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THE PRIMARY STRUCTURE OF CROTALASE, A THROMBIN-LIKE VENOM ENZYME, EXHIBITS CLOSER HOMOLOGY TO KALLIKREIN THAN TO OTHER SERINE PROTEASES

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SUMMARY

Amino acid sequences of crotalase, totaling 98 residues or about 37% of the molecule, were determined by Edman degradation and were compared with the published sequences of nine serine proteases. Homologous alignment could be found for all crotalase sequences except one decapeptide. Comparison between crotalase and porcine pancreatic kallikrein showed the largest number of identical amino acids (36%). This finding has led to experiments which demonstrate that crotalase has specific enzymatic properties resembling kallikrein.

INTRODUCTION

Crotalase is one of a group of enzymes from snake venoms which shares with the blood clotting enzyme, thrombin, very narrowly specific proteolytic actions (1). Like thrombin, crotalase cleaves a single arginyl bond in the Aα chain of fibrinogen (2) and in prothrombin (3), but, unlike thrombin, crotalase neither releases fibrinopeptide B from fibrinogen nor activates factor XIII (4). Crotalase has molecular weight of 32,700 and is inhibited by diisopropyl phosphofluoridate and by the chloromethyl ketone of tosyl-L-lysine (5). Since several of the thrombin-like enzymes possess different, though overlapping, sets of thrombin-like proteolytic specificities (1-3), they offer rich opportunity for comparative studies on the structural basis for narrowly limited proteolysis.

The amino acid sequences of crotalase reported here are the first for a thrombin-like venom enzyme. They show clear homology with the serine proteases, especially with porcine glandular kallikrein.

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MATERIALS AND METHODS

Preparation of Crotalase Crotalase was isolated from pooled, freeze-dried venom from our own Eastern diamondback rattlesnakes (Crotalus adamanteus) as previously described (5,6). A 100 mg sample of purified enzyme was digested with 10 units of neuraminidase (Sigma Chemical Co., type IX) in 0.2 M sodium acetate (pH 5.5) at 37°C. Release of sialic acid reached completion at about 60 min (7). The active serine was labelled with (^{14}C) diisopropyl phosphofluoridate (DFP) in approximately equimolar amount, followed by about a hundredfold molar excess of cold DFP. The reaction was carried out in 0.1 M sodium acetate, pH 7.4, for 4.5 h. The sample was then dialyzed, freeze-dried, dissolved in 6M guanidine•HCl, and reduced at pH 8.6 with dithiothreitiol in a fiftyfold molar excess with respect to sulfhydryl groups (8). Next, sulfhydryl groups of 100 mg of protein were carboxymethylated, first with 2 mg (^{3}H) iodoacetic acid and then with 268 mg of cold iodoacetic acid (9). The reaction was stopped by adding 0.1 ml of 2-mercaptoethanol to 9 ml of reaction mixture. The mixture was then brought to pH 3.2 with formic acid and dialyzed against 0.2 M formic acid for 3 h followed by water for 22 h. Freeze-drying yielded a fluffy, white product.

Cyanogen Bromide Cleavage The reduced, carboxymethylated protein (100 mg) was dissolved in 7.5 ml of 70% formic acid and reacted with 200 mg of CNBr under nitrogen in the dark for 22 h (10). The mixture was then diluted with water (5 vol), reduced to 10 ml by rotary evaporation, again diluted with water (5 vol), and freeze-dried. The resulting peptides were dissolved in 30% acetic acid and fractionated on a 3.5 x 110 cm column of Sephadex G-25 superfine. Some fractions were further purified on DEAE-cellulose or CM-cellulose in an ammonium acetate buffer, ph 4.9/8 M decyanated urea, using a NaCl gradient.

