Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1688–1692, March 1982
Biochemistry

Kallikrein-like activity of crotalase, a snake venom enzyme that clots fibrinogen

(thrombin-like activity/kininogen–kinin/plasminogen/serine esterase/chloromethyl ketone)

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Communicated by George A. Olah, October 19, 1981

ABSTRACT **During the amino acid sequence determination of crotalase (EC 3.4.21.30), the thrombin-like enzyme from the venom of *Crotalus adamanteus* (eastern diamondback rattlesnake), we found that, in addition to the expected structural homology with bovine thrombin (EC 3.4.21.5), there was even greater homology with porcine pancreatic kallikrein (EC 3.4.21.8). In exploring further the similarities between crotalase and kallikrein, several striking observations were made. First, crotalase was rapidly and specifically inhibited by the tripeptide affinity labeling derivative prolylphenylalanylarginine chloromethyl ketone, which is known to be a specific inhibitor of kallikrein. Second, NaDodSO$_4$/acrylamide gel electrophoresis revealed that crotalase cleaves the plasma kallikrein-susceptible bonds in human high molecular weight kininogen, producing an intermediate with procoagulant activity. Crotalase-catalyzed cleavage of high molecular weight kininogen also liberates kinin as evidenced by rat blood pressure bioassay. Finally, crotalase exhibits substrate specificity not only for the thrombin chromogenic substrate S-2238 but also for the kallikrein substrates S-2302 and S-2266. Interestingly, one of the other reactions catalyzed by plasma kallikrein, the activation of plasminogen, was not one of the activities exhibited by crotalase.**

Crotalase (EC 3.4.21.30), the thrombin-like enzyme from the venom of *Crotalus adamanteus* (eastern diamondback rattlesnake) has been purified to homogeneity (1–4). Physicochemical characterization of crotalase indicated that it contains a single polypeptide chain with a molecular weight of 33,000 (1). Although analysis for carbohydrate indicated that the enzyme is a sialoglycoprotein, removal of the sialic acid does not appear to alter its enzymatic activity (2). Crotalase clots fibrinogen and plasma anticoagulated with EDTA or heparin (5). In contrast to thrombin (EC 3.4.21.5), crotalase cleaves only fibrinopeptide A from fibrinogen (6) and it does not activate factor XIII (4). There are also other subtle differences in specificity between these two enzymes (4, 7).

The enzymatic properties of crotalase have been investigated, and it has been found that the enzyme exhibits esterase activity on small, basic amino acid esters (1). Both coagulant and esterase activities are inhibited rapidly and simultaneously by diisopropyl fluorophosphate. These findings are consistent with crotalase being a member of the serine protease family (1, 3, 4). Interestingly, the chloromethyl ketone of *N*-α-tosyl-L-lysine also inhibits both esterase and coagulant activities but only partially and with a very slow reaction rate.

During recent investigations aimed at elucidating the primary structure of crotalase (8, 9), it became evident that there was significant homology between the sequence of crotalase (9) and the known primary structure of thrombin (10). Of more interest, however, was the even greater degree of homology between crotalase (9) and glandular kallikrein (EC 3.4.21.8) (11). Although these findings were unexpected, there are several reports in the literature showing that snake venoms have kinin-releasing (kallikrein-like) activity. Mitchell and Reichert in the late 1800s reported a hypotensive effect in dogs after envenomation by members of the Crotalidae family (12). More recently, Russell has shown that several Crotalidae venoms possess kinin-releasing activity (13).

In view of the reports of kinin-releasing activity in snake venoms and our finding of structural homology between crotalase and kallikrein, we initiated a series of experiments to further characterize the relationship between these enzymes. It is the purpose of this report to detail our studies on the remarkable similarities we have identified between crotalase and kallikrein.

MATERIALS AND METHODS

Crotalase Preparation. Crotalase was prepared from eastern diamondback rattlesnake venom by a previously described procedure (2). Lyophilized venom was obtained from the Miami Serpentarium (Miami, FL).

