Sone, N., Yanagita, Y., Hon-nami, K., Fukumori, Y., & Yamanaka, T. (1983) *FEBS Lett. 155*, 150–154.
Sreekrishna, K., Jones, C. E., Guetzow, K. A., Prasad, M. R., & Joshi, V. C. (1981) *Anal. Biochem. 103*, 55–57.
Tanaka, T., Kawano, N., & Oshima, T. (1981) *J. Biochem. (Tokyo) 89*, 677–682.
Tracy, R. P., & Chan, S. H. P. (1979) *Biochim. Biophys. Acta 576*, 109–117.
Van De Bogart, M., & Beinert, H. (1967) *Anal. Biochem. 20*, 325–334.
Wang, C. S., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414–417.
Weber, K., & Osborn, M. J. (1969) *J. Biol. Chem. 244*, 4406–4412.
Yamanaka, T., & Fujii, K. (1980) *Biochim. Biophys. Acta 591*, 53–62.
Yamanaka, T., & Fukumori, Y. (1981) *Plant Cell Physiol. 22*, 1223–1230.
Yamakawa, T., Irie, R., & Iwanage, M. (1960) *J. Biochem. (Tokyo) 48*, 490–507.
Yamanaka, T., Kamita, Y., & Fukumori, Y. (1981) *J. Biochem. (Tokyo) 89*, 265–273.
Yonetani, T. (1960) *J. Biol. Chem. 235*, 845–852.

Anticoagulant Proteases from Western Diamondback Rattlesnake (*Crotalus atrox*) Venom†

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ABSTRACT: *Crotalus atrox* venom contains agents that render human fibrinogen and plasma incoagulable by thrombin. To elucidate the mechanism of alteration of fibrinogen clotting function by the venom, four immunochemically different proteases, I, II, III, and IV, were purified from the venom by anion-exchange chromatography and column gel filtration. All four proteases had anticoagulant activity rendering purified fibrinogen incoagulable. Proteases I and IV do not affect fibrinogen in plasma but in purified fibrinogen cleave the Aα chain first and then the Bβ and γ chains. Both enzymes are metalloproteases containing a single polypeptide chain with 1 mol of zinc, are inhibited by (ethylenedinitrilo)tetraacetate and human α_2-macroglobulin, and have an optimal temperature of 37 °C and an optimal pH of 7. Protease I has a molecular weight (M_r) of 20 000 and is the most cationic. Protease IV has an M_r of 46 000 and is the most anionic glycoprotein with one free sulfhydryl group. Proteases II and III degrade both purified fibrinogen and fibrinogen in plasma, cleaving only the Bβ chain and leaving the Aα and γ chains intact. Both enzymes are alkaline serine proteases, cleave chromogenic substrates at the COOH terminal of arginine or lysine, are inhibited by diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride, and have an optimal temperature of 50–65 °C. Protease II is a single polypeptide chain glycoprotein with an M_r of 31 000. Protease III is a two polypeptide chain protein with an M_r of 24 000, each of the two chains having an M_r of 13 000; its activity is not affected by major protease inhibitors of human plasma. Proteases II and III are enzymes with unique and limited substrate specificity by cleaving only the Bβ chain, releasing a peptide of M_r 5000 and generating a fibrinogen derivative of M_r 325 000, with intact Aα and γ chains and poor coagulability. Since the two enzymes are active in human plasma and serum, it is postulated that proteases II and III can mediate anticoagulant effects in vivo after envenomation.

Proteases, which in vitro convert fibrinogen to fibrin, have been found in venoms of snakes belonging to Crotalidae and Viperidae families (Iwanaga & Suzuki, 1979). Fibrinogen-clotting enzymes were purified from venoms of *Agkistrodon acutus* (Ouyang et al., 1971), *Agkistrodon rhodostoma* (Esnouf & Tunnah, 1967), *Agkistrodon contortrix contortrix* (Herzig et al., 1970), *Bitis gabonica* (Marsh & Whaler, 1974), *Bothrops atrox* (Stocker & Egberg, 1973), *Trimeresurus gramineus* (Ouyang & Yang, 1974), *Crotalus horridus horridus* (Bonilla, 1975), and *Crotalus adamanteus* (Markland & Damus, 1971). A fibrinogen-clotting activity was also found in the venom of juvenile specimens of *Crotalus atrox*, a western diamondback rattlesnake, but it disappeared after the snake reached the age of about 1 year (Reid & Theakston, 1978). Preliminary reports describing the absence of coagulant activity and the presence of fibrinolytic activity in *C. atrox* venom have been published (Deutsch & Diniz, 1955; Denson, 1969). Column gel filtration of the venom on Sephadex G-100 revealed two protein peaks of M_r 60 000 and 21 500 with fibrinolytic activity, as determined by the fibrin plate assay (Bajwa et al., 1980, 1981). However, the corresponding enzymes have not been purified and characterized.

A variety of proteases have been isolated from *C. atrox* venom. These include three proteases with caseinolytic activity, called α-, β-, and γ-proteases (Pfleiderer & Sumyk, 1961), five hemorrhagic toxins (*a*, *b*, *c*, *d*, and *e*) with proteolytic activity on dimethylcasein and dimethylhemoglobin (Bjarnason & Tu, 1978), and four complement-inactivating proteases (Man & Minta, 1977). Our studies with unfractionated *C. atrox* venom showed that human plasma or purified human fibrinogen was rendered incoagulable. The effect resulted from cleavage of the fibrinogen molecule. The degradation pattern of plasma fibrinogen, however, was different from that of purified fibrinogen, suggesting that the venom contained two different types of fibrinogenolytic enzymes: those that initially cleave the Aα chain of fibrinogen and other proteases that

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preferentially cleave the Bβ chain (Pandya et al., 1983).

Fibrinogen (M_r 340 000) is a dimeric molecule composed of two sets of three different polypeptide chains: Aα (M_r 68 000), Bβ (M_r 58 000), and γ (M_r 47 000) (McKee et al., 1966). Thrombin cleaves fibrinopeptides A and B from the NH_2-terminal of the Aα and Bβ chains, respectively, and the generated fibrin monomers polymerize spontaneously. The interaction between binding regions on fibrin monomer molecules results in clot formation (Doolittle, 1973).

In this work, the question addressed was what is the nature and mechanism of action of anticoagulant proteases in *C. atrox* venom. Fractionation of the venom was undertaken in order to purify fibrinogenolytic enzymes with Aα or Bβ chain cleaving activity. The proteases were characterized, and the effect of plasma protease inhibitors on their activity was studied in order to explain the role of each protease in the degradation of plasma fibrinogen occurring in vivo after a bite of the rattlesnake.

Materials and Methods

Reagents. Three different batches of *C. atrox* venom were obtained from Sigma Chemical Co., St. Louis, MO. Human α-thrombin (2440 units/mg) was a generous gift from Dr. John W. Fenton, II (New York State Department of Health, Albany, NY). Plasmin (12.8 CTA units/mg) was obtained from Dr. D. Aronson (Bureau of Biologics, Food and Drug Administration, Rockville, MD). Trypsin–TPCK[1] (273 units/mg) was from Worthington Biochemical Corp., Freehold, NJ. α-Chymotrypsin–TLCK (49 units/mg) and hog pancreatic elastase (50 units/mg) were obtained from Sigma Chemical Co.

Chromogenic substrates were obtained from the following sources: Bz-Ile-Glu-[γ-OR]-Gly-Arg-pNA (S-2222), Phe-Pip-Arg-pNA (S-2238), Pro-Phe-Arg-pNA (S-2302), and Bz-Phe-Val-Arg-pNA (S-2160) from Ortho Diagnostics, Raritan, NJ; Bz-Pro-Phe-Arg-pNA (Chromozym PK) and Tos-Gly-Pro-Arg-pNA (Chromozym TH) from Boehringer Mannheim Biochemicals, Indianapolis, IN; Val-Leu-Lys-pNA (S-2251) from Kabi Group Inc., Greenwich, CT; Suc-$(Ala)_3$-pNA from Calbiochem-Behring Corp., La Jolla, CA; Bz-Tyr-pNA from Sigma Chemical Co.

EDTA and 1,10-phenanthroline were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ, PMSF was from Sigma Chemical Co., and DFP was from Aldrich Chemical Co., Milwaukee, WI.

Plasma protease inhibitors were obtained from the following sources: α_1PI from Sigma Chemical Co.; AT III, C1-INA, and α_2PI from Dr. M. Wickerhouser, American Red Cross, Bethesda, MD; α_2M from Dr. K. Lonberg-Holm, Du Pont, Wilmington, DE. Tris–saline buffer contained 0.05 M Tris-HCl, 0.10 M NaCl, and 0.02% sodium azide, pH 7.4.

