1	113
5.0	10.7	15.9	89.6

[a] α2-M was incubated with Ht-a, -c, -d, and -e at 22 °C for 2 min at the molar ratios shown. Samples were electrophoresed on SDS–PAGE and the bands quantitated by densitometry.

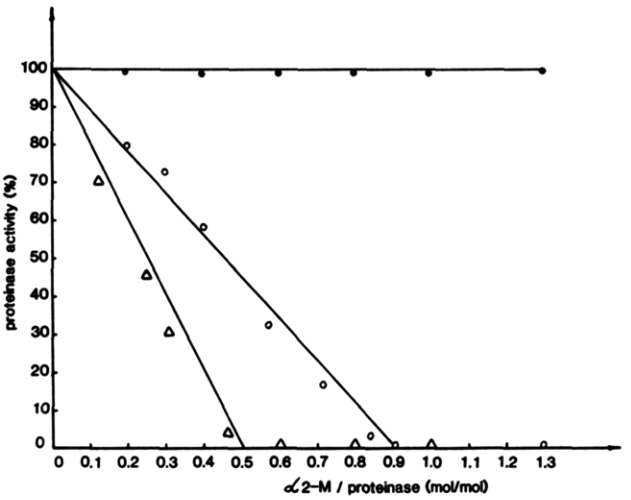

FIGURE 4: Stoichiometry of inhibition of Ht-a, -c, -d, and -e by α2-M. Various amounts of α2-M were incubated with the four proteinases in molar ratios indicated on the abscissa (α2-M:proteinase = 0–1.3). Proteinase activity in the mixture was assayed with hide powder azure as substrate. The ordinate indicates the remaining activity of the proteinases as a percent of the original activity. (●) Ht-a; (○) Ht-c; (△) Ht-e.

Stoichiometry of Ht-a, -c, -d, and -e with α2-M. Increasing amounts of α2-M were incubated with a constant concentration of each metalloproteinase, and the proteinase activity was assayed with hide powder azure as substrate (Figure 4). The proteolytic activities of Ht-c, -d, and -e decreased in proportion to the amount of added α2-M and were completely inhibited. The stoichiometry of inhibition was 2.0 mol of Ht-e, 1.1 mol of Ht-c, and 1.1 mol of Ht-d per mole of α2-M (results not shown for Ht-d). Since the proteolytic activity of Ht-a against hide powder azure was not significantly inhibited by α2-M, the binding ratio was not determined (Figure 4).

Rate of Interaction of α2-M with Ht-a, -c, -d, and -e. The kinetics of reaction of α2-M with Ht-a, -c, -d, and -e were investigated by measuring changes in the intrinsic fluorescence of α2-M caused by each metalloproteinase as a function of time. Figure 5, panel A, shows the relative change in intrinsic