Synthesis of Peptide Chloromethyl Ketones. Tripeptide chloromethyl ketones with carboxyl-terminal arginine were prepared by coupling NH_2-blocked peptides to H-Arg(NO_2)CH_2Cl (NO_2 being in the ω position), using the mixed anhydride procedure (14). Final products were obtained by removing protective groups with anhydrous HF. The synthesis of Pro-Phe-ArgCH_2Cl was described previously (14), as was the synthesis of Ac-Pro-Phe-ArgCH_2Cl (15). [^{3}H]Acetic anhydride (50 mCi/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) was utilized for the synthesis of [^{3}H]Ac-Pro-Phe-ArgCH_2Cl. The radiolabeled peptide chloromethyl ketone had a specific activity of 1.62×10^{13} cpm/mol as determined by measuring the diluted peptide in Aquasol (New England Nuclear), using a Beckman LS 233 scintillation spectrometer.

Assay of Crotalase Activity. Crotalase activity was measured during inhibition studies with the peptide chloromethyl ketone by thioesterase assay as follows. The assay solutions consisted of 0.10 mM Z-Lys-SBzl and 5 μl of Ellman's reagent (16) (52 mg/ml in dimethylformamide) in 1.90 ml of 0.20 M Tris buffer (pH 8.0) containing 0.20 M NaCl. To start the reaction, 100 μl of the crotalase solution from the inhibition reaction was added. The increase in absorbance was monitored at 412 nm on a Beckman DB spectrophotometer.

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Abbreviations: [^{3}H]Ac-Pro-Phe-ArgCH_2Cl, [^{3}H]acetylprolylphenylalanylarginine chloromethyl ketone; H-Arg(NO_2)CH_2Cl, ω-nitroarginine chloromethyl ketone; Z-Lys-SBzl, thiobenzyl ester of *N*-α-carbobenzoxy-L-lysine; HMWK, high molecular weight kininogen; Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid.

Clotting activity of crotalase was determined by using a buffered solution of human fibrinogen (10 mg/ml) as described (3). Activity is expressed in National Institutes of Health equivalent thrombin clotting units.

Kinetic Analysis of Crotalase Inhibition by Peptide Chloromethyl Ketones. Inactivation of crotalase was studied at 25°C in 1.00 ml of 50 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) buffer (pH 7.0), containing 0.20 M NaCl and the arginine chloromethyl ketone. The inactivation reaction was initiated by the addition of 5 μl of crotalase (8.5 μM), and 100-μl aliquots were removed at 3-min intervals and assayed for residual thioesterase activity by using Z-Lys-SBzl as described above. The apparent pseudo-first-order rate constant, k_{app}, for the inactivation reaction was determined from the slope of the semilogarithmic plot of esterase activity versus time. Values of K_i, the reversible binding constant, and k_2, the first-order rate constant for the alkylation reaction, were determined by the equation described by Kitz and Wilson (17):

$$\frac{1}{k_{app}} = \frac{K_i}{k_2}\frac{1}{I} + \frac{1}{k_2}.$$

At least four values of k_{app} were determined at different concentrations of the affinity label, I, and kinetic constants were determined from double-reciprocal plots of k_{app} versus I after determining the best line by the method of least squares.

Affinity Labeling of Crotalase by [^{3}H]Ac-Pro-Phe-ArgCH$_2$Cl. [^{3}H]Ac-Pro-Phe-ArgCH$_2$Cl (10 μl of a 10 mM solution) was added to 0.023 μmol of crotalase in 0.45 ml of 40 mM Tris·HCl buffer (pH 7.4) containing 0.1 M NaCl. After 95 min at 25°C, no thioesterase activity could be detected. The solution was applied to a 1.3 × 61.5 cm column of Sephadex G-25 to remove excess radiolabeled peptide chloromethyl ketone. The column was eluted with 0.10 M Tris·HCl buffer (pH 7.4), and 1.0-ml fractions were collected. Protein and radioactivity were eluted in fractions 18–21 and radioactivity was also eluted in fractions 30–51. The radioactivities of aliquots of the fractions were measured in Aquasol, using the LS 233 scintillation counter.