Fibrinogen was purified from fresh human citrated plasma (1 L) by precipitation with ammonium sulfate at 0.85 M final concentration. The precipitate was washed twice with 0.85 M ammonium sulfate, dissolved in water, dialyzed in 0.15 M NaCl, and precipitated at 4 °C with glycine at 2.2 M final concentration. The precipitate was dissolved in water and gel filtered on a Sepharose CL-6B (Pharmacia, Piscataway, NJ) column (2 × 150 cm). The main peak containing fibrinogen was collected and precipitated with ammonium sulfate at 0.65 M final concentration. The precipitate was dissolved in water to an approximate protein concentration of 20 mg/mL and dialyzed 3 times in 100 volumes of 0.15 M NaCl–0.025 M sodium citrate, pH 7.4. Thrombin-coagulable protein was 95% of the total. This preparation of purified fibrinogen was free of plasminogen, as determined by a lack of lysis of streptokinase-supplemented fibrin clots. $NaDodSO_4$ electrophoresis in 3.5% polyacrylamide gels of 10-μg samples demonstrated the presence of one homogeneous fibrinogen band and the absence of plasma fibronectin. Electrophoretic patterns of reduced samples separated in 7% gels demonstrated undegraded Aα, Bβ, and γ chains and the absence of any other bands.

Labeling of Fibrinogen. Human fibrinogen was labeled with ^{125}I by using a modified iodine monochloride method (Knight et al., 1981) and carrier-free $Na^{125}I$ (New England Nuclear, Boston, MA). The radioactivity of the labeled protein was counted in a γ counter (automatic γ counter, Nuclear Chicago, Chicago, IL). The labeled protein contained approximately 0.8 atom of iodine per molecule and had a specific radioactivity of 100 μCi/mg of protein.

DEAE-cellulose Ion-Exchange Chromatography. A 100-mg sample of the venom dissolved in 5 mL of 0.01 M potassium phosphate buffer, pH 8.0, was applied on a 1.7 × 24 cm DEAE-cellulose column (DE-52, Whatman Inc., Clifton, NJ) equilibrated with the same buffer and operated in a cold room. The unbound proteins were eluted by the same buffer until the A_{280} of the fractions was less than 0.02. The bound proteins were eluted with a linear gradient from 0.01 to 0.3 M potassium phosphate buffer, pH 8.0. Fractions of 3.2 mL were collected at an elution rate of 40 mL/h. At the end of the linear gradient, the column was washed with 0.3 M potassium phosphate buffer containing 1.0 M NaCl, pH 8.0. This treatment did not result in elution of any more protein peaks. The fractions were assayed for their anticoagulant effect on purified fibrinogen and plasma. The anticoagulant effect was defined by prolongation of the thrombin clotting time of the coagulable substrate.

Gel Filtration on Ultrogel AcA 54. The fractions pooled from the DEAE-cellulose column were concentrated by vacuum ultrafiltration, dialyzed against 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.02% NaN_3, pH 7.4, and applied on a 1.7 × 95 cm Ultrogel AcA 54 (LKB, Rockville, MD) column equilibrated with the same buffer. The column was developed at a flow rate of 18 mL/h, and fractions of 1.5 mL were collected. The absorbance of each fraction at 280 nm and the anticoagulant activity were determined.

Thrombin Clotting Time. Equal volumes of purified human fibrinogen (2 mg/mL) or normal human citrated plasma (containing 2 mg/mL of fibrinogen) and a fraction from DEAE-cellulose or Ultrogel AcA 54 columns were mixed and incubated at 37 °C for 10 min. Then a 0.2-mL aliquot was mixed with 0.1 mL of human thrombin (20 units/mL) and the clotting time determined at 37 °C by using the fibrometer (BBL, Division of Becton, Dickinson and Co., Cockeysville, MD). The results were calculated as the ratio of sample clotting time to that of the control, where the latter contained buffer instead of the sample and was adjusted to 15 ± 3 s.

Determination of Protein Concentration. The concentration of purified fibrinogen was determined at 280 nm by using an absorption coefficient of 1.5 for a 1 mg/mL solution. The concentration of fibrinogen in plasma was determined by radial

[1] Abbreviations: DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); α_1PI, α_1-protease inhibitor; AT III, antithrombin III; α_2M, α_2-macroglobulin; C1-INA, C1 inactivator; α_2PI, α_2-antiplasmin; pNA, *p*-nitroanilide; Suc, succinyl; Bz, benzoyl; Tos, toluenesulfonyl; Pip, pipecolic acid; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

immunodiffusion (Mancini et al., 1965) using monospecific polyclonal rabbit antiserum against human fibrinogen obtained in our laboratory by routine immunization. The protein concentrations of *C. atrox* venom, chromatographic fractions, and purified proteases were determined with a Coomassie Brilliant Blue G-250 binding assay (Bradford, 1976) by using human serum albumin as a standard.

Degradation of Fibrinogen by Purified Proteases. Purified fibrinogen (2 mg/mL) was mixed with an equal volume of protease I (24 μg/mL), protease II (2 μg/mL), protease III (2 μg/mL), or protease IV (54 μg/mL) and incubated at 37 °C. Different protease concentrations were chosen to render fibrinogen incoagulable in 75 min. At different times, a 100-μL aliquot of the reaction mixture was withdrawn and inhibited with 400 μL of 9 M urea and 3% $NaDodSO_4$ with or without 3% β-mercaptoethanol. Degradation of the fibrinogen molecule and its polypeptide chains was followed by polyacrylamide gel electrophoresis using 3 or 6 μg of total protein for nonreduced or reduced samples, respectively.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in cylindrical tubes (0.5 × 9 cm) in a polyacrylamide gel in the presence of 0.1% $NaDodSO_4$ (Weber & Osborn, 1969). The samples applied to 3.5% gels were unreduced; those applied to 7% gels were reduced at 90 °C for 5 min in a solution containing 3% β-mercaptoethanol, 9 M urea, and 3% $NaDodSO_4$. The gels were stained with Coomassie Brilliant Blue R-250 (Fairbanks et al., 1971). For the determination of molecular weight, plasmin degradation products of fibrinogen [fragment X (M_r 250 000), fragment Y (M_r 150 000), fragment D (M_r 103 000), fragment E (M_r 50 000), and fibrinogen (M_r 340 000)] were used as standards for 3.5% gels. Bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 500), α-chymotrypsinogen (M_r 23 500), myoglobin (M_r 17 000), and lysozyme (M_r 14 000) were standards for 7% gels.

Chromogenic Substrate Assay. All enzymes and substrates except Bz-Tyr-pNA were dissolved in 0.05 M Tris-HCl buffer, pH 7.4. The substrate Bz-Tyr-pNA was insoluble in aqueous solutions due to its hydrophobicity; it was dissolved first in dimethylformamide and then diluted to an appropriate concentration with 0.05 M Tris-HCl buffer, pH 7.4. The cleavage of all chromogenic substrates by plasmin, trypsin, thrombin, α-chymotrypsin, and elastase was determined to establish optimal concentrations of substrates and enzymes and especially incubation times.

An aliquot of 250 μL of a substrate (0.5 or 1.0 mM) was mixed with 250 μL of a 1 μM solution of the enzyme. This reaction mixture was incubated at room temperature for 6 min for the substrates Val-Leu-Lys-pNA, Pro-Phe-Arg-pNA, Bz-Ile-Glu-[γ-OR]-Gly-Arg-pNA, Bz-Pro-Phe-Arg-pNA, Phe-Pip-Arg-pNA, and Bz-Phe-Val-Arg-pNA, 15 min for the substrates Suc-$(Ala)_3$-pNA and Tos-Gly-Pro-Arg-pNA, and 40 min for the substrate Bz-Tyr-pNA. At the end of the incubation time, 50 μL of 50% acetic acid was added to stop the reaction. The cleavage of the substrate, evidenced by the generation of pNA, was measured by determination of the absorbance at 405 nm. When a molar extinction coefficient of 10 600 at this wavelength and pH was used, the pNA released was determined and expressed as moles of pNA cleaved per minute per mole of the enzyme.

Fibrinogenolysis Assay. The assay was based on degradation of ^{125}I-labeled human fibrinogen and separation of the soluble cleaved products from undegraded fibrinogen or its high molecular weight degradation products. The fibrinogenolysis reaction mixture (500 μL) contained fibrinogen (1 mg/mL = 3 μM) supplemented with 1 μg of ^{125}I-labeled fibrinogen (approximately 30 000 cpm/μg) and 0.5 μM protease. The reaction mixture was incubated at room temperature for 90 min. The reaction was stopped by the addition of 215 μL of saturated ammonium sulfate containing 30 mM EDTA (final concentrations of ammonium sulfate and EDTA are 1.23 M and 10 mM, respectively). Fibrinogen incubated with Tris–saline buffer served as a control. The precipitates were separated from the supernate by centrifugation at 2000*g* for 15 min and washed with 1 mL of 1.23 M ammonium sulfate, and the wash was combined with the supernate after centrifugation at 2000*g* for 15 min. The radioactivity in the supernate and precipitate was counted in a γ counter. The protease activity was expressed as the percent cpm in the supernate after subtracting the percent cpm in the supernate found for a control. Alternatively, for experiments determining the effect of various inhibitors on proteases, the proteolytic activity was expressed as the percent activity, where 100% activity was in the absence of any inhibitor.

Effect of Inhibitors. Each protease, at 1 μM concentration, was incubated with EDTA, 1,10-phenanthroline, PMSF, or DFP, each at 10 mM final concentration at room temperature for 30 min. A parallel incubation with Tris–saline buffer was used as a control. The activity of proteases I and IV was determined by fibrinogenolysis assay. The activity of protease II was determined by the cleavage of the chromogenic substrate Pro-Phe-Arg-pNA, while the activity of protease III was determined with Bz-Pro-Phe-Arg-pNA.

