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ENZYMATIC INACTIVATION OF HUMAN ANTITHROMBIN III

LIMITED PROTEOLYSIS OF THE INHIBITOR BY SNAKE VENOM PROTEINASES IN THE PRESENCE OF HEPARIN

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Summary

Incubation of human plasma antithrombin III with Crotalid, Viperid and Colubrid snake venoms resulted in the enzymatic inactivation of the inhibitor, as evidenced by a gradual loss of inhibitory activity against trypsin and thrombin. This indicates that proteinases which selectively inactivate antithrombin III are widespread among the families of poisonous snakes. The inactivation was due to metalloproteinases present in the venoms, since the reaction could be terminated by the addition of EDTA. Elapid venoms were tested and shown to be devoid of activity on antithrombin III.

Preincubation of the anthrombin III with heparin accelerated the reaction, and less venom was required to achieve total inactivation. Several venoms had very little effect on antithrombin III in the absence of heparin, but inactivated the inhibitor completely within 2 h when heparin was present. Optimal rates of inactivation were observed with antithrombin III: heparin ratios of approx. 3 : 1. When heparin was present in excess, the inactivation was retarded.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the antithrombin III/venom proteinase reaction mixtures indicated that intact antithrombin III (63 000 daltons) was converted to an inactive form (57 500 daltons) by limited proteolysis. No complex formation between antithrombin III and venom proteinases was detectable. The inactivating cleavage occurred within a disulfide loop of the antithrombin III molecule, since the lower molecular weight species was detected only under reducing conditions.

Abbreviations: BAPA, benzoyl-DL-arginine *p*-nitroanilide · HCl.

Introduction

Most naturally occurring proteinase inhibitors have been studied in terms of their ability to form stoichiometric enzyme-inhibitor complexes with proteinases [1]. However, several reports [2—7] indicate that inhibitors such as α_1 proteinase inhibitor can also be enzymatically digested by various proteinases. Snake venom has been shown [8] to contain metalloproteinases which enzymatically inactivate α_1 proteinase inhibitor and also all detectable trypsin and chymotrypsin inhibitory activity of human serum.

This observation of a novel function of venom proteinases may be of significance in assessing the biochemical events associated with snakebite pathogenesis, in view of the wide range of physiological processes involving the plasma proteinase inhibitors [9].

The initial report [8] indicated that all serum inhibitory activity against trypsin and chymotrypsin was lost during incubation with venoms. Since the molarity of α_1 proteinase inhibitor in serum exceeds that of other inhibitors by at least 10-fold [9], the majority of the loss of serum inhibitory activity could be attributed to the enzymatic inactivation of α_1 proteinase inhibitor. However, the possibility could not be excluded that inactivation of the less concentrated serum inhibitors, such as antithrombin III, might be due to enzyme-inhibitor complex formation rather than enzymatic digestion.

Antithrombin III is a plasma proteinase inhibitor which forms complexes with virtually all the serine proteinases involved in the coagulation-fibrinolysis system, and is considered critical for the regulation of the hemostatic mechanism [10]. Enzyme-antithrombin III complex formation has been shown to be greatly accelerated in the presence of heparin [11]. This effect of heparin in the system antithrombin III/heparin/thrombin has been considered to involve a conformation change in antithrombin III [12,13], but interaction of heparin with thrombin has also been reported [14,15].

The present investigation was undertaken to determine the effect of venom metalloproteinases on antithrombin III. This is the initial report of the enzymatic inactivation of antithrombin III without detectable complex formation. Data are presented which indicate that the inactivation occurs by limited proteolysis of the antithrombin III molecule, and that the reaction is accelerated by prior exposure of the antithrombin III to heparin.

Materials and Methods

Snake venoms and heparin (Lot No. 736-0160; 158 U.S.P. units per mg) were obtained from Sigma; human thrombin (Fibrindex) from Ortho; bovine trypsin from Worthington; α-*N*-benzoyl-L-arginine ethyl ester · HCl from Sigma; BAPA from Mann; and D-Phe-Pip-Arg-*p*-nitroanilide · 2 HCl (S-2238) from KABI (U.S.A.); α_1 proteinase inhibitor was prepared in this laboratory [16]. The antithrombin III was provided by the American Red Cross National Fractionation Center with the partial support of NIH Grant HL 13881.

