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Inactivation of Human Plasma Serine Proteinase Inhibitors (Serpins) by Limited Proteolysis of the Reactive Site Loop With Snake Venom and Bacterial Metalloproteinases

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Human plasma serine proteinase inhibitors (serpins) gradually lost activity when incubated with catalytic amounts of snake venom or bacterial metalloproteinases. Electrophoretic analyses indicated that antithrombin III, $C\bar{1}$-inhibitor, and α_2-antiplasmin had been converted by limited proteolysis into modified species which retained inhibitory activity. Further proteolytic attack resulted in the formation of inactivated inhibitors; α_1-proteinase inhibitor (α_1-antitrypsin) and α_1-antichymotrypsin were also enzymatically inactivated, but active intermediates were not detected. Sequence analyses indicated that the initial, noninactivating cleavage occurred in the amino-terminal region of the inhibitors. Inactivation resulted in all cases from the limited proteolysis of a single bond near, but not at, the reactive site bond in the carboxy-terminal region of the inhibitors. The results indicate that the serpins have two regions which are susceptible to limited proteolysis—one near the amino-terminal end and another in the exposed reactive site loop of the inhibitor.

Key words: plasma proteinase inhibitor, serpin, reactive site

Investigations in this laboratory have been concerned with a systematic study of the effect of snake venom proteinases on human plasma serine proteinase inhibitors (serpins), following the initial reports that human serum gradually lost all detectable inhibitory activity against trypsin and chymotrypsin during incubation with crotalid, viperid, or colubrid venoms [1] and that human α_1-proteinase inhibitor (α_1-PI) was inactivated by limited proteolysis of an exposed segment of the native α_1-PI molecule [2]. The enzymatic inactivation of the serpins by *Pseudomonas aeruginosa* protease and elastase and by several other bacterial proteinases has also been investigated [3,4].

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This report summarizes the effects of venom and bacterial metalloproteinases on five members of the serpin superfamily—namely, α_1-PI, antithrombin III (AT III), C$\bar{1}$-inhibitor (C$\bar{1}$-Inh), α_1-antichymotrypsin (α_1-AC), and α_2-antiplasmin (α_2-AP). Data are also presented indicating that an α_1-PI analog isolated from opossum (*Didelphis virginiana*) serum is resistant to inactivation under conditions in which human α_1-PI is totally inactivated. The results are discussed in relation to the recent proposal that enzymatic inactivation of serpins involves proteolysis within the exposed reactive site loop which converts the inhibitors from a stressed to a relaxed conformation [5].

MATERIALS AND METHODS

Inhibitors were prepared as described: α_1-PI [6]; C$\bar{1}$-Inh [7]; α_1-AC by a slight modification of [8] as described in [9]. AT III was provided by the American Red Cross Blood Services Laboratory, α_2-AP by Dr. D. Collen, University of Leuven, *P aeruginosa* protease and elastase by Dr. K. Morihara, Toho Pharmaceutical Co. (Kyoto, Japan), and human leucocyte elastase by Dr. Dave Johnson, East Tennessee State University. Inactivation of α_1-AC and C$\bar{1}$-Inh by leucocyte elastase was performed essentially according to the procedures used for inactivation of these serpins by the *P aeruginosa* enzymes [3]. *Crotalus adamanteus* proteinase II and *Crotalus atrox* α-proteinase were prepared according to [2,10], respectively. Serpin inactivation and analyses of the reaction intermediates and products were performed as described: α_1-PI [11]; AT III [12]; C$\bar{1}$-Inh [3, 7]; α_1-AC [3]; and α_2-AP [9]. Frozen opossum serum was obtained from Research Biogenics (Bastrup, TX). The 45–80% $(NH_4)_2SO_4$ fraction was chromatographed on DEAE-Sepharose (0.02 M Na phosphate, pH 6.5; 0–0.3 M NaCl gradient). The material which eluted between 0.13 and 0.16 M NaCl and which inhibited both trypsin and chymotrypsin was chromatographed on phenyl-Sepharose (0.1 M Na phosphate-1.0 M $(NH_4)_2SO_4$, pH 7.0; 1.0–0 M $(NH_4)_2SO_4$ gradient). Opossum α_1PI eluted between 0.5 and 0.4 M $(NH_4)_2SO_4$.

