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ENZYMATIC INACTIVATION OF HUMAN SERUM PROTEINASE INHIBITORS BY SNAKE VENOM PROTEINASES*

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SUMMARY: Snake venoms of the Crotalid, Viperid, and Colubrid families possess proteinases which catalytically inactivate purified human α_1-proteinase inhibitor. Incubation with these venoms also results in the gradual loss of all detectable trypsin and chymotrypsin inhibitory activity present in human serum. The venom proteinases involved are metal dependent and are unaffected by phenyl methyl sulfonyl fluoride. The Elapid and Hydrophid snake venoms tested were devoid of this activity.

Recent work in this laboratory (1) has resulted in the purification from *Crotalus adamanteus* venom of two proteinases which catalytically inactivate human α_1-proteinase inhibitor (α_1PI). The proteinases cleave α_1PI (54,000 daltons) by limited proteolysis of a single region of the inhibitor molecule, resulting in the appearance of an inactive α_1PI fragment (50,000 daltons) and a peptide (4,000 daltons). Disappearance of native α_1PI results in the corresponding loss of inhibitory activity against trypsin and chymotrypsin. Other results (Kress, unpublished) indicated that *Crotalus adamanteus* venom catalytically inactivated all human serum trypsin and chymotrypsin inhibitors detectable by the assay systems employed (see Methods).

Although snake venom proteinases are known to act upon various plasma proteins (2), the enzymatic inactivation of human serum proteinase inhibitors (PI) represents a previously unde-

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scribed action of snake venom. Since the serum PI are believed to regulate various physiological processes (3), knowledge of their inactivation by snake venom proteinases could be of importance in assessing venom toxicology. The present study was undertaken to determine the distribution, general properties, and activity of the serum PI inactivating proteinases among the various families of poisonous snakes. Evidence is presented that inhibitor inactivation is a property not only of Crotalid, but also of Viperid and Colubrid venoms, and is therefore widely distributed. Also, the inhibitor inactivation is not restricted to α_1PI but extends to other chymotrypsin and trypsin inhibitors present in human serum.

MATERIALS AND METHODS

Bovine trypsin and α-chymotrypsin were obtained from Worthington. Phenylmethylsulfonyl fluoride (PMSF) was from Sigma. Human α_1PI was prepared as previously described (4). Fresh human serum was obtained through the courtesy of Dr. Elias Cohen, Plasmapheresis Department, Roswell Park Memorial Institute. Snake venoms were obtained from Sigma, except for *Crotalus adamanteus* venom which was from the Miami Serpentarium. Chymotrypsin was assayed using benzoyl-L-tyrosine ethyl ester (5); trypsin was assayed using benzoyl-L-arginine ethyl ester (6). Inhibitor activity was determined according to the modification of Kassell et al. (7).

Stock solutions of α_1PI were prepared by dissolving the inhibitor in 0.05 M Tris-HCl-0.002 M $CaCl_2$, pH 8.0, and adjusting to a final concentration of 1.0 mg/ml. Venom was dissolved in the same buffer, centrifuged, and adjusted to a level of 12 A_{280} units/ml. (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 absorbancy of one). The inactivation of α_1PI was followed by incubation at 23° of 100 µl of α_1PI with 15 µl of venom plus buffer to a final volume of 200 µl. Samples (30 µl) were withdrawn at given intervals and added to an equal amount of 0.05 M Tris-HCl-0.01 M EDTA, pH 8.0, to end the reaction. The samples were kept at 4°, and suitable aliquots were assayed for residual inhibitory activity against chymotrypsin. Control experiments showed that with the exception of *V. russelli* and *D. angusticeps* none of the venoms tested interfered with the assay system itself or with formation of the complex between α_1PI and chymotrypsin. Specific activity was defined as µg α_1PI inactivated/A_{280} unit of venom/min.

A similar protocol was followed for monitoring the inactivation of serum chymotrypsin inhibitors. Serum (100 µl; 55.0 A_{280}

TABLE I. Enzymatic Inactivation of α_1PI by Various Venoms

	α_1PI Inactivation (μgm α_1PI inactivated/A_{280} unit of venom/min)	Proteolytic Activity[1] (Casein substrate)
Crotalid		
Agkistrodon piscivorus leukostoma	64.7	
Crotalus basiliscus	59.6	77.0
Crotalus adamanteus	49.6	13.8
Crotalus atrox	43.5	59.0
Bothrops atrox	24.9	17.8
Trimeresurus flavoviridis	21.2	22.5
Lachesis muta	15.9	
Viperid		
Bitis arietans	201.4	10.0
Causus rhombeatus	13.3	3.1
Echis carinatus	7.0	23.5
Colubrid		
Dispholidus typus	22.1	
Elapid		
Opiophagus hannah	0	6.4
Naja naja kaouthia	0	
Bungarus fasciatus	0	0
Hydrophid		
Laticauda semifasciata	0	