Temperature Dependence. Each protease and substrate (in separate tubes) was preincubated for 5 min at 1, 12, 25, 37, 50, and 65 °C. After preincubation, the substrate and enzyme were mixed, and the protease activity was determined at the corresponding temperature either by the fibrinogenolysis assay (proteases I and IV) or by the cleavage of Pro-Phe-Arg-pNA (proteases II and III). The substrates incubated with Tris–saline buffer at corresponding temperatures served as a control. The activity of proteases I and IV was expressed as the percent cpm in the supernate and that of proteases II and III as the absorbance determined at 405 nm.

pH Dependence. The dependence of the activity of the proteases on pH was determined in the following buffers: 0.1 M glycine hydrochloride at pH 3.0 and 3.5; 0.1 M sodium acetate at pH 4.0, 4.5, 5.0, and 5.5; 0.1 M sodium phosphate at pH 5.5, 6.0, 6.5, and 7.0; 0.1 M Tris-HCl at pH 7.0, 7.5, 8.0, 8.5, and 9.0; 0.1 M glycine–NaOH at pH 9.0, 9.5, 10.0, 10.5, and 11.0. The proteolytic activity of proteases I and IV was determined by degradation of ^{125}I-fibrinogen, while that of proteases II and III by cleavage of Pro-Phe-Arg-pNA. The activity change was expressed in the same way as in temperature-dependence experiments.

Amino Acid Composition. Approximately 25 nmol of protease was mixed with 25 nmol of norleucine and hydrolyzed in 0.2 mL of a hydrochloric acid–propionic acid mixture (1:1) (Pierce Chemical Co., Rockford, IL) in sealed ampules under vacuum at 110 °C for 18, 36, and 54 h. The hydrolysates were analyzed in an automatic amino acid analyzer (Model 119, Beckman, Palo Alto, CA). The number of nanomoles of each amino acid was calculated for each hydrolysis time, and the value obtained by extrapolation to zero time (serine and threonine) or the highest value was accepted. Cysteine was determined as cysteic acid after performic acid oxidation according to the method of Moore (1963). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967).

Carbohydrate Staining. Each protease (40 μg) was reduced in the presence of 3% $NaDodSO_4$, 9 M urea, and 3% 2-

mercaptoethanol and electrophoresed in 7% polyacrylamide gels. The gels were stained with fuchsin–sulfite stain (McGuckin & McKenzie, 1958) after treatment with 1% periodic acid (Zacharius et al., 1969).

Determination of Sulfhydryl Groups. Free sulfhydryl groups present in proteases were determined by using DTNB (Ellman, 1959) according to a previously described method (Anderson & Wetlaufer, 1975). Briefly, 900 μL of an approximately 10 μM protease solution in 0.08 M sodium phosphate buffer with 0.5 mg/mL EDTA, pH 8.0, with or without 2% $NaDodSO_4$, was mixed with 100 μL of a 5 mM solution of DTNB and incubated at room temperature for 20 min. The concentration of DTNB reacting with the protease (concentration of 2-nitro-5-thiobenzoate anion generated) was determined from the absorbance at 412 nm by using a molar extinction coefficient of 13 600 at this wavelength and at pH 8.0.

Atomic Absorption Spectroscopy for Determination of Zn^{2+}. Zinc was determined at 213.7 nm by using a Perkin-Elmer atomic absorption spectrometer (Model 4000) with an O_2/acetylene flame (Evenson & Anderson, 1975). A standard curve was constructed by using a solution of $ZnSO_4 \cdot 7H_2O$ in double-distilled deionized water at 0.95, 1.9, 3.8, 7.6, and 15 μM zinc. A 10 μM solution of each protease either in double-distilled water or in 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.02% NaN_3, pH 7.4, was used for zinc analysis. The Tris buffer contained a very small amount of zinc that was subtracted from the samples to determine the concentration of the metal in the proteases.

Double Immunodiffusion. A standard procedure (Ouchterlony, 1958) and 1% agarose (SeaKem, Bausch and Lomb, Rochester, NY) were used. The concentration of individual proteases was 250 μg/mL. A polyvalent equine antiserum against Crotalidae venoms (Criley, 1956), antivenin, was obtained from Wyeth Laboratories, Marietta, PA. The slides were stained with Coomassie Brilliant Blue R-250 (Fairbanks et al., 1971).

Interaction of C. atrox Proteases with Plasma Protease Inhibitors. Five human plasma protease inhibitors were tested: α_1PI (M_r 54 000), AT III (M_r 65 000), α_2M (M_r 720 000), C1-INA (M_r 104 000), and α_2PI (M_r 70 000). Each plasma protease inhibitor was mixed with each protease at different molar ratios of inhibitor to enzyme and incubated at room temperature for 30 min. The control samples contained Tris–saline buffer instead of an inhibitor. The activity of proteases I and IV, in the presence of plasma inhibitor, was determined by the degradation of ^{125}I-fibrinogen and that of proteases II and III by the cleavage of Pro-Phe-Arg-pNA. The activity of proteases II and III, in the presence of α_2M, was also determined by the degradation of ^{125}I-fibrinogen. The protease activity measured in the absence of inhibitors was considered to be 100%.

Inhibition of C. atrox Proteases by Human Serum. A 1 μM solution of each protease was incubated with various amounts of human serum at room temperature for 30 min. Since human serum contained 3.6 μM α_2M (Laskowski & Kato, 1980), the highest molar ratio of α_2M in serum to the protease was 3.6, when equal volumes of serum and protease were mixed. The activity of all four proteases was determined by fibrinogenolysis assay, since the chromogenic substrate assay could not be used due to a very high absorbance of the serum at 405 nm.

Results

Purification of Anticoagulant Proteases. After anion-exchange chromatography on DEAE-cellulose, eight protein fractions were collected as indicated in Figure 1. Pools I, V, VI, and VIII degraded purified fibrinogen, rendering the substrate incoagulable by thrombin after sufficiently long incubation, and were called proteases I, II, III, and IV. When human citrated plasma was used as the substrate, only two proteases, II and III, abolished its coagulability (data not shown). It was concluded from this result that proteases I and IV may not degrade fibrinogen in plasma, probably because of inhibition by plasma protease inhibitors. Each protease eluted from the DEAE-cellulose column was concentrated by vacuum ultrafiltration, dialyzed against 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.02% NaN_3, pH 7.4, and then chromatographed on an Ultrogel AcA 54 gel filtration column. The fractions containing anticoagulant activity were pooled and analyzed for homogeneity and polypeptide chain composition by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2).

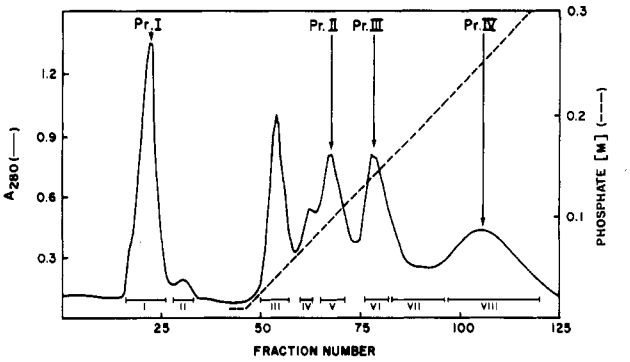

FIGURE 1: Fractionation of *C. atrox* venom on DEAE-cellulose. The elution profile of the whole venom fractionated by anion-exchange chromatography was monitored by A_{280} (—). After elution of non-bound material, pools I and II, a linear gradient of potassium phosphate buffer (---) was used for elution of bound proteins, pools III–VIII. The arrows indicate peaks containing anticoagulant proteases, Pr. I–Pr. IV. The horizontal bars below protein peaks show the fractions that were pooled for further purification.

A single polypeptide chain was found in proteases I (M_r 19 500), II (M_r 30 500), and IV (M_r 46 000). However, protease III consisted apparently of two chains, each of approximate M_r 13 000, with a total M_r of 26 500.

The molecular weight of each protease shown in Figure 2 refers to the nonreduced preparation. The mobility of reduced proteases I, II, and IV was slightly slower than that of the nonreduced counterpart, indicating that the actual molecular weights of the proteases may be slightly higher than those shown in the figure. However, we chose to give the values of the nonreduced proteases for the sake of uniformity. Additionally, the molecular weight of each protease was determined later from the amino acid composition.

Degradation of Fibrinogen. The unique feature of fibrinogen degradation by *C. atrox* venom was that only the Bβ chain was degraded when plasma was used as the substrate (Pandya et al., 1983). Having purified four proteases, we next addressed the question of which enzyme was responsible for such a specific attack on the fibrinogen molecule. Proteases I and IV cleaved purified fibrinogen virtually in the same manner, implying functional similarity of these two enzymes which attacked the Aα chain first and, after its complete degradation, then the Bβ chain (Figure 3, lower panel). The nonreduced electrophoretic patterns (Figure 3, upper panel) were very similar to those observed with the whole venom. The generation of a fibrinogen derivative of M_r 280 000, at the digestion time of 50 min, correlated with the degradation of both the Aα and Bβ chains.