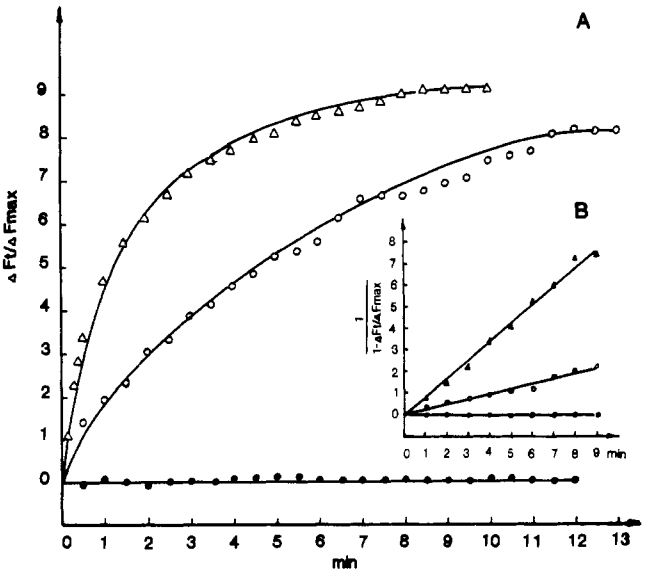

FIGURE 5: Kinetics of change in intrinsic fluorescence when Ht-a, -c, -d, and -e interact with α2-M. Panel A: The relative fluorescence change $\Delta F_t/\Delta F_{max}$, where ΔF_{max} is the fluorescence change obtained at the end of the reaction and ΔF_t is the fluorescence change at time t, is plotted against time. Panel B: The relative amount of unreacted product $a_t/a_0 = 1 - (\Delta F_t/\Delta F_{max})$, where a_0 is the initial amount of substrate and a_t is the amount of substrate at time t, is plotted against time: (●) Ht-a; (○) Ht-c; (△) Ht-e.

fluorescence obtained as a function of time. The rate constants are calculated from the slopes of the lines in panel B. The rate constants of the reactions between α2-M and Ht-c and -d were both 0.62×10^5 M^{-1} s^{-1} (results not shown for Ht-d). Ht-e reacted more rapidly, with a rate constant of 2.3×10^5 M^{-1} s^{-1}. The interaction of Ht-a with α2-M was very slow, and no changes in α2-M intrinsic fluorescence were observed during 30 min of incubation.

Identification of Cleavage Sites of Ht-a, -c, -d, and -e in the Bait Region of α2-M. After incubation of α2-M with each metalloproteinase, the digestion fragments were electrophoresed, electroblotted onto a poly(vinylidene difluoride) membrane, and subjected to amino-terminal sequence analysis. Each of the gel bands with a M_r of 90000 contained two or three peptide chains; one was the amino terminus of α2-M and the others were derived from the proteinase cleavage site of the α2-M bait region. The amino-terminal sequences of the peptides were compared with the complete primary structure of α2-M (Sottrup-Jensen et al., 1984; Kan et al., 1985). Sequence data presented in Chart I show that Ht-c and -d produce a single new protein fragment with an amino terminus corresponding to Leu_{697} in the bait region of α2-M. Ht-a cleaves α2-M at two positions in the bait region. The primary cleavage site is at the Arg_{696}–Leu_{697} peptide bond, producing the same protein fragments as Ht-c and -d. The secondary cleavage site is at the His_{694}–Al_{695} peptide bond. Ht-e, like Ht-a, cleaves the α2-M bait region at two sites; however, both are different from those hydrolyzed by Ht-a, -c, and -d. The primary cleavage site is at the Val_{689}–Met_{690} peptide bond and the secondary one at the Gly_{694}–His_{694} peptide bond.

Figure 6 shows the gels of the M_r 220000 and 130000 complexes, produced by the interaction of Ht-e with α2-M. In the M_r 130000 band, two peptide fragments were identified, one corresponding to the amino terminus of Ht-e (Bjarnason and Fox, unpublished data) and the other to a fragment beginning at Met_{690} in the bait region of α2-M. From the molecular weight and sequence data, it appears that the M_r 220000 band contains two identical α2-M fragments, possibly cross-linked by one molecule of Ht-e.

Chart I: N-Terminal Sequence Analysis of Fragments of α2-M Produced by Ht-a, -c, -d, and -e[a]

Enzyme: Ht-a
Mr of Band: 85 000-95 000
Sequences:

1				5		
S	-	V	-	S	-	G	-	K	-	P
(3.5)	(2.6)	(2.8)	(5.5)	(3.2)	(2.9)	
697				701		
L	-	V	-	H	-	V	-	E	-	E
(1.7)	(2.6)	(0.2)	(0.5)	(1.1)	(1.4)	
695				699		
A	-	R	-	L	-	V	-	H	-	V
(0.6)	(-)	(1.0)	(0.5)	(0.1)	(0.1)	

Enzyme: Ht-c
Mr of Band: 85 000-95 000
Sequences:

1					5					10
S	-	V	-	S	-	G	-	K	-	P	-	Q	-	Y	-	M	-	V
(4.7)	(7.2)	(4.7)	(10.6)	(9.1)	(11.3)	(8.6)	(8.4)	(7.87)	(2.0)	
697					701					706
L	-	V	-	H	-	V	-	E	-	E	-	(P)	-	H	-	T	-	E
(1.7)	(7.2)	(0.4)	(4.2)	(5.7)	(2.4)	-	(1.5)	(3.9)	(1.3)	

Enzyme: Ht-d
Mr of Band: 85 000-95 000
Sequences:

1					5					
S	-	V	-	S	-	G	-	K	-	P	-	Q	-	Y		
(6.3)	(6.8)	(1.2)	(9.0)	(1.4)	(7.6)	(4.3)	(2.1)			
697					701					
L	-	V	-	(H)	-	V	-	E	-	E	-	P	-	(H)		
(6.4)	(6.8)	(-)	(4.2)	(0.5)	(2.7)	(-)	(-)			

Enzyme: Ht-e
Mr of Band: 85 000-95 000
Sequences:

1					5					10
S	-	V	-	S	-	G	-	K	-	P	-	Q	-	Y	-	M	-	V
(5.5)	(6.3)	(3.4)	(5.0)	(3.3)	(7.0)	(6.5)	(4.3)	(4.4)	(2.2)	
690				694						699
M	-	G	-	R	-	G	-	(H)	-	A	-	R	-	L	-	V	-	H
(1.5)	(2.9)	(0.6)	(5.0)	(-)	(2.6)	(1.6)	(2.6)	(3.9)	(-)	
694					698					703
H	-	A	-	R	-	L	-	V	-	H	-	V	-	E	-	E	-	P
(0.3)	(1.5)	(0.6)	(1.6)	(1.1)	(0.2)	(2.0)	(-)	(2.1)	(0.1)	

Covalent complexes of Ht-e and α2-macroglobulin
Mr of Band: 130,000

690		694		
M	-	G	-	R	-	G		α2-M
5.4		6.2		1.85		4.7		
1								
N	-	P	-	E	-	H		Ht-e
3.4		5.1		3.2		0.59		

[a] After incubation of the proteinase and α2-M, the bands in the molecular weight ranges indicated were electroblotted and sequenced. Because the bands were not resolved, two or three bands were sequenced simultaneously. The top numbers indicate the position of the amino acid residue in the intact α2-M sequence. Numbers in parentheses are the net picomoles of phenylthiohydantoin amino acid (yield$_n$ – yield$_{n-1}$) recovered in each step of Edman degradation; a dash indicates that the amino acid was not quantitated. Where the same residue appears in the same position in two sequences, the yield is split between the two sequences. Letters in parentheses are residues postulated from the known sequences but not seen either because of loss of the amino acid or because of a high background in the previous cycle.

DISCUSSION

Hemorrhagic toxins a and c–e isolated from the *C. atrox* crude venom are metalloproteinases, with proteolytic activity against gelatin type I, collagen type IV, and some other components of extracellular matrix (Shannon et al., 1989; Baramova et al., 1989). α2-M, the major plasma metalloproteinase

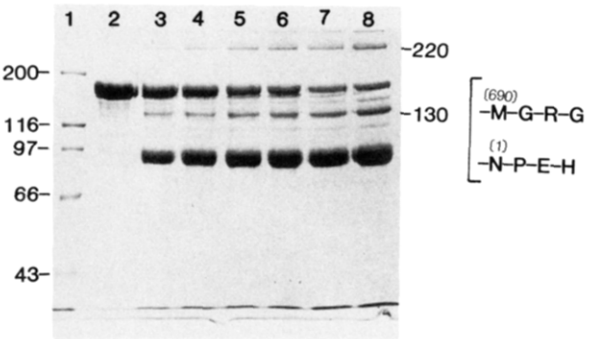

FIGURE 6: Digestion of α2-M and formation of covalent complexes of Ht-e and α2-M. Varying amounts of Ht-e were incubated with α2-M for 2 min at 22 °C. Lane 1, molecular weight markers; lane 2, α2-M control; lane 3, Ht-e:α2-M = 0.77; lane 4, Ht-e:α2-M = 1; lane 5, Ht-e:α2-M = 1.25; lane 6, Ht-e:α2-M = 1.66; lane 7, Ht-e:α2-M = 2.5; lane 8, Ht-e:α2-M = 5. Sequence data for the M_r 220000 and 130000 bands are shown.