[1] Data taken from Ref. 9.

units/ml) was incubated with 50 μl of venom. The reaction was terminated and loss of chymotrypsin inhibitory activity followed as described above. The venoms tested did not possess detectable activity against benzoyl-L-tyrosine ethyl ester (BTEE) and crude venom could therefore be studied directly for its effects on inhibitory activity in systems involving this artificial substrate. However, since the crude venoms possessed activity against benzoyl-L-arginine ethyl ester (BAEE), they were pretreated with 5×10^{-4} M phenyl-methylsulfonyl fluoride (8) and dialyzed against 0.05 M Tris-HCl-0.002 M $CaCl_2$, pH 8.0. This effectively removed all serine proteinase activity and allowed loss of serum trypsin inhibition to be monitored following incubation with venom, as described above.

RESULTS

The effect of various venoms on human α_1PI is shown in Table I. Levels of proteolytic activity for several of the venoms studied are also given for comparative purposes, since

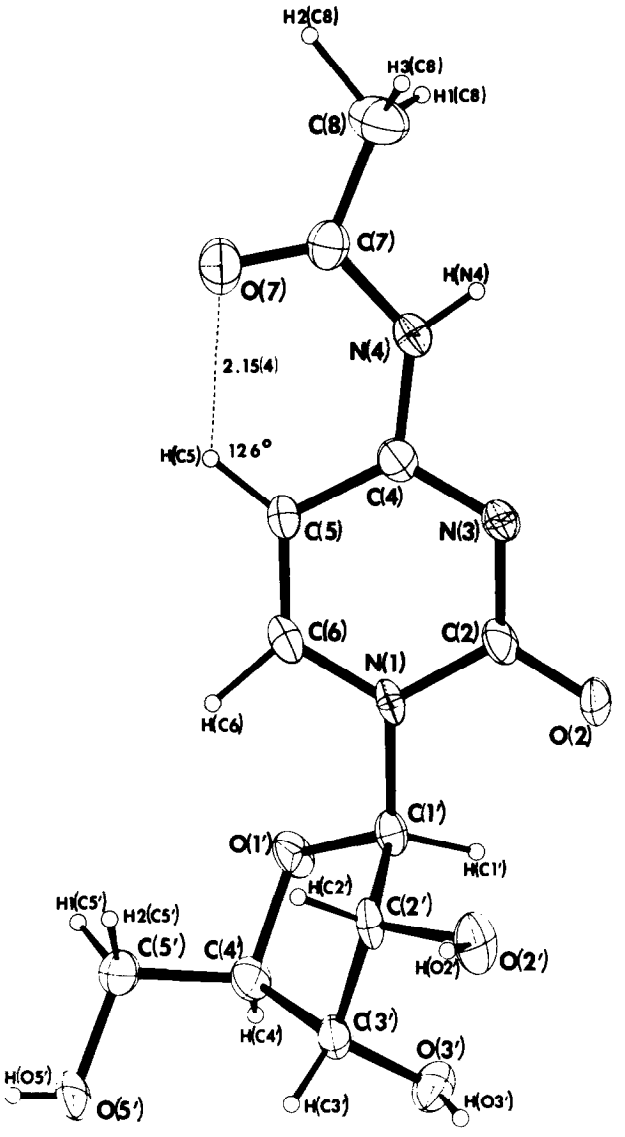

Figure 1. Conformation of N^4-acetylcytidine. Note that the substituent on N(4) is oriented proximal to C(5). The conformation across C(4')-C(5') is gauche$^-$.

and individual isotropic thermal parameters were included in the refinement.

Results and Discussion. Figure 1 illustrates the conformation of the molecule. The 'proximal' conformation of the molecule with

only representative venom reactions from each snake family are plotted. However, all active venoms listed in Table I digested α_1PI in a manner similar to those shown in Fig. 1. The α_1PI inactivation could be effectively terminated by the addition of 0.01 M EDTA, as shown in Fig. 1 for *Crotalus adamanteus* venom. This effect of EDTA was obtained with all active venoms. Several of the active venoms contained esterases active on BAEE. Treatment of these venoms with PMSF, followed by dialysis to remove excess reagent, did not affect the inactivation reactions. Proteolytic inactivation of α_1PI was demonstrated by the gradual loss of α_1PI inhibitory activity against trypsin. The PMSF-treated venoms contained no residual esterase activity in the BAEE/trypsin assay system.