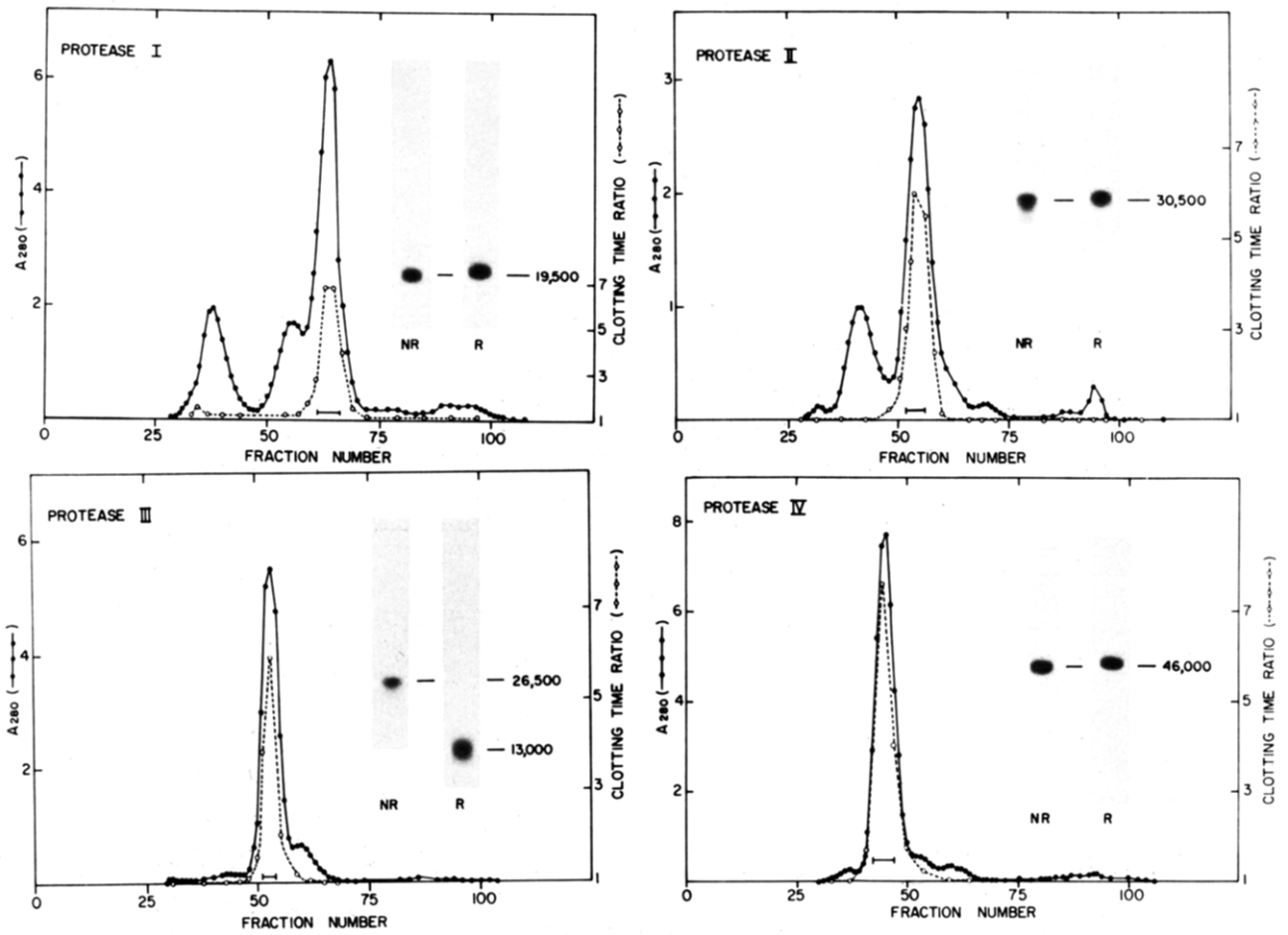

FIGURE 2: Purification of *C. atrox* anticoagulant proteases I, II, III, and IV by column gel filtration on Ultrogel AcA 54. The elution profile was monitored by A_{280} (—), and anticoagulant activity measured on fibrinogen is shown as the clotting time ratio (---). The horizontal bars under the peaks of anticoagulant activity show the fractions that were pooled and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis in nonreduced (NR) and reduced (R) forms. The insert gels show the polypeptide chain composition and molecular weights of the nonreduced enzymes calculated from the electrophoretic mobility of protein standards.

Proteases II and III had a similar mechanism of action, cleaving only the Bβ chain but leaving the Aα and γ chains apparently undegraded (Figure 4). Similar high molecular weight fibrinogen derivatives of M_r 325 000 were generated by these proteases (Figure 4, upper panel). The results were consistent with the formation of an M_r 325 000 derivative and the degradation of only the Bβ chain when fibrinogen in plasma was digested with the whole venom (Pandya et al., 1983).

Substrates and Inhibitors. A variety of synthetic oligopeptides with the chromophore group pNA covalently linked to the peptide have been tested in order to establish kinetic parameters of several serine proteases (Svendsen et al., 1972; Mattler & Bang, 1977). The chromogenic substrates have been preferred over macromolecular protein substrates, since the former provided an answer as to the specificity of the studied proteases.

Proteases I and IV did not have a high affinity for any of the chromogenic substrates tested. Protease I cleaved Pro-Phe-Arg-pNA slowly, and protease IV cleaved Suc-$(Ala)_3$-pNA at an even slower rate. Protease II hydrolyzed all substrates with a COOH-terminal arginine and lysine but did not cleave those with alanine or tyrosine, suggesting that the enzyme functioned as a serine protease with a trypsin- or plasmin-like activity. Protease III cleaved all substrates with a COOH-terminal arginine except Bz-Ile-Glu-(γ-OR)-Gly-Arg-pNA, but not those with lysine, alanine, or tyrosine (Table I). Thus, protease III also resembled a serine protease. A difference between the substrate specificity of proteases II and III was established when the cleavage of two substrates, Pro-Phe-Arg-pNA and Bz-Pro-Phe-Arg-pNA, was compared. Even though Pro-Phe-Arg-pNA was cleaved by protease III at a rate 14-fold lower than by protease II, the cleavage rates of Bz-Pro-Phe-Arg-pNA by both enzymes were essentially the same. Thus, introduction of a benzoyl group increased the hydrolysis rate for protease III by about 1 order of magnitude. The results indicated that proteases II and III have different affinities for substrates with a COOH-terminal arginine.

Table I: Substrate Specificity of *C. atrox* Proteases

chromogenic substrate	mol of pNA released min^{-1} (mol of enzyme)$^{-1}$ for protease			
	I	II	III	IV
Bz-Ile-Glu-(γ-OR)-Gly-Arg-pNA	0	4.8	0	0
Phe-Pip-Arg-pNA	0	12	1.7	0
Pro-Phe-Arg-pNA	0.4	10.1	0.7	0
Bz-Pro-Phe-Arg-pNA	0	12.8	12.0	0
Bz-Phe-Val-Arg-pNA	0	21.0	2.6	0
Tos-Gly-Pro-Arg-pNA	0	2.0	0.44	0
Val-Leu-Lys-pNA	0	17.4	0	0
Suc-$(Ala)_3$-pNA	0	0	0	0.2
Bz-Tyr-pNA	0	0	0	0

In order to evaluate which class of proteolytic enzymes the *C. atrox* proteases belong to, the effect of certain class-specific inhibitors was tested. The activity of proteases I and IV was

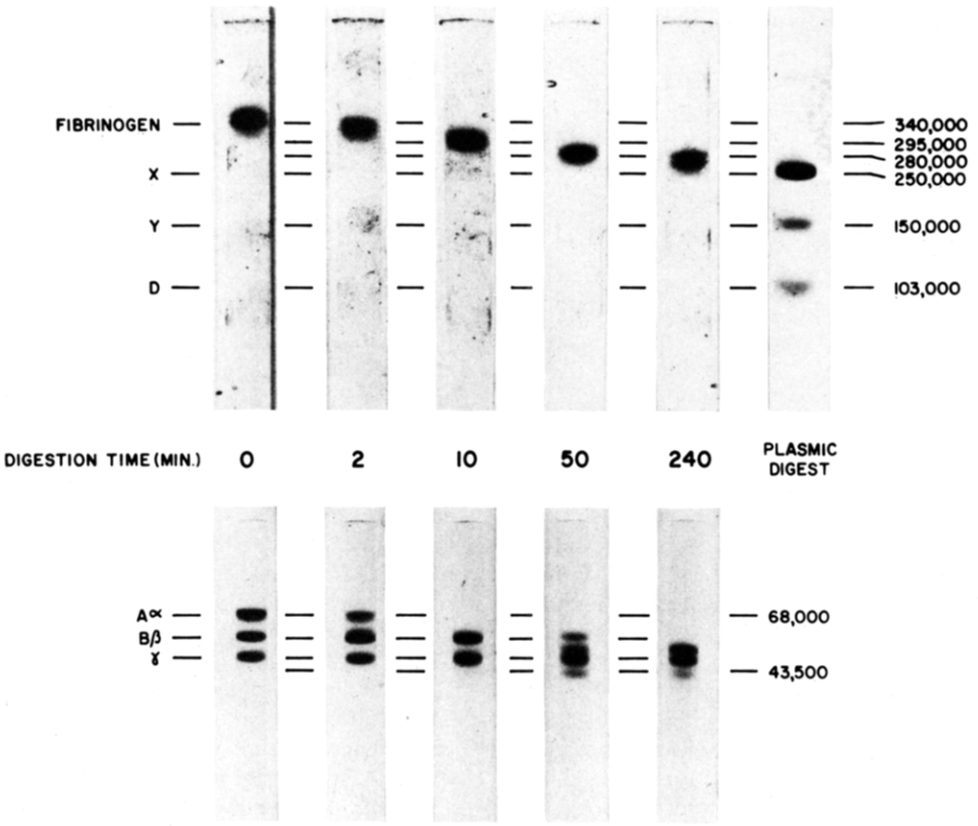

FIGURE 3: Degradation of human fibrinogen by *C. atrox* proteases. At the indicated times, an aliquot of the digest was withdrawn and mixed with 9 M urea containing 3% $NaDodSO_4$ without or with 3% 2-mercaptoethanol. The nonreduced samples (upper panel) were analyzed in 3.5% gels and the reduced samples (lower panel) in 7% gels. The composition of the original fibrinogen is shown at zero digestion time. Plasmic digest of fibrinogen contained, as protein standards for nonreduced gels, the following: fragment X (M_r 250 000); fragment Y (M_r 150 000); fragment D (M_r 103 000). Fibrinogen (M_r 340 000) at a final concentration of 1 mg/mL was digested. Protease I concentration was 12 μg/mL; essentially the same degradation patterns were obtained by using protease IV at 27 μg/mL.