Antithrombin III activity was measured using trypsin and BAPA [17] or thrombin and S-2238 [18]. Inhibitor inactivation was monitored as previously described [8,19]. Reaction products of antithrombin III/venom incubation mixtures were analyzed by SDS-polyacrylamide gel electrophoresis [20].

Results

The effect of two representative venoms on antithrombin III is shown in Fig. 1. No loss of inhibitor activity was observed after a 2 h incubation with *Crotalus adamanteus* venom in the absence of heparin. However, with prior exposure of antithrombin III to heparin, digestion of the inhibitor by the same venom occurred rapidly and complete loss of activity was noted at 2 h. On the other hand, *Bitis arietans* venom was capable of slowly digesting antithrombin III in the absence of heparin, and a 50% loss of activity was observed at 2 h. However, with heparin present the loss of antithrombin III activity occurred rapidly and was complete at 2 h. In a separate experiment antithrombin III/heparin was incubated with *B. arietans* venom and the reaction allowed to proceed for 30 min, at which time EDTA was added to a final concentration of 0.01 M. It can be seen (Fig. 1) that EDTA addition effectively terminated the inactivation of antithrombin III by *B. arietans* venom. Similar experiments involving addition of EDTA were performed for all venoms tested, and in all instances in which an initial loss of antithrombin III was occurring, the digestion was terminated by EDTA. This was true for incubations containing heparin and those without heparin. It was concluded that in all cases, the inactivation of antithrombin III was due to metalloproteinases present in the venoms.

The results for all the venoms tested on antithrombin III are given in Table I. In general, the venoms which react with antithrombin III digest the inhibitor comparatively slowly in the absence of heparin and varying amounts of initial antithrombin III activity still remained after 2 h incubation. In contrast, a rapid digestion leading to total inactivation of the inhibitor occurred within 2 h when antithrombin III was reacted with heparin prior to the addition of venom. The effect of heparin was most pronounced with several Crotalid venoms (*C. adamanteus*, *Bothrops atrox* and *Trimeresurus flavoviridis*) which produced essen-

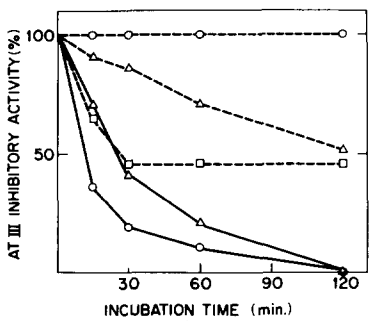


Fig. 1. The effect of heparin and EDTA on the enzymatic inactivation of antithrombin III (AT III). Antithrombin III (150 μg per 100 μl of 0.05 M Tris-HCl/0.002 M $CaCl_2$, pH 8.0) was incubated alone or in the presence of 10 μg of heparin (Sigma, 158 U.S.P. units per mg) for 3 min at 25°C in a total volume of 195 μl. Venom (5 μl) diluted in buffer was added. At the designated times aliquots were withdrawn, diluted with an equal amount of 0.05 M Tris-HCl/0.02 M EDTA, pH 8.0, and assayed for residual antithrombin III activity against trypsin using BAPA as substrate [17]. The amounts of venom in the incubation mixtures are listed in Table I. ○- - - - -○, antithrombin III plus *C. adamanteus*; △- - - - -△, antithrombin III plus *B. arietans*; ○———○, antithrombin III/heparin plus *C. adamanteus*; △———△, antithrombin III/heparin plus *B. arietans*; □- - - - -□, antithrombin III/heparin plus *B. arietans* to which EDTA was added after 30 min incubation.

TABLE I

ENZYMATIC INACTIVATION OF ANTITHROMBIN III BY SNAKE VENOM METALLOPROTEINASES

For experimental details see legend to Fig. 1. Residual antithrombin III inhibitory activities are all normalized to the activity of a control sample containing trypsin and antithrombin III only