Tryptic Digestion Reduced, carboxymethylated crotalase (100 mg) was dissolved in 15 ml of 50 mM N-ethylmorpholine/5 mM EDTA, pH 8.2. Citraconic anhydride (120 µl or a 30 fold molar excess with respect to lysine) was added over 4 h (11) while the pH was held constant by automatic addition of NaOH. The sample was dialyzed against 0.1 M NH_4HCO_3, pH 8.0, concentrated two-fold, and then digested with 1 mg of TPCK-trypsin (Worthington) for 45 min at 37°C, followed by an additional 1 mg of trypsin for 90 min. The preparation was next decitraconylated by lowering the pH to 2.5 with acetic acid for 6 h at 22°C and 16 h at 4°C. After freeze-drying the sample was dissolved in 30% acetic acid and fractionated on a 3.5 x 110 cm column of Sephadex G-25 fine using 30% acetic acid. The peptides were further purified on a 1.6 x 90 cm column of Dowex 50-2X, at 40°C, using pyridine/acetic acid buffers with a gradient from pH 3.3 to pH 4.9 or by high voltage paper electrophoresis at pH 6.5, pH 3.5, or pH 1.9.

Analysis for Homogeneity Fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12) and to combined paper electrophoresis (pH 6.5, 3.5 or 1.9/pyridine and acetic acid or formic acid and acetic acid) and paper chromatography using 1-butanol, pyridine, acetic acid, and water (15:10:3:12).

Amino Acid Analysis Peptides were hydrolyzed for 24 h in 6 M HCl containing 2% mercaptoacetic acid and 0.04% phenol. Analyses were done with a Durrum D500 analyzer (ninhydrin monitoring) or with a Dionex analyzer (o-phthalaldehyde monitoring).

Amino Acid Sequence Determination Automatic Edman degradation was carried out with Beckman Model 890B sequencer using a program rewritten from Hunkapiller and Hood (13). The coupling buffer was 0.25 M Quadrol added at the beginning and in the middle of the coupling stage. Benzene and ethyl acetate/acetic acid were delivered simultaneously in one 400 sec step. Before application of sample, polybrene (5 mg) and glycylglycine (100 nmole) in 0.5 ml of water were placed in the cup and subjected to three complete degradation cycles. Phenylthiohydantoin (PTH) amino acids were identified by thin-layer chromatography on polyamide plates (14) and by high-performance liquid chromatography (HPLC) using three DuPont Zorbax ODS columns (0.46 x 15 cm) in tandem, an increasing gradient of acetonitrile in 0.02 M sodium acetate, pH 5.0, a flow rate of 1.2ml/min, and a column temperature of 54°C. All PTH-amino acids, including arginine and histidine in the water phase, were resolved by HPLC. Arginine was also identified by a phenanthrenequinone spot test (15) and histidine by the Pauly reaction (16). Identification of PTH-serine and PTH-threonine was assisted by the use of a HPLC detector which measures at both 254 nm and 313 nm (Waters 440).

RESULTS AND DISCUSSION

Some amino acid sequences of crotalase are given in Fig. 1. On comparison with several serine proteases, homology was evident by inspection. Of 13 positions invariant among vertebrate serine proteases (17), 11 are occupied in crotalase by the same amino acids. However, the two differences which were found (Arg 13 and AsN 161, each in place of an invariant Pro) were somewhat radical from both physicochemical and evolutionary standpoints (24). Table 1 compares the crotalase sequences with other serine proteases with respect to percent of positions occupied by identical residues and shows that porcine pancreatic kallikrein has the greatest percentage.

The kallikreins (glandular and plasma) are central enzymes in systems with wide-ranging actions, including effects on blood coagulation, fibrinolysis, complement, inflammation, and cardiovascular function. Although the presence of kallikrein-like activity in the venom of certain snakes has been known for some time (18,19), there was no particular reason to expect such properties of crotalase until the similarity of amino acid sequences was found. This finding prompted us to examine the interaction of crotalase with tripeptide p-nitroanilide substrates specific for the kallikreins, a specific tripeptide chloromethyl ketone kalli-