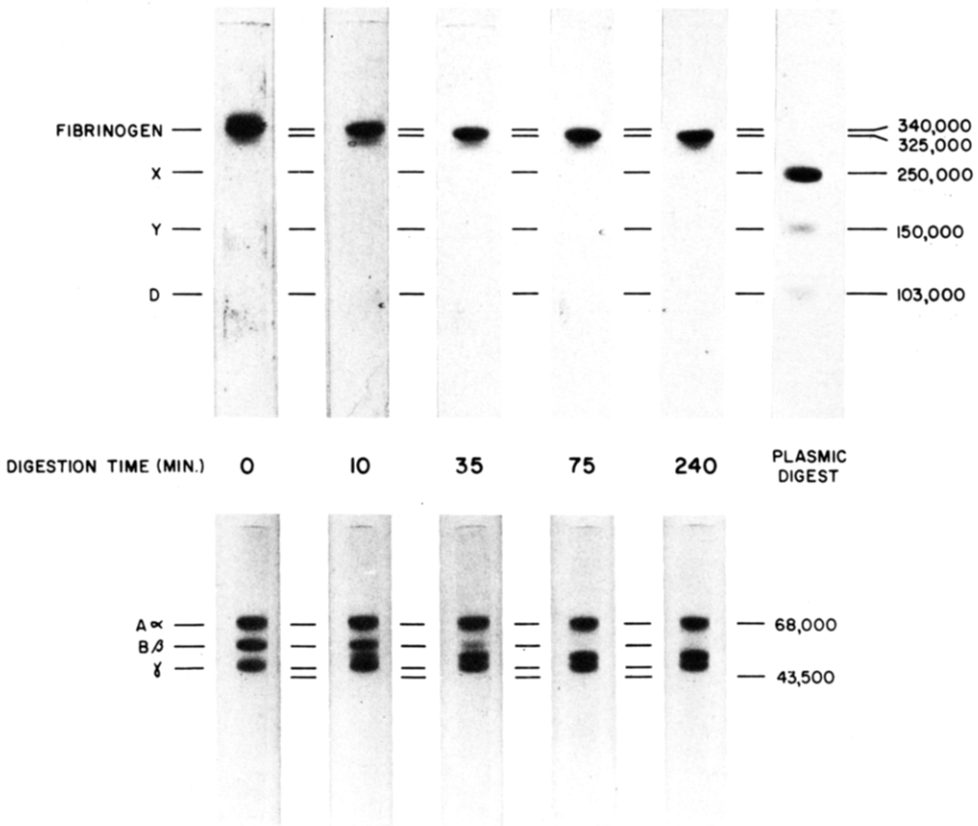

FIGURE 4: Degradation of human fibrinogen by *C. atrox* proteases. Conditions were the same as those given for Figure 3 except protease III (1 μg/mL) was used. Essentially the same degradation patterns were obtained by using protease II at 1 μg/mL.

Table II: Effect of Inhibitors on the Activity of *C. atrox* Proteases[a]

inhibitor	% activity			
	protease I	protease II	protease III	protease IV
EDTA	3.9	85.8	85.4	0.8
1,10-phenanthroline	68.9	105.0	55.4	7.1
PMSF	108.0	61.1	0.0	80.0
DFP	103.3	0.0	0.0	102.6

[a] Protease and inhibitor were mixed at final concentrations of 1.0 μM and 10 mM, respectively. The following substrates were used for the determination of the enzyme's activity: protease I, ^{125}I-fibrinogen; protease II, Pro-Phe-Arg-pNA; protease III, Bz-Pro-Phe-Arg-pNA; protease IV, ^{125}I-fibrinogen.

determined by the fibrinogenolysis assay; the activity of proteases II and III was determined by cleavage of Pro-Phe-Arg-pNA and Bz-Pro-Phe-Arg-pNA, respectively. Proteases I and IV were completely inhibited by EDTA, but not by PMSF or DFP (Table II). Thus, proteases I and IV were metalloenzymes. Protease I was not completely inhibited by 1,10-phenanthroline, but protease IV was. The hydrophobic nature of 1,10-phenanthroline may have contributed to the relative inaccessibility of the metal ion in protease I to this reagent. Proteases II and III were not inhibited by EDTA but were completely inactivated by DFP, indicating that these enzymes were serine proteases. Protease II was not inhibited by 1,10-phenanthroline, in contrast to protease III (55.4% activity remaining). PMSF inhibited protease II partially and protease III completely. Lack of complete inhibition of the former was not surprising, since PMSF, unlike DFP, does not inhibit some serine proteases.

Temperature and pH Dependence. In order to determine the optimal conditions for proteases, the enzymes were preincubated at a given temperature, the substrate was added, and proteolytic activity was measured at the same temperature. The dependence of proteases I and IV was similar, both having a maximal activity at 37 °C. Protease IV, though, still retained at 1 °C 40% of optimal activity. Proteases II and III had similar temperature-dependence curves, attaining a maximal activity in the range of 50–65 °C. At 37 °C, the activities of these two proteases were 74 and 62%, respectively, as compared to the maximal one.

The pH dependence of proteases was determined to evaluate if the enzymes were acidic, neutral, or alkaline proteases. Both proteases I and IV had an optimal pH at neutrality (Figure 5). The activities of proteases I and IV in 0.1 M sodium phosphate buffer were lower than those in sodium acetate and Tris-HCl buffers; the phenomenon was particularly evident at pH 5.5 and 7.0. This effect may be due to the inhibition of the proteases by phosphate ions, a feature observed frequently with metalloproteases. Proteases II and III had optimal pHs of 8.5 and 10.5, respectively, suggesting that these enzymes were alkaline proteases. The activity of the two enzymes was not affected by different buffers used in the experiments.

Chemical Properties. The amino acid composition of the proteases was in agreement with the net charge of each enzyme deduced from DEAE-cellulose column elution patterns since the ratio of acidic to basic residues increased in the order of proteases from I and IV. All enzymes had a high content of sulfur-containing amino acids. Protease I, the most cationic of the four enzymes, had the highest content of arginine and lysine, 11.93 residues per 100 residues (Table III). Protease III had a high proline and tryptophan content. The high content of tryptophan would account for the high absorption coefficient at 280 nm of protease III of 1.9, as compared to 0.84, 1.4, and 1.1 for proteases I, II, and IV, respectively, at 1 mg/mL. Protease IV was distinguished by the lowest content of hydroxyamino acids; in addition, it had the highest content of aspartate and a low content of arginine which would account for the most acidic nature of protease IV among the four enzymes. Protease IV had the highest content and an odd number of cysteine residues. The minimum molecular weights of the four proteases calculated from the amino acid composition, after multiplication by a factor of 2, 4, 2, and 5, respectively (Table III), were in good agreement with those determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). The molecular weights based on amino acid composition were probably slightly underestimated for proteases II and IV, since the contribution of the carbohydrate moiety has not been taken into the consideration.

Table III: Amino Acid Composition of *C. atrox* Proteases

amino acid	protease I		protease II		protease III		protease IV	
	residues/ molecule	residues/ 100 residues	residues/ molecule	residues/ 100 residues	residues/ molecule	residues/ 100 residues	residues/ molecule	residues/ 100 residues
Asp	23	13.07	32	11.27	20	10.10	76	18.72
Thr	8	4.55	18	6.34	11	5.56	16	3.94
Ser	10	5.68	21	7.39	12	6.06	15	3.69
Glu	14	7.95	28	9.86	22	11.11	43	10.59
Pro	8	4.55	16	5.63	22	11.11	32	7.88
Gly	11	6.25	23	8.10	15	7.58	28	6.90
Ala	8	4.55	17	6.00	12	6.06	18	4.43
Cys-O_3H	6	3.41	16	5.63	12	6.06	29	7.14
Val	11	6.25	19	6.69	9	4.55	20	4.93
Met	5	2.84	4	1.41	2	1.01	10	2.46
Ile	10	5.68	14	4.93	8	4.04	17	4.19
Leu	17	9.66	18	6.34	9	4.55	20	4.93
Tyr	8	4.55	8	2.82	9	4.55	17	4.19
Phe	7	3.98	12	4.23	9	4.55	11	2.71
His	7	3.98	9	3.17	5	2.53	11	2.71
Lys	11	6.25	15	5.28	10	5.05	27	6.65
Arg	10	5.68	9	3.17	5	2.53	11	2.71
Trp	2	1.14	5	1.76	6	3.03	5	1.23
total residues	176		284		198		406	
M_r[a]	20 163		31 245		22 113		45 857	

[a] Calculated from the minimum molecular weight.