RESULTS

Incubation of human α_1-PI with crotalid, viperid, or colubrid venoms resulted in inactivation of the inhibitor [1]. The reaction could be terminated with EDTA and was unaffected by addition of phenylmethylsulfonyl fluoride. Two metalloproteinases, designated proteinases I and II, were isolated from *C adamanteus* (Eastern diamondback rattlesnake) venom [2]. Both proteinases I and II catalytically inactivated α_1-PI, and electrophoretic analysis revealed that intact α_1-PI (54k) was cleaved by limited proteolysis to yield inactive α_1-PI (50k) and a peptide (4k), as shown diagrammatically in Figure 1. Sequence analysis [11] indicated (Fig. 2) that the inactivating cleavage had occurred at the Ala_{350}-Met bond eight residues removed from the Met_{358}-Ser reactive site bond of α_1-PI which is cleaved by trypsin, papain, elastase, chymotrypsin [13], and *Serratia marcescens* metalloproteinase [14]. *P aeruginosa* elastase inactivated α_1-PI by cleavage of the Pro_{357}-Met bond [15].

In contrast to the above, an α_1-PI analog isolated from opossum *(Didelphis virginiana)* serum resisted inactivation by proteinase II and *C atrox* α-proteinase as shown in Table I. Similar results were also obtained for incubation of these inhibitors with several crude venoms. Electrophoretic analysis indicated the expected cleavage

Fig. 1. Schematic representation of serpin inactivation by snake venom and bacterial proteinases. The schematic indicates the molecular weights and inhibitory activity (a = active, i = inactive) of the major products of the inhibitor digests, and, where known, the origin of the peptide(s) released. The metalloproteinases used and the references giving the experimental details upon which this schematic is based are as follows: *C adamanteus* proteinase II (α_1-PI [2,11], AT III, AT III/heparin [12]); *P aeruginosa* elastase ($C\bar{1}$-Inh [3]); *C atrox* α-proteinase (α_1-AC [10]); and *C basiliscus* crude venom or *P aeruginosa* elastase (α_2-AP [9], (unpublished)). Additional proteinases which cleave the individual inhibitors in the manner depicted above are mentioned in the text and in Table II.

Fig. 2. Location of bonds cleaved during limited proteolysis of serpins by metalloproteinases. P_1-P_1' designates the inhibitor reactive site [28]. The location of the cleavages and the effect on inhibitory activity were as follows: open arrows, noninactivating cleavages; closed arrows, inactivating cleavages; dashed arrow, minor cleavage subsequent to inactivation. The metalloproteinases used and references giving details of the structural analyses were as follows: *C adamanteus* proteinase II (α_1-PI [11], AT III, AT III/heparin [12]); for $C\bar{1}$-Inh [7], *C atrox* α-proteinase cleaved P_{36}-I and *P aeruginosa* elastase cleaved S-V in the isolated $C\bar{1}$-Inh intermediate (see text for details). Residue numbering for α_1-PI and AT III is from [29], and for the amino terminal region of $C\bar{1}$-Inh from [30]. The reactive site sequence and carboxy terminal residue of $C\bar{1}$-Inh are from A.E. Davis (personal communication).

TABLE I. Effect of Venom Proteinases on Human and Opossum α_1-PI*

Incubation mixture	Ratio (A_{280} units)[a]	% α_1-PI inactivated
1. Opossum α_1-PI + *C atrox* α-proteinase	12:1	0
2. Human α_1-PI + *C atrox* α-proteinase	115:1	100
3. Opossum α_1-PI *C adamanteus* proteinase II	55:1	5
4. Human α_1-PI + *C adamanteus* proteinase II	55:1	96

*Opossum α_1-PI (340 A_{280} units) or human α_1-PI (215 A_{280} units) was incubated with the venom metalloproteinases to give the ratios noted at 23°C in 0.05 M Tris-HCl-0.002 M $CaCl_2$, pH 8.0, in a final volume of 250 μl. Aliquots were assayed after 1-hr incubation for residual α_1-PI inhibitory activity against bovine trypsin (BAPA substrate).

[a]One A_{280} unit is that amount of protein which if dissolved in 1 ml and read in a 1-cm light path at 280 nm will give an absorbance of one.