Sequence Analysis of Crotalase. Portions of the amino acid sequence have been reported separately (9).

Reaction of Crotalase with High Molecular Weight Kininogen (HMWK). HMWK was prepared as described (18). The preparations used in these experiments had an activity of about 8 units/ml with a specific activity of 15 units/mg. There was no detectable factor XI, factor XII, or Fletcher factor activity. Human plasma kallikrein, prepared as described (19), had a Fletcher factor activity of about 0.04 unit/ml at the time of use. It was purified about 80-fold over normal plasma and had no factor XI, factor XII, or HMWK activity. Crotalase was purified to homogeneity and had a specific activity of 470 National Institutes of Health equivalent thrombin clotting units/mg. HMWK was dialyzed against 0.05 M 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes)/0.10 M NaCl pH 7.35 buffer prior to use, and kallikrein was dialyzed against 0.017 M citrate/0.125 M NaCl. Mixtures of 5 μl of HMWK and either 10 μl of the Tes/NaCl buffer, 10 μl of crotalase (80 units/ml in 0.04 M Tris·HCl/0.10 M NaCl, pH 7.35), or 20 μl of kallikrein were incubated for 3 hr at 37°C and an additional 16 hr at 20°C. Then 7.5 μl (or 12.5 μl to the kallikrein incubation mixture) of 10% (vol/vol) 2-mercaptoethanol in 6% NaDodSO$_4$ was added to each reaction mixture and the contents were heated at 37°C for 1 hr. Each reduced mixture was then subjected to NaDodSO$_4$/acrylamide gel electrophoresis on 10% slab gels (140 × 95 × 0.75 mm) essentially as described by Laemmli (20). Gels were run at 13 mA for 4 hr with tap water cooling, and after the run gels were stained with Coomassie brilliant blue. Proteins of known molecular weight were run as standards. Gels were scanned with a Gilford spectrophotometer and quantitated with an integrating recorder.

Crotalase-Catalyzed Kinin Release from HMWK. HMWK (10 μl of the preparation containing about 8 units/ml in 0.05 M Tes/0.10 M NaCl pH 7.35 buffer) was incubated with either 20 μl of crotalase (80 units/ml in 0.04 M Tris·HCl/0.10 M NaCl, pH 7.35) or 20 μl of Tes/NaCl buffer for 3 hr at 37°C and an additional 18 hr at 20°C. The reaction was stopped by the addition of 15 μl of 10% 2-mercaptoethanol in 6% NaDodSO$_4$ to each tube followed by incubation for 1 hr at 37°C. A buffer control and a crotalase control (in which the Tes/NaCl buffer was substituted for HMWK) were similarly treated. After addition of 2-mercaptoethanol/NaDodSO$_4$, incubation mixtures containing crotalase-treated HMWK, HMWK alone, crotalase control, and buffer control were lyophilized and the residue was dissolved in 100 μl of physiological saline. Bioassays of bradykinin release were carried out essentially as described (15). Samples were injected separately into the right carotid artery of 260-g Fisher female rats anesthetized with urethane (1.6 g per kg). All injections were in a final volume of 100 μl of physiological saline, and samples were washed in with an additional 100 μl of saline. Rats were tested before the experimental protocols by injecting 20 ng of bradykinin (Sigma) two to five times to establish sensitivity. Blood pressure was monitored by a Grass polygraph connected to a Statham pressure transducer that was inserted through a cannula in the left femoral artery.