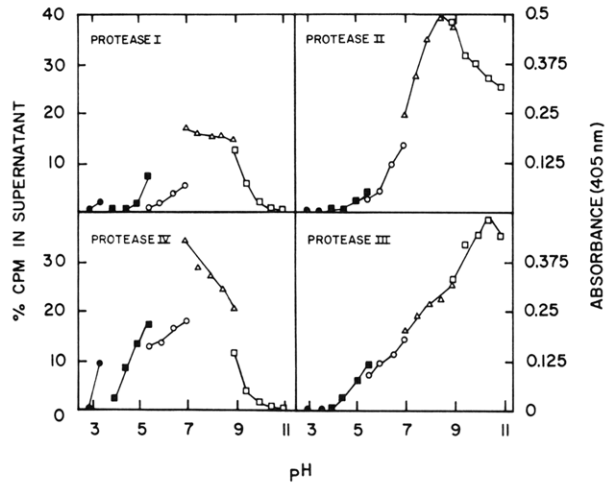

FIGURE 5: pH dependence of the proteolytic activity of *C. atrox* proteases. The activity of proteases I and IV was determined by the degradation of ^{125}I-fibrinogen (left ordinate). The activity of proteases II and III was measured by the cleavage of Pro-Phe-Arg-pNA (right ordinate). The buffers were as follows: 0.1 M glycine hydrochloride at pH 3.0 and 3.5 (●); 0.1 M sodium acetate at pH 4.0, 4.5, 5.0, and 5.5 (■); 0.1 M sodium phosphate at pH 5.5, 6.0, 6.5, and 7.0 (○); 0.1 M Tris-HCl at pH 7.0, 7.5, 8.0, 8.5, and 9.0 (△); 0.1 M glycine–NaOH at pH 9.0, 9.5, 10.0, 10.5, and 11.0 (□).

To determine which proteases contained carbohydrates, the enzymes were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis and stained with basic fuchsin. A preparation of reduced fibrinogen containing fibronectin was tested as a positive control. As expected, the Bβ and γ chains of fibrinogen (Iwanaga et al., 1968; Nishibe & Takahashi, 1981; Townsend et al., 1982; Watt et al., 1979) and the fibronectin subunit with an M_r of 220 000 were stained for carbohydrate, whereas the Aα chain of fibrinogen was not. Only proteases II and IV were stained for carbohydrate; the latter reacted more heavily. It was concluded from this experiment that proteases II and IV were glycoproteins, while proteases I and III did not seem to carry a carbohydrate moiety.

The presence of an odd number of cysteine residues in the amino acid composition of protease IV suggested that this enzyme may have at least one sulfhydryl group. It was also interesting to determine the number of these groups in all tested enzymes since some proteases do contain sulfhydryl groups (Bradshaw et al., 1969; Reeck & Neurath, 1972). Only protease IV reacted with DTNB in both the presence and absence of NaDodSO$_4$, giving 0.86 and 0.93 mol of SH per mol of enzyme, respectively. Thus, protease IV has one free sulfhydryl group, while proteases I, II, and III have all cysteine residues involved in disulfide bonds. Since protease IV showed the same reactivity with DTNB in the presence and absence of NaDodSO$_4$, it can be concluded that the sulfhydryl group in protease IV is accessible on the surface of the molecule.

The effects of class-specific inhibitors (Table II) suggested that proteases I and IV were metalloenzymes. A more definitive proof for this property was provided by the demonstration of a metal associated with proteases I and IV. Protease I had 0.85 mol of zinc per mol of the enzyme; protease IV contained 1.05 mol of zinc per mol of enzyme. Proteases II and III did not contain metal ions.

A lack of an immunochemical relationship between all four proteases was demonstrated by double immunodiffusion (Figure 6). All immunoprecipitation lines, formed between proteases I, II, III, or IV and polyvalent anti-Crotalidae antiserum, were intersected, showing the dissimilarity between all proteases and suggesting that the two pairs of proteases, I/IV and II/III, were not related as a precursor and product.

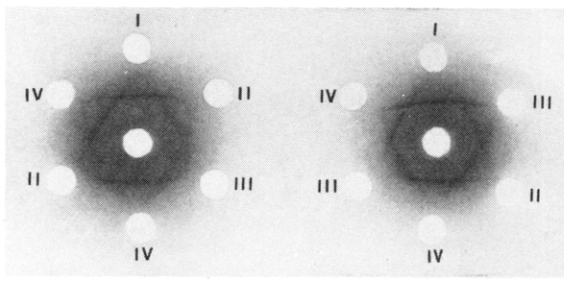

FIGURE 6: Double immunodiffusion of proteases I, II, III, and IV (outer wells) against polyvalent anti-Crotalidae antiserum (center wells).

Table IV: Effect of Plasma Protease Inhibitors on *C. atrox* Proteases I and IV

enzyme	inhibitor/protease molar ratio	% activity				
		α_1PI	AT III	α_2M	C1-INA	α_2PI
protease I	0.25	113	108	48	103	107
	0.5	107	100	10	107	107
	1.0	108	108	2	108	98
	p^a	104	103	0.5	102	
protease IV	0.25	96	99	93	97	97
	0.5	92	97	85	94	96
	1.0	98	96	58	99	80
	p^a	134	91	27	103	

[a] p designates the molar ratio of the inhibitor to the protease which would occur if normal human plasma were supplemented with 1 μM enzyme: 55.8 for α_1PI, 3.7 for AT III, 3.6 for α_2M, and 2.3 for C1-INA.

Interaction with Plasma Protease Inhibitors. Different cleavage patterns of fibrinogen in plasma and in the purified system implied that proteases I and IV were inhibited by plasma protease inhibitors, whereas proteases II and III were resistant. The effect of purified human plasma protease inhibitors on purified proteases was determined to evaluate this hypothesis.

The effect of α_1PI, AT III, α_2M, C1-INA, and α_2PI on proteases I and IV was measured at different molar ratios of inhibitor to enzyme. Protease I was inhibited only by α_2M, and the inhibition was dependent on the amount of α_2M in the reaction mixture (Table IV). Similarly, protease IV was also inhibited only by α_2M in a concentration-dependent manner, but its activity was less affected than that of protease I. At a molar ratio of α_2M to the enzyme of 1.0, protease I was completely inhibited, whereas there was only 42% inhibition of protease IV.

The effect of the same plasma protease inhibitors on the activity of proteases II and III was also determined, and it was found that none had any effect. This was true even when the molar ratio of the inhibitor to the protease was as high as would be expected to be achieved in plasma if 1 μM protease were present. This ratio was calculated to be 55.8, 3.7, 3.6, 2.3, and 1.0 for α_1PI, AT III, α_2M, C1-INA, and α_2PI, respectively. The lack of inhibition of proteases II and III by α_2M was not conclusively proven in this experiment, since the proteases were assayed by the cleavage of a chromogenic substrate, rather than a macromolecular substrate like fibrinogen. Therefore, the effect of α_2M was tested by the fibrinogenolysis assay. Protease III activity was not affected, but the inhibition of protease II was proportional to the concentration of α_2M, and at a molar ratio of α_2M to the protease of 3.6, there was 40% inhibition. Thus, protease II was partially inhibited by α_2M although to a much lesser extent than either protease I or protease IV.

Inhibition by Human Serum. Human serum contains plasma protease inhibitors but does not contain fibrinogen, the substrate for proteases. Therefore, the effect of human serum on their activity was tested. A 1 μM solution of each protease was incubated with various amounts of human serum (pH 7.4) at room temperature for 30 min. Assuming that serum contained essentially the same amount of α_2M as plasma, the molar ratio of α_2M in serum to the protease was varied. The protease activity was determined by the fibrinogenolysis assay. At a molar ratio of α_2M in serum to the protease of 3.6, proteases I and IV were completely inhibited, protease II was partially inactivated (40% inhibition), and protease III was not affected. The results confirmed those obtained with purified plasma protease inhibitors, and there was an excellent correlation with the data shown in Table IV.

Discussion

The venom of the western diamondback rattlesnake, *C. atrox*, contains anticoagulant proteases which render both plasma and purified fibrinogen incoagulable (Figures 1 and 2). This fact is in contrast with most rattlesnakes whose venoms have potent clotting rather than fibrinolytic action on blood. Our observation corroborates the data of other investigators who did not find fibrinogen-clotting enzyme in *C. atrox* venom (Denson, 1969; Deutsch & Diniz, 1955) and showed that the venom contained a variety of proteases, including fibrinolytic enzymes (Bajwa et al., 1980, 1981; Bjarnason & Tu, 1978; Man & Minta, 1977; Pfleiderer & Sumyk, 1961).