Venom	Amount (A_{280} units)	antithrombin III (% activity remaining)			antithrombin III + heparin (% activity remaining)		
		15 (min)	60	120	15 (min)	60	120
Crotalid							
Crotalus adamanteus	0.095	100	100	100	37	12	0
Crotalus atrox	0.083	100	91	87	53	12	0
Crotalus basiliscus	0.032	94	63	43	65	26	0
Bothrops atrox	0.065	100	100	100	53	20	0
Trimeresurus flavoviridis	0.165	97	92	92	69	31	0
Agkistrodon piscivorus	0.084	81	67	37	69	23	0
Lachesis muta	0.185	91	74	57	54	16	0
Viperid							
Bitis arietans	0.036	91	71	51	71	23	0
Echis carinatus	0.196	90	65	48	30	15	0
Causus rhombeatus	0.005	98	71	36	71	24	0
Vipera ammodytes	0.088	93	78	74	72	48	0
Colubrid							
Dispholidus typus	0.060	91	67	52	40	7	0
Elapid							
Naja naja kaouthia	1.105	100	100	100	100	100	100
Bugarus fasciatus	0.980	100	100	100	100	100	100

tially no loss of antithrombin III activity in the absence of heparin, but completely digested the inhibitor when heparin was present.

The amounts of venom required to achieve total inactivation of antithrombin III within 2 h varied (Table I). However, the activity on antithrombin III did not correlate with the levels of caseinolytic activity [22,23], indicating that some of the venoms tested possess high levels of proteinases specific for antithrombin III inactivation. *Causus rhombeatus* was the most active venom tested, with only 0.005 A_{280} units * of venom required to achieve total inactivation of antithrombin III with heparin present (Table I).

Neither of the Elapid venoms tested showed any activity on antithrombin III even when comparatively large amounts of venom were employed (Table I). This corresponds to the the results with another inhibitor, α_1 proteinase inhibitor, which was also not inactivated by Elapid venoms [8].

It should be noted that the data in Fig. 1 and Table I were obtained by measuring residual antithrombin III activity towards trypsin using BAPA as substrate. However, several venoms were also tested using thrombin and S-2238 as substrate. The latter assay required 15 min incubation of antithrombin III with thrombin to achieve maximal complex formation in the absence of heparin, whereas a 1 min incubation sufficed for the trypsin/BAPA assay. In

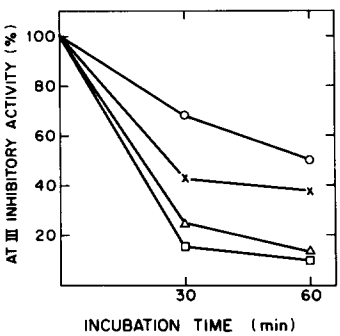
* 1 A_{280} unit is that amount of protein which if dissolved in 1 ml and read in a 1 cm light path at 280 nm will give an absorbance of one.

addition, many of the venoms tested showed considerable activity on S-2238, but had very low activity on BAPA [24]. The rate of inactivation of antithrombin III was similar regardless of which enzyme the inhibitor was tested against, and the accelerated inactivation due to heparin was noted in both systems.

The data in Table I indicate that proteinases capable of selectively inactivating antithrombin III are widespread among the families of poisonous snakes. While most of the venoms tested showed some inactivation of the inhibitor, the reaction was in all cases accelerated by heparin.

The effect of heparin concentration on the reaction was studied, and the results are shown in Fig. 2. Inhibitor inactivation was slightly more rapid at an antithrombin III/heparin ratio of 3 : 1 than at a 1 : 1 ratio. Therefore, in order to avoid possible interference due to excess heparin, the 3 : 1 ratio was chosen for all experiments shown in Fig. 1 and Table I. Increasing the heparin concentration so that it exceeded the antithrombin III concentration resulted in a slowing of the inactivation (Fig. 2). At an antithrombin III/heparin ratio lower than 0.4 : 1, the excess heparin interfered significantly with the trypsin or thrombin assays and interpretation of results became ambiguous. The effect of heparin is shown for the inactivation of antithrombin III by *Echis carinatus* venom. A similar effect was noted with other venoms in which heparin concentration was varied. The acceleration of antithrombin III inactivation by venom proteinases in the presence of heparin appears due primarily to an effect of heparin on the inhibitor, rather than on the proteinases. As stated above, excess heparin actually retards the inactivation reaction. In separate experiments (not shown) it was observed that the presence of heparin also decreased the rate of inactivation of α_1 proteinase inhibitor by *C. adamanteus* proteinases. It was concluded that heparin is inducing a conformational change in the antithrombin III molecule which renders the inhibitor more susceptible to inactivation by venom proteinases.