TABLE II. Activity of Proteinases on the Serpins*

Proteinase	α_1-PI	AT III	AT III/ heparin	α_1-AC	C$\bar{1}$-Inh	α_2-AP
C adamanteus proteinase II	3.3×10^3	33	580	140	0	0
C atrox α-proteinase	4.4×10^3	0.7	4.4	2.2×10^5	0	4.2
D angusticeps proteinase	—	—	—	5.1×10^3	—	—
P aeruginosa elastase	[a]	—	2.2	1.7×10^5	6.0×10^4	166
P aeruginosa protease	[a]	—	1.9	1.6×10^5	131	0.9
Human leucocyte elastase	[d]	[b]	—	2.3×10^3	8.4×10^3	[c]

*The values listed are specific activities expressed as μg inhibitor inactivated/min/A_{280} of proteinase, calculated from the residual inhibitory activity after 30-min incubation. Details of the experimental results from which the above calculations were taken can be found in the references listed in Figure 1. Dashes indicate that activity has not yet been determined in this laboratory. References in which inhibitor inactivation by the proteinases listed above has been noted by others are as follows: [a][25]; [b][5]; [c][26]. Inactivation of C$\bar{1}$-Inh by leucocyte elastase was also reported in [26].

[d]Inactive enzyme/inhibitor complex formed [27].

of human α_1-PI (54k) into the inactive (50k) form. No cleavage of opposum α_1-PI was noted, and no proteinase/inhibitor complex band was detected. Venom proteolytic activity was retained, and chromatography of the incubation mixtures on Mono Q showed no evidence of complex formation. In all instances, opossum α_1-PI resisted digestion under conditions in which human α_1-PI was enzymatically inactivated.

C adamanteus proteinase II also inactivated AT III [12], and the presence of heparin caused several important differences, including an acceleration of the inactivation process (Table II). Incubation of AT III (60k) with proteinase II in the absence of heparin resulted in a slow loss of inhibitory activity against thrombin, and electrophoretic analysis showed bands corresponding to inactive inhibitor (56k) and a peptide

(Fig. 1). Sequence analyses indicated that the inactivating cleavage had occurred at Ala_{375}-Ser and that no cleavage had occurred in the amino-terminal region of AT III in the absence of heparin (Fig. 2).

Incubation of AT III with heparin followed by catalytic amounts of proteinase II resulted initially in the conversion of intact inhibitor (60k) into an intermediate species (56k) which retained inhibitory activity against thrombin (Fig. 1). Further proteolysis resulted in the gradual appearance of a third band (50k) and the corresponding loss of inhibitory activity against thrombin. Characterization of the peptide initially released and of the intermediate species indicated that the initial cleavage occurred at Glu_{37}-Gln. Analysis of the final reaction products indicated that the inactivating cleavage had occurred at Ala_{375}-Ser. Slight cleavage of the Ala_{378}-Val bond was also noted. The reactive-site Arg_{384}-Ser bond, which is cleaved by thrombin during complex formation with AT III [16], was not cleaved by proteinase II either in the presence or in the absence of heparin.

Human $C\bar{1}$-Inh was cleaved only in the amino-terminal region by *C atrox* (Western diamondback rattlesnake) α-proteinase [10], whereas *P aeruginosa* elastase enzymatically inactivated the inhibitor [3]. Advantage was taken of these observations to elucidate the reactive site sequence of $C\bar{1}$-Inh. Incubation of intact $C\bar{1}$-Inh (104k) with α-proteinase resulted in formation of modified $C\bar{1}$-Inh (89k) (Fig. 1). The modified inhibitor was fully active and could be obtained in homogeneous form by gel filtration. Sequence analysis (Fig. 2) indicated that the proteinase had cleaved the native inhibitor at the Pro_{36}-Ile bond in the amino-terminal region of the molecule. Modified $C\bar{1}$-Inh (89k) was then incubated with *P aeruginosa* elastase, resulting in formation of inactive $C\bar{1}$-Inh (83k) (Fig. 1). The amino-terminal sequence of inactivated $C\bar{1}$-Inh (83k) was identical to that of modified $C\bar{1}$-Inh, indicating that no further cleavage had occurred in the amino-terminal region of the inhibitor molecule. The sequence of the released peptide showed that the inactivating cleavage had occurred at an X-Val bond (Fig. 2). The X residue is now known to be Ser (A.E. Davis, personal communication). The peptide overlapped the reactive site peptide released during complex formation with $C\bar{1}$-esterase and established Arg-Thr as the reactive site P_1 and P'_1 residues of human $C\bar{1}$-Inh [7].