Snake Venom Metalloproteases. It has been shown in this study that proteases I and IV are neutral metalloendoproteases that cleave the Aα chain of fibrinogen first and then the Bβ chain. Enzymes with similar chemical and enzymatic properties have recently been isolated from the venom of *A. contortrix mokasen* (Moran & Geren, 1981), *A. acutus* (Ouyang & Huang, 1976), *T. mucrosquamatus* (Ouyang & Teng, 1976; Ouyang et al., 1977), and *T. gramineus* (Ouyang & Huang, 1979). These enzymes are metalloproteases with an M_r of 22 000 ± 2000, similar to that of *C. atrox* protease I. Since *Agkistrodon*, *Trimeresurus*, and *Crotalus* genera belong to the same subfamily, Crotalinae, the presence of zinc-containing neutral metalloendoproteases in venom may be a common feature of snakes belonging to this subfamily.

Five hemorrhagic toxins designated *a*, *b*, *c*, *d*, and *e* with molecular weights of 68 000, 24 000, 24 000, 24 000, and 25 700, respectively, have been isolated from *C. atrox* venom (Bjarnason & Tu, 1978). All toxins were proteases containing one molecule of zinc per molecule of the enzyme. The isolation in this work of only two zinc-containing anticoagulant metalloproteases, proteases I and IV, can most probably be attributed to the inability of all five hemorrhagic toxins to cleave fibrinogen and render it incoagulable. Since the hemorrhagic toxins *a*, *c*, and *d* were present in the venom at very low concentrations, these may not be detected by the anticoagulant activity assay. Protease I (mean M_r 20 000) is similar in many respects to the hemorrhagic toxin *b*, being an abundant basic protein (Figure 1) and zinc-containing metalloenzyme with the absence of sulfhydryl groups, of comparable molecular weight (Figure 2 and Table III), and having a well-correlated amino acid composition (Table III). Protease I is probably the same enzyme as the fibrinolytic protease of M_r 21 500 (Bajwa et al., 1980, 1981) and the α-protease with caseinolytic activity (M_r 23 000) that was isolated by Pfleiderer & Sumyk (1961) 22 years ago. α-Protease had a specificity for cleaving peptide bonds at the NH_2-terminal side of leucine and isoleucine (Pfleiderer & Krauss, 1965), and protease I did not cleave well any chromogenic substrates (Table I). Protease I is similar to complement-inactivating protease Q1 (Man & Minta, 1977) as both have very similar molecular weights and are inhibited by EDTA.

Protease IV is dissimilar to all enzymes isolated from *C. atrox* venom and described previously. The protease differs significantly from all *C. atrox* hemorrhagic toxins, especially toxin *a* with an M_r of 68 000 (Bjarnason & Tu, 1978), by having an M_r of 46 000 (Figure 2 and Table III), a dissimilar amino acid composition (Table III), a carbohydrate moiety, and a single sulfhydryl group. Since both proteases I and IV are inhibited by α_2M (Table IV) and serum, it seems that in envenomated humans the apparent anticoagulant effect resulting from the two metalloproteases will be minor.

Snake Venom Serine Proteases That Cleave the Bβ Chain of Fibrinogen. Many proteases cleave preferentially in fibrinogen the COOH-terminal of the Aα chain. A novel observation in this work is the selective cleavage of the Bβ chain by proteases II and III (Figure 4) which, in contrast to proteases I and IV, are alkaline serine proteases (Figure 5 and Table II). Proteases II and III do not resemble the enzyme from *A. contortrix contortrix* venom which cleaves from fibrinogen fibrinopeptide B at a higher rate than fibrinopeptide A (Herzig et al., 1970).

The *C. atrox* venom has been shown to contain a trypsin-like activity determined by the cleavage of Tos-Arg methyl ester; this activity did not degrade collagen (Simpson, 1971) and was inhibited by DFP, but not EDTA (Simpson, 1972). In retrospect, it seems evident that the trypsin-like activity of the venom was due to proteases II and III. The failure of the earlier workers to isolate these enzymes can be attributed to the unique specificity. Among the many substrates tested, proteases II and III cleaved rapidly only the Bβ chain of fibrinogen, having a very potent anticoagulant effect on fibrinogen and plasma.

The evidence supporting the view that proteases II and III are serine proteases was provided by the following observations. Both enzymes cleaved chromogenic substrates at the COOH-terminal side of arginine and lysine, and arginine, respectively (Table I), were inhibited by DFP, but not EDTA (Table II), and did not contain zinc. Since proteases II and III did not have any sulfhydryl groups, they do not fit into the category of sulfhydryl proteases.

Proteases II and III are characterized by a high optimal temperature in the range of 50–65 °C. Interestingly, β-fibrinogenase, but not α-fibrinogenase, of *T. gramineus* venom has been shown to be remarkably heat resistant (Ouyang & Huang, 1979). By comparison, the optimal temperature for *C. atrox* proteases I and IV is 37 °C.

The venoms of *T. mucrosquamatus* and *T. gramineus* contain a protease, called β-fibrinogenase, that cleaves the Bβ chain of fibrinogen (Ouyang & Huang, 1979; Ouyang & Teng, 1976; Ouyang et al., 1977) in addition to the Aα chain cleaving metalloprotease. Like *C. atrox* proteases II and III, β-fibrinogenases are sensitive to inhibition by PMSF, but not EDTA, and thus probably are serine proteases. The presence of two different classes of fibrinogenolytic enzymes in the venoms of *C. atrox*, *T. gramineus*, and *T. mucrosquamatus* suggests that the search for Bβ chain cleaving enzymes in venoms of other snakes from the Crotalinae subfamily may be quite rewarding. An important difference between the venoms of *C. atrox* and *Trimeresurus* genera is that in this study two Aα chain cleaving enzymes, proteases I and IV, and two Bβ chain cleaving enzymes, proteases II and III, have been isolated from *C. atrox* venom, while the *Trimeresurus* venoms

seem to contain only one α-fibrinogenase and one β-fibrinogenase.

Regulation of Snake Venom Proteases by Plasma Protease Inhibitors. The question of the regulatory functions of plasma protease inhibitors on *C. atrox* proteases was addressed, since it was important for the understanding of the response to envenomation in vivo (Budzynski et al., 1984). In view of our data showing that in serum or plasma proteases I and IV were inactivated by plasma protease inhibitors, but proteases II and III were active (Table IV), it can be concluded that most probably α-fibrinogenases of *A. contortrix mokasen* (Moran & Geren, 1981), *T. mucrosquamatus* (Ouyang & Teng, 1976; Ouyang et al., 1977), and *T. gramineus* (Ouyang & Huang, 1979) and the fibrinolytic enzymes of *A. acutus* (Ouyang & Huang, 1976) and *C. atrox* (Bajwa et al., 1980, 1981), all of which are metalloendoproteases, would be inhibited by plasma protease inhibitors, most likely by α_2M. On the other hand, it seems probable that β-fibrinogenases of *T. mucrosquamatus* and *T. gramineus* may be resistant to inhibition in plasma.

Kress and colleagues have recently shown that metalloendoproteases from *C. adamanteus* venom, called proteinases I and II, catalytically inactivated human α_1PI (Kress et al., 1979; Kurecki et al., 1978) and AT III (Kress & Catanese, 1981a) by a limited proteolytic cleavage. The inactivation of α_1PI by *C. adamanteus* proteinase II is probably not a physiological process, since these studies have been performed with purified inhibitors, wherein α_2M, a most probable inhibitor of the protease, was absent. The resistance of proteases II and III against inhibition by α_1PI and AT III does not seem to be due to inactivation of the inhibitors by proteases. The experiments with purified protease inhibitors showed that proteases II and III were not affected at all.

The total inhibition of proteases I and IV and the partial inhibition of protease II by human α_2M (Table IV) are in agreement with a report showing a complete inhibition of the collagenolytic activity of *C. atrox* venom by α_2M (Werb et al., 1974). Also, the proteolytic activity of all venoms of the Crotalidae family is inhibited by α_2M (Kress & Catanese, 1981b).

Recently, an arginine ester hydrolase (a trypsin-like enzyme) has been separated from a caseinolytic and thrombin-like enzyme present in *C. adamanteus* venom. In agreement with our observation that trypsin-like enzymes from *C. atrox* venom, proteases II and III, are not inhibited by α_1PI, the arginine ester hydrolase of *C. adamanteus* venom is also not inhibited by α_1PI inhibitor (Kress et al., 1978).

Proteases II and III render fibrinogen incoagulable by the cleavage of an M_r 5000 peptide from the Bβ chain. The loss of coagulability may be associated with the cleavage of a fibrin polymerization site (Olexa & Budzynski, 1980) in the fibrinogen molecule. The isolation and characterization of the peptide cleaved from the Bβ chain may provide further insight into the mechanism of fibrin clot assembly. Along this investigative line, proteases II and III may serve as important experimental tools to study the correlation of structure with function of the fibrinogen molecule.

Registry No. *Crotalus atrox* venom metalloproteinase, 86161-99-1; *Crotalus atrox* venom serine proteinase, 87434-92-2.

References

Anderson, W. L., & Wetlaufer, D. B. (1975) *Anal. Biochem. 67*, 493–502.

Bajwa, S. S., Markland, F. S., & Russell, F. E. (1980) *Toxicon 18*, 285–290.

Bajwa, S. S., Markland, F. S., & Russell, F. E. (1981) *Toxicon 19*, 53–59.