Previously, it was shown that a purified proteinase from *C. adamanteus* venom inactivated α_1 proteinase inhibitor by limited proteolysis at a bond near the reactive site of the inhibitor [25]. In order to determine whether the antithrombin III inactivation by crude venom was due to random digestion or limited proteolysis, the incubation products were analyzed electrophoretically. The results are shown in Fig. 3. After 5 min incubation with venom only a single band which migrates to the position of intact antithrombin is detectable. Aliquots taken immediately after venom addition and after 1 min and 3 min of incubation also showed only this single band. No higher molecular weight bands were visible, indicating that the reaction proceeded without complex formation. After 2 h incubation the inactivation of antithrombin III is complete (Table I) and gel electrophoresis reveals the presence of a single band of approx. 57 500. This lower molecular weight species of antithrombin III was visible only in aliquots which had been reduced with mercaptoethanol. The peptide(s) released was not detectable in the gel system used. For reaction aliquots run under nonreducing conditions, a single band migrating in the position of intact antithrombin III was detected (Fig. 3E). The gels for *Crotalus basiliscus* venom are shown in Fig. 3. Aliquots from incubation mixtures of antithrombin III and other venoms (*Crotalus atrox, Dispholidus typus*) were also analyzed electrophoretically. The pattern in all instances was similar, and



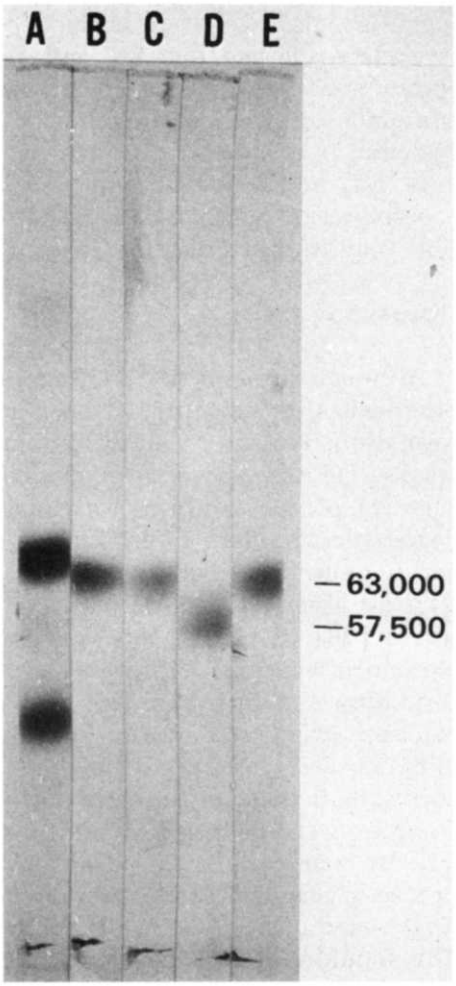


Fig. 2. Effect of varying heparin concentration on the rate of inactivation of antithrombin III (AT III). Antithrombin III (130 μg in 100 μl of 0.05 M Tris-HCl/0.002 M $CaCl_2$, pH 8.0) was incubated with increasing amounts of heparin (9—62 μg in 25 μl buffer) for 3 min at 25°C in a total volume of 175 μl. *E. carinatus* venom (0.2 A_{280} units in 25 μl buffer) was added. Residual antithrombin III activity was measured as detailed in the legend to Fig. 1. The molar ratios of antithrombin III/heparin as as follows: ○———○, no heparin; X———X, 0.4 : 1; △———△, 1 : 1; □———□, 2.8 : 1. The ratios were calculated on the basis of molecular weights of 58 000 for antithrombin III [21] and 11 000 for heparin [15].