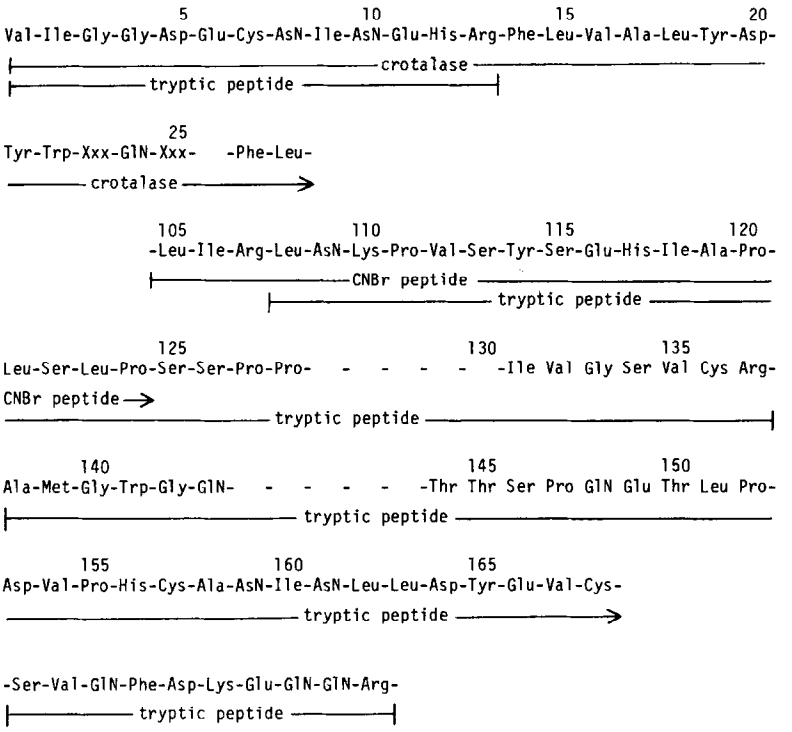

Fig. 1. Amino acid sequences of crotalase. N-Terminal sequence is numbered starting with 1. Internal peptides are aligned by homology with several serine proteases and numbered in register with chymotrypsin (17). Segment marked "crotalase" was established by Edman degradation of the intact molecule. Vertical lines indicate beginning of peptide or end of peptide. Arrows mean end of analysis but peptide continues. Xxx indicates uncertain results. Gaps used to maximize homology are represented as spaces between dash marks. Junction between Arg 137 and Ala 138 was inferred by homology. Tryptic peptide at bottom of diagram was not detectably homologous with any part of 10 serine proteases searched visually.

krein inhibitor, and human high molecular weight kininogen. In each case kallikrein-like activity was demonstrated (Markland, Shaw, Kettner, Schiffman, Bajwa, Reddy, Kirakossian, Theodor, and Pirkle, unpublished work).

The sequences of crotalase which are homologous to bovine trypsin appeared to fit well a stereo-pair model of trypsin (17), taking into account the expected effects of primary structure on conformation (20). Substantial portions of crotalase sequence are consistent with inclusion in β-sheet groupings such as those which form two major core regions of trypsin, while other more polar portions are homologous to surface segments of trypsin. Compara-

Table 1

Comparison of amino acid sequences of crotalase with other serine proteases

Crotalase compared with	Identities/positions compared N-terminal[a]	Internal[b]	Identical residues (total %)
Kallikrein[c]	11/25	21/64	36
Trypsin	6/25	22/62	32
Thrombin	5/25	23/72	29
Chymotrypsin	6/25	19/64	28
Elastase	6/26	17/64	26
Bacterial trypsin	7/25	13/65	22
Plasmin	5/25	15/64	22
Factor X	5/25	14/67	21
Factor IX[d]	5/26	10/67	16

[a]Positions 1-28 in Fig. 1.

[b]Positions 105-168 in Fig. 1.

[c]Reference (22)

[d]Reference (23)

The remaining sequences for comparison are from reference (17) and are bovine except for elastase (porcine), bacterial trypsin (S. griseus) and plasmin (human).

tive considerations also suggest that salt bridges probably exist in crotalase between the amino terminus and a buried acidic residue and between the carboxy terminus and Arg 107. Thus, it appears that the amino acid sequence of crotalase may be amenable to detailed model building, using the comparative method of Greer (21).

The apparently nonhomologous sequence given at the bottom of Fig. 1 may have some role in crotalase's unique set of enzymatic specificities, but since it contains several polar residues it is more likely a surface segment similar to those variably present in other serine proteases and presumed to have little or no functional significance (21).

Further studies on crotalase and related enzymes are in progress in our laboratory.

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