A similar pattern of inactivation occurred when $C\bar{1}$-InH was incubated with *P aeruginosa* proteinase (Fig. 3). The rather high amount of proteinase (inhibitor/enzyme molar ratio, 22:1) led to an almost immediate generation of the 89k intermediate (Fig. 3C,D) due to rapid cleavage in the amino-terminal region of the molecule. However, no activity losses were detected until the 83k product began to appear, indicating that the intermediate was fully active and that inactivation was correlated with a subsequent cleavage near the reactive site.

Incubation of *P aeruginosa* proteinase or elastase with α_1-AC (Fig. 1) resulted in limited proteolysis of the native inhibitor (64k) into inactive α_1-AC (60k) and a peptide [3]. Similar results were obtained when α_1-AC was incubated with pronase, subtilopeptidase A, subtilisin BPN′, thermolysin [4], α-proteinase [10], proteinase II, or human leucocyte elastase (Table II).

Incubation of α_2-AP with catalytic amounts of *C basiliscus* venom resulted in cleavage of the intact inhibitor (68k) to form a 61k intermediate (Fig. 1). A second cleavage produced a 53k product. Loss of activity correlated with the appearance of the 53k form, and it was concluded that the 61k intermediate was active [9]. Similar results were obtained with *P aeruginosa* protease and elastase [3] (Table II). However,

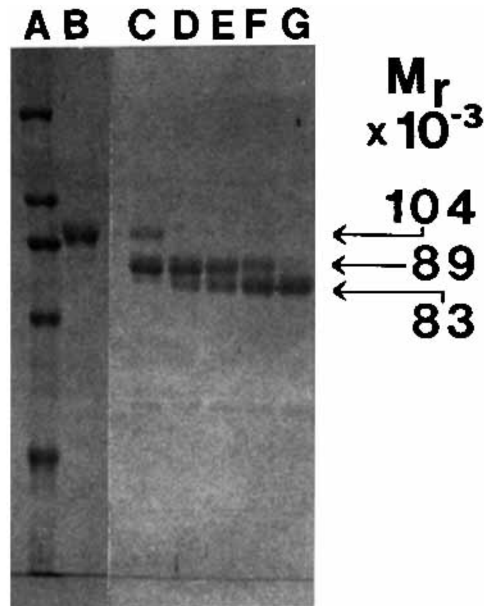

Fig. 3. Enzymatic inactivation of human C$\bar{1}$-Inh by *P aeruginosa* proteinase. C$\bar{1}$-Inh (250 μg) was incubated at room temperature with *P aeruginosa* proteinase (5.4 μg) in a total volume of 260 μl of 0.05 M Tris-HCl/0.2 M NaCl (pH 7.8) to give an inhibitor/enzyme molar ratio of 22:1. Aliquots (18 μl) of the mixture were withdrawn at various times. A portion (15 μl) was added to 15 μl of 0.125 M Tris-HCl/0.1 M EDTA (pH 6.8) containing 4% SDS/10% β-mercaptoethanol, and heated at 100°C for 5 min. Electrophoresis was performed in a 7.5% separating gel at pH 8.8 with a 4.5% stacking gel at pH 6.8. Protein bands were stained with 0.125% Coomassie brilliant blue R-250 and diffusion-destained. The remainder of each aliquot (3 μl) was added to buffer containing EDTA and assayed for residual inhibitory activity against plasmin using S-2251 substrate. Gel channels contained: A, molecular weight standards (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin); B, C$\bar{1}$-Inh; C-G, inhibitor plus proteinase after 1-, 15-, 30-, 60-, and 120-min reactions. At these time points the residual C$\bar{1}$-Inh activity was 100, 85, 70, 40, and 15%, respectively. Details of similar analyses, including assay details, can be found in the references given in Figure 1.

with *C adamanteus* proteinase II, only the active intermediate was generated and no α_2-AP inactivation occurred (Table II). No structural analyses have been performed as yet on either the α_1-AC or the α_2-AP digest products. However, by analogy to the results with AT III and C$\bar{1}$-Inh, it seems likely that the initial noninactivating cleavage in α_2-AP occurred in the amino-terminal region, followed by an inactivating cleavage near the reactive sites of both α_2-AP and α_1-AC in the carboxy-terminal regions of the inhibitor molecules.