Bjarnason, J. B., & Tu, A. T. (1978) *Biochemistry 17*, 3395–3404.

Bonilla, C. A. (1975) *Thromb. Res. 6*, 151–169.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bradshaw, R. A., Ericsson, L. H., Walsh, K. A., & Neurath, H. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1389–1394.

Budzynski, A. Z., Pandya, B. V., Rubin, R. N., Brizuela, B. S., Soszka, T., & Stewart, G. J. (1984) *Blood 63*, 1–14.

Criley, B. R. (1956) in *Venoms* (Buckley, E. E., & Porges, N., Eds.) pp 373–380, American Association for the Advancement of Science, Washington, DC.

Denson, K. W. E. (1969) *Toxicon 7*, 5–11.

Deutsch, H. F., & Diniz, C. R. (1955) *J. Biol. Chem. 216*, 17–26.

Doolittle, R. F. (1973) *Adv. Protein Chem. 27*, 1–109.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Esnouf, M. P., & Tunnah, G. W. (1967) *Br. J. Haematol. 13*, 581–590.

Evenson, M. A., & Anderson, C. T., Jr. (1975) *Clin. Chem. (Winston-Salem, N.C.) 21*, 537–543.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Herzig, R. H., Ratnoff, O. D., & Shainoff, J. R. (1970) *J. Lab. Clin. Med. 76*, 451–465.

Iwanaga, S., & Suzuki, T. (1979) in *Snake Venoms* (Lee, C. Y., Ed.) pp 61–158, Springer-Verlag, New York.

Iwanaga, S., Blombäck, B., Gröndahl, N. J., Hessel, B., & Wallén, P. (1968) *Biochim. Biophys. Acta 160*, 280–283.

Knight, L. C., Budzynski, A. Z., & Olexa, S. A. (1981) *Thromb. Haemostasis 46*, 593–596.

Kress, L. F., & Catanese, J. J. (1981a) *Biochemistry 20*, 7432–7438.

Kress, L. F., & Catanese, J. J. (1981b) *Toxicon 19*, 501–507.

Kress, L. F., DaRoza, D., & Laskowski, M., Sr. (1978) *Toxins: Anim., Plant Microb., Proc. Int. Symp., 5th*, 141–146.

Kress, L. F., Kurecki, T., Chan, S. K., & Laskowski, M., Sr. (1979) *J. Biol. Chem. 254*, 5317–5320.

Kurecki, T., Laskowski, M., Sr., & Kress, L. F. (1978) *J. Biol. Chem. 253*, 8340–8345.

Laskowski, M., Jr., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593–626.

Man, D. P., & Minta, J. O. (1977) *Immunochemistry 14*, 521–527.

Mancini, G. A., Carbonara, O., & Heremans, J. (1965) *Immunochemistry 2*, 235–254.

Markland, F. S., & Damus, P. S. (1971) *J. Biol. Chem. 246*, 6460–6473.

Marsh, N. A., & Whaler, B. C. (1974) *Br. J. Haematol. 26*, 295–306.

Mattler, L. E., & Bang, N. U. (1977) *Thromb. Haemostasis 38*, 776–792.

McGuckin, W. F., & McKenzie, B. F. (1953) *Clin. Chem. (Winston-Salem, N.C.) 4*, 476–483.

McKee, P. A., Rogers, L. A., Marler, E., & Hill, R. L. (1966) *Arch. Biochem. Biophys. 116*, 271–279.

Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.

Moran, J. B., & Geren, C. R. (1981) *Biochim. Biophys. Acta 659*, 161–168.

Nishibe, H., & Takahashi, N. (1981) *Biochim. Biophys. Acta 661*, 274–279.

Olexa, S. A., & Budzynski, A. Z. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1374–1378.

Ouchterlony, Ö. (1958) *Prog. Allergy 5*, 1–78.

Ouyang, C., & Yang, F. Y. (1974) *Biochim. Biophys. Acta 351*, 354-363.
Ouyang, C., & Huang, T. (1976) *Biochim. Biophys. Acta 439*, 146-153.
Ouyang, C., & Teng, C. (1976) *Biochim. Biophys. Acta 420*, 298-308.
Ouyang, C., & Huang, T. (1979) *Biochim. Biophys. Acta 571*, 270-283.
Ouyang, C., Hong, I. S., & Teng, C. M. (1971) *Thromb. Diath. Haemorrh. 24*, 224-234.
Ouyang, C., Teng, C., & Chen, Y. (1977) *Biochim. Biophys. Acta 481*, 622-630.
Pandya, B. V., Rubin, R. N., Olexa, S. A., & Budzynski, A. Z. (1983) *Toxicon 21*, 515-526.
Pfleiderer, G., & Sumyk, G. (1961) *Biochim. Biophys. Acta 51*, 482-493.
Pfleiderer, G., & Krauss, A. (1965) *Biochem. Z. 342*, 85-94.
Reeck, G. R., & Neurath, H. (1972) *Biochemistry 11*, 3947-3955.
Reid, H. A., & Theakston, R. D. G. (1978) *Am. J. Trop. Med. Hyg. 27*, 1053-1057.
Simpson, J. W. (1971) *Comp. Biochem. Physiol. B 40B*, 633-636.
Simpson, J. W. (1972) *Int. J. Biochem. 3*, 243-245.
Stocker, K., & Egberg, N. (1973) *Thromb. Diath. Haemorrh., Suppl. No. 54*, 361-370.
Svendsen, L., Blombäck, B., Blombäck, M., & Olsson, P. I. (1972) *Thromb. Res. 1*, 267-278.
Townsend, R. R., Hilliker, E., Li, Y., Laine, R. A., Bell, W. R., & Lee, Y. C. (1982) *J. Biol. Chem. 257*, 9704-9710.
Watt, K. W. K., Takagi, T., & Doolittle, R. F. (1979) *Biochemistry 18*, 68-76.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406-4412.
Werb, Z., Burleigh, M. C., Barret, A. J., & Starkey, P. M. (1974) *Biochem. J. 139*, 359-368.
Zacharius, R. M., Zell, T. E., Morrison, J. H., & Woodlock, J. J. (1969) *Anal. Biochem. 30*, 148-152.

Structure of the Divalent Metal Ion Activator Binding Site of *S*-Adenosylmethionine Synthetase Studied by Vanadyl(IV) Electron Paramagnetic Resonance†

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ABSTRACT: The structure of the divalent metal ion binding site of *S*-adenosylmethionine synthetase from *Escherichia coli* has been studied by using the vanadyl(IV) ion (VO^{2+}) as probe. VO^{2+} binds at a single site per subunit in the presence or absence of substrates. Single turnover experiments measuring *S*-adenosylmethionine (AdoMet) formation from methionine and the ATP analogue 5'-adenylyl imidodiphosphate show that complexes containing VO^{2+} and either Mg^{2+} or Ca^{2+} as a second metal ion are catalytically active, while a complex containing VO^{2+} alone is inactive. Electron paramagnetic resonance spectra of the enzyme–VO^{2+} complex, as well as complexes also containing AdoMet or methionine, indicate the coordination of two water molecules and at least two protein ligands to the VO^{2+}. In complexes with polyphosphate substrates or products (e.g., enzyme–VO^{2+}–ATP–methionine, enzyme–VO^{2+}–PP_i–Mg^{2+}), EPR spectral changes reveal ligand substitutions on the VO^{2+}, and 8.5-G isotropic superhyperfine coupling to two ^{31}P nuclei can be resolved. ^{17}O superhyperfine coupling from [^{17}O]pyrophosphate indicates coordination of two oxygen atoms of PP_i to the VO^{2+} ion. Thus the polyphosphate compounds are bidentate ligands to the VO^{2+}, demonstrating that the VO^{2+} binds at the active site and suggesting a catalytic role for the protein-bound metal ion.

S-Adenosylmethionine is the primary alkylating agent in biological systems and occupies a central role in cellular metabolism (Cantoni, 1975; Tabor & Tabor, 1976). The biosynthesis of *S*-adenosylmethionine (AdoMet)[1] occurs in a unique enzymatic reaction catalyzed by *S*-adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase) (Mudd, 1973). In the biosynthetic reaction, a substitution at C5' of the ribose of ATP results in formation of the sulfonium compound AdoMet; the tripolyphosphate formed is then hydrolyzed to pyrophosphate and orthophosphate before products are released (Mudd, 1963; Markham et al., 1980). The tetrameric AdoMet synthetase from *Escherichia coli* requires two divalent metal ions (e.g., Mg^{2+}, Mn^{2+}, Ca^{2+}) as well as a single monovalent cation (e.g., K^+) per active site for catalytic activity (Markham et al., 1980; Markham, 1981). One of the divalent metal ion activators binds to the protein in the absence of substrates, while the second divalent metal ion binds in conjunction with the nucleotide substrate or the product PP_i. EPR studies using Mn^{2+}, which binds at both sites, showed that in complexes of enzyme with Mn^{2+}, AdoMet, and either the product PP_i or imidotriphosphate (an analogue of the tripolyphosphate intermediate) the two metal ions were coupled by spin exchange (Markham, 1981). The exchange coupling

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AMPPNP, 5'-adenylyl imidodiphosphate; PPNP, imidotriphosphate (O_3P–O–PO_2–NH–PO_3); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PP_i, pyrophosphate; PPP_i, tripolyphosphate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

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