Hydrolysis of Chromogenic Substrates by Crotalase. A 0.1-ml solution of the chromogenic substrate (S-2238, S-2302, or S-2266, from Kabi Diagnostica, Stockholm) in distilled water was placed in a quartz semimicro cuvette, followed by 0.86 ml of 50 mM Tris·HCl/0.05 M NaCl at pH 7.35 (or pH 8.0 for S-2302). Substrate concentrations varied between 5 and 800 μM. Reaction mixtures were preincubated at 37°C for 5 min in the jacketed sample compartment of a Beckman model 25 spectrophotometer with a recorder attachment. The reaction was started by adding 40 μl of crotalase solution containing 8 National Institutes of Health clotting units/ml and the absorbance at 405 nm was followed for 5 min against a blank containing buffer and chromogenic substrate. The spontaneous hydrolysis rate was subtracted from the rate of enzyme-catalyzed hydrolysis.

Crotalase Action on Plasminogen. Activation of human plasminogen by crotalase was determined by the azocaseinolytic activity of the plasmin generated as described (21). NaDodSO$_4$/acrylamide gel electrophoretic analysis (22) was also carried out on the activation samples (after reduction of the disulfide bridges with 2-mercaptoethanol) to detect the formation of the polypeptide chains of plasmin (21). In control experiments plasminogen was activated by streptokinase.

RESULTS

Inhibition of Crotalase by Peptide Chloromethyl Ketones. Our earlier studies (1) showed that crotalase was inhibited rather poorly by the chloromethyl ketone of tosyl-L-lysine. Recently, however, we found that crotalase is inhibited quite rapidly by Pro-Phe-ArgCH$_2$Cl. Pro-Phe-Arg is the tripeptide sequence at the carboxy terminus of bradykinin that is cleaved by kallikrein, and Pro-Phe-ArgCH$_2$Cl is an effective and specific inhibitor of plasma kallikrein (14). As shown in Fig. 1, the time for 50% inactivation of crotalase by 5 μM Pro-Phe-ArgCH$_2$Cl is 3.8 min. From the reciprocal plot (17) of the apparent pseudo-first-order rate constant (k_{app}) against Pro-Phe-ArgCH$_2$Cl concentration (I), the affinity of crotalase for the chloromethyl ketone inhibitor (K_i) was determined as 11 μM, whereas the rate constant for the irreversible step of the affinity labeling reaction, k_2, was found to be 0.57 min^{-1}.

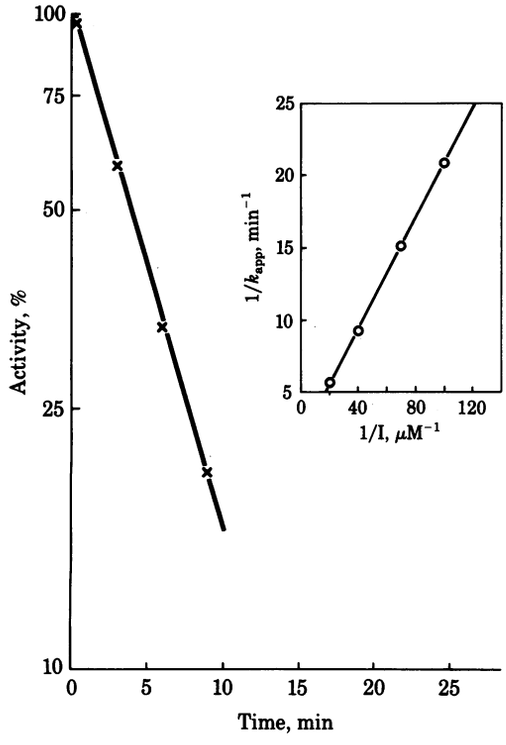

FIG. 1. Inhibition of crotalase by 5.0 μM Pro-Phe-ArgCH_2Cl at 25°C, pH 7.0, in 0.05 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) buffer containing 0.20 M NaCl. The semilogarithmic plot shows percentage of remaining thiobenzyl esterase activity versus reaction time. (*Inset*) Double-reciprocal plot of k_{app} versus inhibitor concentration (I), which was used to determine the reversible binding constant, K_i, and the first-order rate constant, k_2, for the alkylation reaction of Pro-Phe-ArgCH_2Cl with crotalase. Four values of k_{app} were determined at different concentrations of Pro-Phe-ArgCH_2Cl. The slope and intercept were used to calculate K_i and k_2 from the equation of Kitz and Wilson (17) given in the text. K_i = 10.8 μM; k_2 = 0.567 min^{-1}.