Fig. 3. Limited proteolysis of antithrombin III. Aliquots of the incubation mixture of antithrombin III/heparin and *C. basiliscus* venom were withdrawn after 5 min and 120 min, added to SDS/mercaptoethanol (final concentration 1%) and heated for 3 min at 100°C. A separate aliquot was taken at 120 min and heated with SDS only. Electrophoresis was performed in 5% gels for 17 h at 3.5 mA per gel [20]. The position of the bromophenol blue tracking dye was marked with India ink, the gels stained for 4 h in 0.125% Coomassie brilliant blue R250, and destained by diffusion in water/methanol/acetic acid (5 : 4 : 1). The gels shown are as follows: A, bovine albumin and ovalbumin standards; B, antithrombin III control; C and D, incubation mixture at 5 min and 120 min respectively; E, incubation mixture at 120 min without mercaptoethanol. The molecular weight values were calculated from separate experiments (not shown) using soybean trypsin inhibitor (Kunitz) and lysozyme in addition to the standards shown above.

suggested cleavage of a single peptide bond. However, the possibility of a secondary cleavage at a bond near the primary cleavage could not be eliminated. The latter mechanism would result in loss of a small peptide containing only a few amino acids, and would not be detected in the present system.

It was concluded that incubation with snake venom results in limited proteolysis of antithrombin III into an inactive form by cleavage of a susceptible bond within a disulfide loop of the inhibitor molecule.

Discussion

Although proteolytic modification of antithrombin III has previously been observed, this has usually been associated with enzyme-inhibitor complex formation. Passage of antithrombin III through Sepharose-thrombin columns resulted in the appearance of small amounts of an inactive derivative, and cleavage of the inhibitor was increased by the presence of heparin [12]. A degradation product of antithrombin III having a lowered affinity for heparin and a molecular weight of 60 000 (compared to 63 000 for intact antithrombin III) was observed during isolation of antithrombin III complexes from human serum [26]. Recently, it was reported that exposure of antithrombin III to equimolar amounts of thrombin resulted in both enzyme-inhibitor complex formation and in proteolysis of a portion of the antithrombin III [27] in a reaction which appears to resemble the interaction between α_1 proteinase inhibitor and elastase [5]. In the examples cited, major electrophoretic bands corresponding to antithrombin III-thrombin complex or to partially degraded complex were detected in addition to enzymatically-inactivated antithrombin III. By contrast, inactivation of antithrombin III by venom proteinases proceeds enzymatically with no detectable complex formation and only a single band corresponding to inactivated antithrombin III is observed (Fig. 3E). This should simplify analysis of the inactivated antithrombin III species.

The characteristics of the antithrombin III enzymatically modified by thrombin [27] are similar to those reported in the present investigation for antithrombin III digested by venom metalloproteinases. In both instances a proteolytic cleavage occurred within a disulfide loop of antithrombin III and the inhibitor was inactive against thrombin. It was postulated [27] that the inactivation involved cleavage of one or at most a few adjacent bonds 30—60 residues from the end of the antithrombin III molecule. A similar conclusion can be drawn from the data in Fig. 3, although the molecular weight of the inactivated antithrombin III in the present study would indicate a cleavage approx. 50—55 residues from the terminus (Fig. 3D). Since disulfide loops encompass regions of this size at both ends of the antithrombin III molecule [28], it cannot be determined from the present data whether cleavage occurred in the amino or carboxy terminal region. Previous reports on the inactivation of α_1 proteinase inhibitor [25,29] and the postulated structural homology between α_1 PI and antithrombin III [28] would favor cleavage in the amino terminal region. Further characterization and comparison of these enzymatically-inactivated inhibitor species will be of interest to determine whether the bond cleaved by venom proteinases in the absence of complex formation is

identical to, or in the region of, that cleaved by thrombin in conjunction with complex formation. Previously, it was found that inactivation of α_1 proteinase inhibitor by a purified venom proteinase [19] involved cleavage of a single bond [25] which was eight residues removed from the reactive site bond in α_1 proteinase inhibitor cleaved by other enzymes [29]. The venom proteinases selective for antithrombin III inactivation may, therefore, prove useful in delineating the reactive site region of this inhibitor and also in determining the structural requirements for antithrombin III-enzyme complex formation.

The observation that venoms possess proteinases which selectively catalyze the inactivation of antithrombin III may have significance in terms of the in vivo functioning of other venom proteinases. For example, thrombocytin, a platelet-activating enzyme from *B. atrox* venom, is inactivated by complex formation with antithrombin III [30]. In addition, several venom enzymes generate activated coagulation factors [31] which can in turn be inhibited at antithrombin III [10,11]. The presence in venom of metalloproteinases which enzymatically inactivate antithrombin III would, therefore, complement the in vivo activity of other venom enzymes, thereby amplifying the disruption of the hemostatic balance noted with Crotalid and Viperid snakebite.

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