inhibitor, effectively inhibited the activities of Ht-c, -d, and -e against gelatin type I and collagen type IV. The activity of Ht-a against gelatin type I was diminished but not completely inhibited even after longer incubation times at elevated temperature (37 °C).

These results indicate that Ht-c, -d and -e, which have M_r of approximately 25000, interact with α2-M more rapidly than Ht-a (M_r 68000). It is possible that the relatively large size of Ht-a inhibits reversible association of the proteinase with α2-M. Size is not sufficient to cause a slow reaction with α2-M because the large proteinase, plasmin (M_r 90000) (Christensen & Sottrup-Jensen, 1984), reacts with α2-M at a rate comparable with that found here for Ht-e, M_r 25000. An alternative explanation may involve the primary structure of the α2-M bait region. We suggest that the bait region of α2-M may be a poor substrate for Ht-a, compared with the other enzymes studied here.

The cleavage pattern of α2-M complexes with Ht-a, -c, -d, and -e shows that the small metalloproteinases produce M_r 90000 fragments from α2-M and large (M_r 220000 and 130000), covalent complexes with α2-M more rapidly than Ht-a. Furthermore, Ht-e forms complexes with α2-M more rapidly than Ht-c and -d. Ht-e gives rise to M_r 90000 fragments within 1 min of incubation at 4 °C (results not shown). Thus, at equimolar ratios of enzymes to α2-M the reactivities of the four metalloproteinases with α2-M can be arranged in the following decreasing order; Ht-e > Ht-d = Ht-c ≫ Ht-a. It is not surprising that Ht-c and Ht-d react comparably with α2-M since it has been shown that they are isoenzymes with very similar characteristics (Bjarnason & Fox, 1987).

The sequence analysis of the M_r 220000 and 130000 bands, produced by the interaction of Ht-e with α2-M, shows that both bands contain two peptide chains; one starts at the amino terminus of Ht-e and the other at methionine 690 in the bait region of α2-M. Apparently, the M_r 130000 band is formed by the covalent binding of Ht-e to one α2-M subunit which has been cleaved in the bait region. The M_r 220000 band most probably represents a bis-cross-linked product, formed by the covalent binding of one Ht-e molecule with two cleaved α2-M subunits. The formation of similar complexes has been reported for trypsin (Howell et al., 1983).

The maximum binding capacities of α2-M to Ht-a, -c, -d, and -e are different. One mole of α2-M can inhibit 2 mol of Ht-e. Similar molar ratios have been reported for the serine proteinase trypsin and cysteine proteinase papain (Howell et al., 1983). Ht-c and -d are inhibited at molar ratio of proteinase:α2-M = 1.1:1; a similar stoichiometry has been reported for chymotrypsin (Howell et al., 1983). Ht-a interacts with α2-M very slowly, like the collagenase of *Clostridium histolyticum* (Werb et al., 1974; Sottrup-Jensen & Birkedal-Hansen, 1989), so it was difficult to estimate its binding ratio with α2-M. These results demonstrate that the rate of α2-M bait region cleavage by each metalloproteinase correlates with the ability of the inhibitor to bind more than 1 mol/mol of that enzyme.