When a 4-fold molar excess of [^{3}H]Ac-Pro-Phe-ArgCH_2Cl was used to affinity label crotalase, thioesterase activity was completely lost within 90 min. After removal of unreacted affinity labeling reagent by gel filtration on Sephadex G-25, aliquots were analyzed for radioactivity and protein concentration; there was stoichiometric incorporation of the affinity labeling reagent. $NaDodSO_4$/acrylamide gel electrophoresis (22) of the inhibited enzyme revealed exact correspondence of the position of the peak of tritium and of the single Coomassie blue staining band on the gel. During a separate reaction carried out in almost identical fashion (but with a 7-fold molar excess of inhibitor), aliquots were removed for assay of fibrinogen clotting activity and hydrolysis of the chromogenic substrates S-2238 and S-2302 (both at 1 mM). In the untreated sample crotalase clotting and chromogenic substrate activity remained unaltered during the 90-min course of the reaction. However, within 30 min after addition of the affinity labeling reagent to crotalase, both clotting activity and thrombin and kallikrein chromogenic substrate activities were completely inhibited.

Table 1 lists the values of k_2/K_i for inhibition of crotalase, kallikrein, urokinase, thrombin, and plasmin by Pro-Phe-ArgCH_2Cl. The k_2/K_i values have the dimension of the rate constant for a second-order reaction. It can be seen that the arginine chloromethyl ketone is a very effective inhibitor of plasma kallikrein and is about 1/30th as active with crotalase, primarily due to differences in binding constants between the two enzymes. The arginine chloromethyl ketone is a more effective inhibitor of crotalase, however, than of any of the other enzymes studied. The inhibitor has weak activity with thrombin.

Table 1. Kinetic constants for inhibition of crotalase and other human trypsin-like proteases by Pro-Phe-ArgCH_2Cl

Enzyme	K_i, μM	k_2, min^{-1}	k_2/K_i, $(M^{-1} min^{-1}) \times 10^{-3}$
Crotalase	11	0.57	52
Plasma kallikrein*	0.24	0.36	1500
Plasmin*	4.2	0.15	37
Urinary kallikrein†	—	—	7.9
Thrombin*‡	—	—	0.4–1.2
Urokinase§	—	—	0.015

* Ref. 14.
† Ref. 15, given as k_{app}/I, which is an estimate of the bimolecular rate constant k_2/K_i.
‡ Ref. 23, given as k_{app}/I.
§ Ref. 24, given as k_{app}/I.

Sequence of Crotalase. Sequence analysis of about 42% of the total structure of crotalase (113 out of about 267 residues) revealed 38% homology with porcine pancreatic kallikrein (9), which was more than with any of the other serine proteases of known sequence used for comparison. Trypsin, with 33% homology, and thrombin, with 29%, were the next closest (9). These values are slightly different from those previously reported (9) due to the recent determination of an additional 16 residues of the sequence.

Crotalase Action on Human HMWK. Kallikrein action on HMWK has been shown to yield a molecule that is composed of a chain of about M_r 65,000 ("heavy" chain) and a smaller "light" chain variously reported as M_r 37,000 (25), M_r 44,000 (26), and M_r 54,000 (18). Crotalase cleaved HMWK to yield heavy and light chains comparable to those formed by kallikrein (Fig. 2). HMWK retained procoagulant activity after treatment with crotalase. Double volumes of duplicates of the kallikrein