The effects of the venom proteinases on human serum chymotrypsin and trypsin inhibitors are shown in Table II. The pattern of inactivation is similar to that observed for the inactivation of α_1PI (Fig. 1). Incubation of serum with Crotalid, Viperid, and Colubrid venoms results in the gradual loss of chymotrypsin and trypsin inhibitory activity. The Elapid and Hydrophid venoms tested were without effect on serum proteinase inhibitors. Testing of *O. hannah* venom for serum chymotrypsin inhibitor inactivation was prevented by the high level of chymotrypsin inhibitor(s) in this venom. However, no inactivation of α_1PI or of serum trypsin inhibitors was noted with this Elapid venom. Addition of EDTA effectively stopped the inactivation of serum PI, and pretreatment of active venoms with PMSF had no effect on the venom proteinases inactivating the serum PI. The differences in the rates of serum inhibitor inactivation shown in Table II are partially a reflection of the differing amounts of venom used to achieve total inactivation. Variations in serum

TABLE II. Effect of Various Venoms on Human Serum Proteinase Inhibitors

Venom	% Chymotrypsin Inhibitory Activity Remaining[1]			% Trypsin Inhibitory Activity Remaining[1]		
	15'	30'	60'	15'	30'	60'
A. piscivorus leukostoma	22	8	0	20	2	0
C. basiliscus	55	6	4	23	7	0
C. adamanteus	95	38	3	47	22	0
C. atrox	34	3	0	45	32	0
B. atrox	50	2	0	29	21	6
T. flavoviridis	36	8	0	68	27	0
L. muta	25	6	0	43	3	1
B. arietans	23	6	0	5	0	-
C. rhombeatus	56	44	0	35	14	0
E. carinatus	28	8	0	11	0	-
D. typus	23	0	-	23	6	0
O. hannah	N.T.[2]			100	-	100
N. naja kaouthia	100	-	100	100	-	100
B. fasciatus	100	-	100	100	98	-
L. semifasciatus	100	-	100	100	98	-

[1] Residual serum chymotrypsin and trypsin inhibitory activities after incubation with venom are all normalized to the activity of a control sample containing serum only.

[2] Not tested due to chymotrypsin inhibitor(s) in venom.

inhibitor capacity and also in venom proteinase activity made standardization of this assay system difficult. Larger amounts of the active venoms were needed to achieve inactivation of serum inhibitors than were employed to inactivate α_1PI. This is due to the fact that venom proteinases form a stoichiometric complex with the α_2 macroglobin (α_2M) proteinase inhibitor present in serum. When amounts of active venoms comparable to those used to inactivate α_1PI are incubated with serum a lag of approximately 15 min occurs before the inactivation of serum chymotrypsin and trypsin inhibitors begins (data not shown). It would appear that the venom proteinase-α_2M complex is transient, and that active proteinase is gradually released from the complex. A similar observation has been made for the interaction between serum α_2M and

the thrombin-like esterase from *Agkistrodon rhodostoma* venom (10). More detailed studies of the interaction between α_2M and purified venom proteases (1) are in progress and will be reported separately.

DISCUSSION

In general, the distribution of venom proteinases inactivating α_1PI and serum PI coincides with the presence or absence of caseinolytic activity (Table I, data of Oshima et al. (9)). The only exception is *O. hannah* which has detectable caseinolytic activity, but shows no inhibitor inactivation. The levels of inhibitor inactivation, however, do not correlate with the level of caseinolytic activity (Table I) within a given family of snakes. This is a reflection of the fact that the proteinases preferentially attack proteinase inhibitors, and show only a limited activity toward other protein substrates such as casein (1). Crotalid venoms also generally possess higher levels of caseinolytic activity than Viperid venom (2,9). However, the highest α_1PI inactivation was noted with a Viperid, *B. arietans*. The enzymes involved are metalloproteinases as shown by their lack of activity in the presence of EDTA (Fig. 1). In addition, they are unaffected by PMSF, a property which distinguishes them from other serine proteinases in venom which act upon specific plasma proteins (2).

With the exception of the serum inhibitor α_2M, the venom proteinases inactivated the proteinase inhibitors by a mechanism involving enzymatic digestion (Fig. 1; Table II). This differs from the usual interaction of proteinases with inhibitors, in which a stoichiometric complex is formed (11). Enzymatic inactivation of α_1PI without complex formation has previously been observed with *Pseudomonas aeruginosa* extracts (12) and with

papain and cathepsin B_1 (13). The present study indicates that proteinases possessing this activity occur in the venoms of several families of poisonous snakes (Table I), and that these venoms are also capable of enzymatically inactivating the chymotrypsin and trypsin inhibitors of serum (Table II). The catalytic mechanism has the obvious biological advantage that small amounts of venom proteinases can effect a widespread inactivation of circulating serum proteinase inhibitors. Envenomation following snakebite would result, therefore, in the alteration or destruction of the victim's major defense mechanism regulating endogenous proteolytic activities. Further knowledge of this enzymatic inactivation of serum proteinase inhibitors by venom proteinases will, therefore, be of significance in assessing the toxicological effects of Crotalid, Viperid, and Colubrid venoms.

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