The rate of interaction of the proteinases with α2-M was studied by measuring the intrinsic fluorescence, carried out at molar ratio 1:1 since it has been shown that the binding of one molecule of proteinase to one molecule of α2-M is sufficient to effect the complete conformational change of the inhibitor (Gonias & Pizzo, 1983; Van Leuven et al., 1981). As assessed by conformational changes, Ht-e interacts 3.7 times faster with α2-M than Ht-c and Ht-d and the rate of reaction of Ht-a was below the sensitivity of our measurements. The range of these results is similar to those obtained for the serine proteinases plasmin and miniplasmin (Christensen & Sottrup-Jensen, 1984; Gonias & Figler, 1988). The fluorescence experiments and stoichiometry studies presented here suggest that the molar binding ratio for metalloproteinases and α2-M may depend on reaction rate, as suggested for serine proteinases by Howell et al. (1983).

Ht-a, -c, -d, and -e cleave α2-M in the bait region. The bait region of human α2-M was initially defined as the region of the protein molecule containing amino acid residues 677–703 (Sottrup-Jensen et al., 1981) and recently enlarged to include amino acid residues 666–706 (Sottrup-Jensen & Birkedal-Hansen, 1989). Ht-c and -d both cleave α2-M at the Arg_{696}–Leu_{697} peptide bond. Previous studies on the substrate specificity of Ht-c and -d have shown that these isoenzymes cleave the oxidized B chain of insulin and synthetic peptides at X–Leu peptide bonds (Fox et al., 1986), consistent with the cleavage of α2-M observed here. Ht-a cleaves primarily the same Arg_{696}–Leu_{697} peptide bond, but a secondary cleavage site at His_{694}–Ala_{695} was also identified. It has been shown that Ht-a, like Ht-c and -d, cleaves the oxidized insulin B chain at X–Leu bonds (Bjarnason et al., 1988), consistent with the site of cleavage of α2-M. It is established that Arg_{696}–Leu_{697} is the cleavage site for plasmin, thrombin, trypsin, and thermolysin (Mortensen et al., 1981; Sottrup-Jensen et al., 1981). Thus, the Arg_{696}–Leu_{697} peptide bond appears to be very susceptible for both serine and metalloproteinases.

Ht-e cleaves α2-M at two sites, different from those of Ht-a, -c, and -d. The primary cleavage site is at the Val_{689}–Met_{690} peptide bond, and the secondary site is at the Gly_{693}–His_{694} peptide bond, consistent with previous data showing that Ht-e cleaves the X–His bond in the oxidized B chain of insulin (Bjarnason & Fox, 1983) and the X–Met peptide bond in Ht-d (Shannon et al., 1989). However, it also cleaves a X–Leu peptide bond in the oxidized B chain of insulin and nidogen (Bjarnason & Fox, 1983; Baramova et al., unpublished data), but it is possible that the conformation of the α2-M bait region makes the Val_{689}–Met_{690} peptide bond the preferred digestion site. The broader substrate specificity of Ht-e in comparison with Ht-a, -c, and -d may perhaps account for its increased reactivity with α2-M. By contrast, stromelysin, which has a broad substrate specificity, reacts with α2-M at a much slower rate than collagenase, which has a substrate specificity limited to interstitial collagens (Enghild et al., 1989).

All proteinases in the circulation are subject to regulation by the plasma proteinase inhibitors. Intrinsic enzymes such as clotting factors and extrinsic enzymes such as the hemorrhagic proteinases may demonstrate little or no activity if the

rate of reaction with inhibitors is rapid (Travis & Salvesen, 1983). In the plasma, α2-M is the major metalloproteinase inhibitor. It is therefore extremely interesting that Ht-a (the most hemorrhagically potent of the four investigated toxins (Bjarnason & Tu, 1978), is relatively resistant to inhibition by α2-M. The other proteinases studied here were inhibited by α2-M, and given the high concentration of α2-M in the plasma, these enzymes are most likely neutralized quickly in vivo as well. Our results are similar to those of Kurecki and Kress (1985), who observed that proteinase H from *Crotalus adamanteus*, which is a large metalloproteinase with a similar hemorrhagic potency to Ht-a, is not inhibited by α2-M even after 20 h of incubation. We propose that the activity of the hemorrhagic proteinases in vivo reflects at least in part their interaction with α2-M.

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