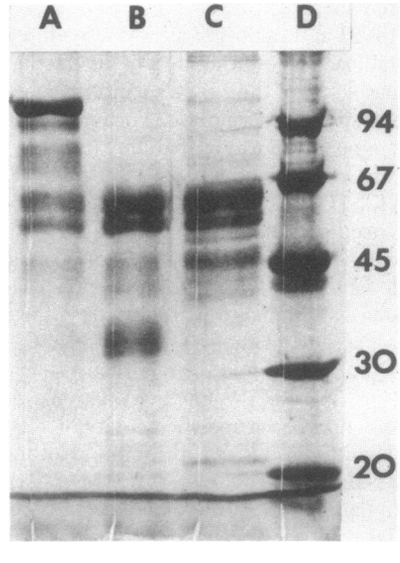

FIG. 2. Cleavage patterns of HMWK catalyzed by crotalase and human plasma kallikrein. Mixtures of 5 μl of HMWK and 10 μl of buffer (channel A), 10 μl of crotalase (channel B), or 20 μl of kallikrein (channel C) were incubated for 3 hr at 37°C and an additional 16 hr at 20°C. Then 10% 2-mercaptoethanol/6% $NaDodSO_4$ was added and the contents were heated at 37°C for 1 hr. Each reduced mixture was then subjected to $NaDodSO_4$/acrylamide gel electrophoresis on a 10% slab gel and stained with Coomassie brilliant blue. Standards are shown in channel D with their respective $M_r \times 10^{-3}$: phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; and soybean trypsin inhibitor, 20,000. The band at about M_r 33,000 in channel B is crotalase. The lighter bands in channel A represent cleavage products of HMWK formed during its isolation.

and crotalase-treated HMWK samples shown in Fig. 2 were subjected to $NaDodSO_4$/acrylamine gel electrophoresis under reducing condition, cut into 1.1-cm slices, and eluted with 0.15 ml buffer, and the eluates were tested for procoagulant activity by measuring their correction of the abnormal activated partial thromboplastin time of Fitzgerald trait plasma. Kallikrein yielded procoagulant activity in two regions corresponding to the heavy and light chains as previously documented for partial cleavage of HMWK (18). Crotalase yielded procoagulant activity at the same two positions, showing that with the incubation conditions used crotalase also produced partial cleavage of HMWK. The HMWK preparation used for these studies (Fig. 2, channel A) shows multiple bands. Quantitation of the different bands with a scanning densitometer showed that the major band of uncleaved HMWK, at about M_r 110,000, represented 53% of the total Coomassie blue-staining material. The bands in the M_r 45,000–65,000 region (heavy and light chains) represent 26% of the total protein and are previously identified cleavage products formed during isolation of HMWK. The intermediate uncharacterized bands in the M_r 70,000–105,000 region (representing 21% of the total protein) appear also to be derived from HMWK, because they are converted to heavy and light chains by both crotalase and kallikrein.

Crotalase-Catalyzed Kinin Release from Human HMWK. To further characterize crotalase action on human HMWK, we measured the release of kinin by using a biological assay, the ability of the released kinin to lower rat arterial blood pressure. Sensitivity of individual rats was monitored by injection of 20 ng of bradykinin, which produced a drop in blood pressure of 26–27.5 mm Hg (1 mm Hg = 133 Pa). A representative tracing (Fig. 3) shows that crotalase-treated HMWK produced a drop in blood pressure of 19 mm Hg, whereas buffer, crotalase alone, or HMWK alone produced virtually no change in arterial pressure. These findings suggest that crotalase releases kinin from HMWK. However, we were not able to definitively establish whether bradykinin or lysylbradykinin was released.

Activity of Crotalase on Chromogenic Substrates. We have already reported that crotalase exhibits esterase activity on small molecular weight, basic amino acid esters (1) and on the thrombin chromogenic substrate (27) D-phenylalanyl-L-pipecoyl-L-arginine *p*-nitroanilide, S-2238 (2). In view of the demonstrated kallikrein-like activity of crotalase, we felt it would be of interest to determine the relative activity of the enzyme on two peptide *p*-nitroanilides that serve as specific substrates for kallikrein. Therefore, we measured the activity of crotalase on D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide, S-2302, a substrate for plasma kallikrein (28), and D-valyl-L-leucyl-L-arginine *p*-nitroanilide, S-2266, a substrate for glandular kallikrein (29). When the kinetic data were plotted according to Lineweaver and Burk (30) to obtain K_m and V_{max}, it was found (Table 2) that crotalase exhibits significant activity with both kallikrein substrates, S-2302 and S-2266. These findings again illustrate the similarities between crotalase and kallikrein.