DISCUSSION

The following generalizations can be made with respect to snake venom and bacterial proteinase inactivation of the serpins. The inhibitors were all inactivated with catalytic amounts of the proteinases, and no stable proteinase-proteinase inhibitor complexes were detected. Bond cleavage appeared to be quite specific, with only one or two high molecular weight inhibitor derivatives being formed. The carboxy-terminal peptide cleavage products in the case of α_1-PI and C$\bar{1}$-Inh remained tightly

bound to the core molecule and could be separated only under denaturing conditions. With AT III the inactivating cleavage occurred within a disulfide loop, and the products were reduced and carboxymethylated prior to attempted separation. However, total separation of inactivated AT III (50k) from the carboxy-terminal peptide occurred only when $NaDodSO_4$ was present in the column buffers [12]. Serpin inactivation by crude snake venoms was in all instances terminated by addition of EDTA and the reaction was unaffected by phenymethylsulfonyl fluoride, indicating that venom metalloproteinases are responsible and that venom serine proteinases do not contribute to the inactivation. However, serpin inactivation by proteolysis within the reactive site loop has been noted with serine proteinases from bacteria [4], by human leucocyte elastase [5] (Table II), and by the cysteinyl proteinase, papain [13].

The inactivating cleavages in α_1-PI, AT III, AT III/heparin, and C$\bar{1}$-Inh all occurred in the exposed reactive site loop at a bond near, but not at, the reactive site bond itself (Fig. 2). Based upon the sequence homology exhibited by the serpin family [17] it seems reasonable to infer that the inactivating cleavages in α_1-AC and α_2-AP (Fig. 1) also occurred near the reactive site bond. In addition, the inactivated serpins were not further digested during the 24-hr incubation with the inactivating proteinases. Nor was random proteolysis of the inactivated serpins noted in the presence of crude snake venoms which contain a mixture of serine and metalloproteinases. These results are consistent with the recent proposal [5] that an exposed loop preceding the reactive site bond serves as a "switch" to allow specific proteolytic cleavage which converts the serpin molecule from a stressed (active) to a relaxed (inactive) conformation.

α_1-AC is the most readily inactivated of the serpins which have been examined in this laboratory. In general, this was true for inactivation by pure proteinases [3, 4, Table II] or by crude venoms [9]. α_1-AC was the only serpin inactivated by Elapid venom metalloproteinases [18], as indicated by the activity of *Dendroaspis angusticeps* proteinase (Table II). α_1-AC is an early stage acute-phase reactant [19], and the rapid mobilization of this serpin is a primary defense against chymotrypticlike proteinases released during inflammation, and a role in protecting membranes against proteolytic damage has been postulated [20,21]. The rapid inactivation of α_1-AC by *P aeruginosa* elastase and protease [3] and by leucocyte elastase (Table II) may be a factor in disrupting this protective mechanism in disease states such as cystic fibrosis in which leucocytosis and pseudomonal infections are frequently found.

Inactivation of AT III/heparin, C$\bar{1}$-Inh, and α_2-AP involved the formation of active intermediates with the initial cleavage occurring in the amino-terminal region of the inhibitor (Figs. 1–3). Cleavage in the amino-terminal region also occurred more rapidly than reactive site region cleavage, as indicated by depletion or disappearance of the native inhibitor species and accumulation of the active intermediate prior to the gradual accumulation of inactivated inhibitor [3,12]. In several reaction systems (α_2-AP plus proteinase II; C$\bar{1}$-Inh plus proteinase II or α-proteinase or Elapid venom metalloproteinases [9]; C$\bar{1}$-Inh plus papain or ficin or bromelain [22]) only the amino-terminal region was cleaved and no loss of inhibitory activity was noted (Table II).

No intermediates were detected during the inactivation of α_1-PI or α_1-AC (Fig. 1), but the electrophoretic system used would not have detected release of small polypeptides [2,3]. Active forms of both these inhibitors lacking an amino-terminal peptide of nine residues (α_1-PI) or 15 residues (α_1-AC) have been reported [23,24].

Therefore, all the serpins listed in Figure 1 contain a bond(s) in their amino-terminal regions that is susceptible to limited proteolytic attack without loss of inhibitory activity. The possible biological significance of this structural characteristic of the serpins has not yet been established. Studies of isolated serpin intermediates (eg, α-proteinase plus C$\bar{1}$-Inh) or site-specific mutagenesis of the proteinase-sensitive amino-terminal region offer two means for delineating the in vivo role of this initial step in the serpin inactivation process.

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