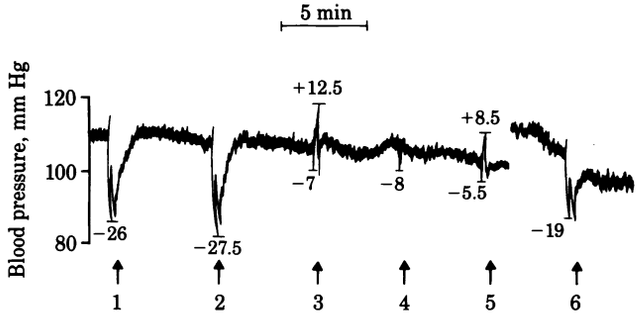

FIG. 3. Response of rat arterial blood pressure to injection of crotalase-treated human HMWK. Arrows indicate the time at which samples were injected and numbers indicate the composition of the samples as follows: 1, 20 ng of bradykinin; 2, 20 ng of bradykinin; 3, crotalase alone; 4, 0.05 M Tes/0.10 M NaCl pH 7.35 buffer alone; 5, HMWK alone; 6, crotalase-treated HMWK. Samples 3, 4, 5, and 6 were lyophilized and dissolved in 100 μl of physiological saline. All injections were in 100 μl of saline and were followed by a 100-μl saline wash.

Table 2. Determination of K_m and V_{max} for crotalase with three chromogenic substrates

Substrate	Kinetic constants: K_m, μM	Kinetic constants: V_{max}, μmol/min per mg protein
S-2238	180 ± 30	48.7 ± 4.1
S-2302	60 ± 10	154.4 ± 41.2
S-2266	140 ± 10	174.3 ± 7.5

Data are given ± SD (three determinations).

Lack of Activation of Human Plasminogen by Crotalase. Human plasma kallikrein weakly activates plasminogen (31) in a stoichiometric reaction (32). Crotalase, on the other hand, failed to activate purified human plasminogen when they were incubated together for up to 60 min, even with equimolar and 4-fold molar excess of crotalase. However, in control experiments plasminogen was completely activated by streptokinase (500 units/mg of plasminogen) in 20 min. Activation was measured both by generation of azocaseinolytic activity and by $NaDodSO_4$/acrylamide gel electrophoresis.

DISCUSSION

Crotalase, the thrombin-like enzyme from eastern diamondback rattlesnake venom, has long been regarded as a serine protease of the trypsin class with both fibrinogen clotting activity and esterase activity on basic amino acid ester derivatives (1). Our preliminary findings that the enzyme exhibits remarkable similarities to kallikrein were surprising (9, 33, 34).

In our original work (1) we found that crotalase exhibited very low reactivity with *N*-α-tosyl-L-lysine chloromethyl ketone. However, in the studies reported here we find that the enzyme reacts quite rapidly with a tripeptide chloromethyl ketone, Pro-Phe-ArgCH_2Cl. This peptide chloromethyl ketone has the sequence of the carboxyl-terminal three residues in bradykinin and is an extremely effective and specific inhibitor of plasma kallikrein (14). Interestingly, when several other chloromethyl ketones, which are effective and specific inhibitors of thrombin, were tested for inhibitory activity with crotalase, they were much less effective than was Pro-Phe-ArgCH_2Cl. Apparently, the topography of the crotalase binding site is more like that of kallikrein than it is like that of thrombin. It should be remembered, however, that crotalase possesses significant clotting activity, 470 National Institutes of Health thrombin clotting units per mg of highly purified protein. Homogeneous thrombin preparations have about 2300 National Institutes of Health thrombin clotting units per mg of protein (35).

That the kallikrein-like activity of crotalase is not due to an impurity was shown by the following. First, the enzyme simultaneously loses fibrinogen clotting and chromogenic substrate activity during inhibition by Pro-Phe-ArgCH_2Cl. Second, on the basis of the protein content of crotalase there is stoichiometric incorporation of [^{3}H]Ac-Pro-Phe-ArgCH_2Cl into the enzyme after 100% inactivation. Third, acrylamide gel electrophoresis by the method of Davis (36) revealed that there is identical migration of native and [^{3}H]Ac-Pro-Phe-ArgCH_2Cl-inhibited crotalase (as shown by Coomassie blue staining) and of the peak of radioactivity of the tritiated chloromethyl ketone-inhibited enzyme and of the peak of clotting activity of native crotalase. Finally, sequence analysis shows a single amino-terminal

residue for crotalase, ruling out the possibility of contaminant unless it has blocked amino-terminal residue. Studies to establish the amino acid sequence of crotalase have revealed a substantial similarity with kallikrein (9, 34). Of the 113 residues of crotalase presently in sequence, there is a greater degree of homology (38%) with kallikrein than with any of the other serine proteases, including trypsin (33%), thrombin (29%), and factor X (23%). The sequence of kallikrein used for comparison is that from porcine pancreas (11); it is quite possible that there is an even greater degree of homology with plasma kallikrein. However, to date this structure remains unknown.

In addition to sequence homology and inhibition by the chloromethyl ketone inhibitor of plasma kallikrein, crotalase exhibits another activity characteristic of plasma kallikrein, cleavage of HMWK. Crotalase cleaves HMWK, producing cleavage products identical to those formed by kallikrein as shown by $NaDodSO_4$/acrylamide gel electrophoresis. Further, the fragments produced by crotalase have procoagulant activity just like the kallikrein-derived fragments. Additionally, crotalase liberates kinin from HMWK, as shown by a drop in rat arterial blood pressure after infusion of crotalase-treated HMWK.

There have been several reports of kinin-releasing enzymes in snake venoms (13, 37), including members of the rattlesnake family (13). However, an association of this activity with the thrombin-like enzyme found in the venom has not been reported, to our knowledge. It is interesting that Viljoen *et al.* (37) reported on the identification of a serine esterase with kinin-releasing activity in the venom of *Bitis gabonica*. This enzyme may be the agent responsible for the marked transient blood pressure drop in dogs after infusion of the crude venom (38). The kinin-releasing enzyme has a slightly different carbohydrate composition and is distinguishable both chromatographically and electrophoretically from the thrombin-like enzyme present in this venom. However, it has an almost identical amino acid composition to the thrombin-like enzyme (37). In eastern diamondback rattlesnake venom, these two activities reside in a single enzyme; however, there may also be a separate kinin-releasing enzyme present in this venom.

Finally, in view of the fact that thrombin-like enzymes from both *Agkistrodon rhodostoma*, ancrod (39, 40), and *Bothrops atrox*, batroxobin (41), are being used clinically in Europe for the treatment of deep vein thrombosis, it will be of interest to determine if these enzymes also exhibit kallikrein-like activity. The finding of kallikrein-like activity should certainly not contraindicate the use of these enzymes. On the contrary, this activity (if present) may be acting to enhance the overall effectiveness of these clinical agents both by an improvement in the blood flow characteristics [Ehrly (42) has shown an improvement in the rheological properties of blood after ancrod-induced fibrinogen depletion with a concomitant reduction in blood viscosity] and by the hypotensive or vasodilatory activity of the enzyme resulting in decreased peripheral resistance.

The authors acknowledge the expert technical assistance of Cathy Mastroni and Rick Baumgartner. This work was supported by Grants HL 22875 (to F.S.M. and H.P.) and HL 17402 (to S.S) from the National Heart, Lung and Blood Institute, by the U.S. Department of Energy (to C.K. and E.S.), and by a Senior Investigatorship from the American Heart Association, Greater Los Angeles Affiliate (to S.S.B.).

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