Degradation of Coagulation Proteins by an Enzyme from Malayan Pit Viper (*Akistrodon rhodostoma*) Venom†

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ABSTRACT: Three hydrolases from the crude venom of the Malayan pit viper (*Akistrodon rhodostoma*) can be differentiated. The first, which we designate ARHα, is the well-known fibrinogenolytic enzyme ancrod. The second, ARHβ, which has not been described previously, is identified by its electrophoretic mobility after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), by its ability to hydrolyze H-D-phenylalanyl-L-piperyl-L-arginyl-*p*-nitroanilide, and by inhibition of its activity by diisopropyl phosphorofluoridate. The third, ARHγ, also previously not described, has been purified by using gel permeation and ion-exchange chromatography and preparative PAGE. Chemical, electrophoretic, and hydrodynamic data indicate that it is a single-chain, nonglobular glycoprotein with a molecular weight of 25 600. ARHγ catalyzes the degradation of several plasma vitamin K dependent coagulation factors, including factor IX, factor X, prothrombin, and protein C. The products are electrophoretically similar to factor IXaβ, factor Xa, thrombin, and activated protein C, respectively. However, these products contain little or no enzymatic activity. ARHγ-degraded factor IX, factor X, prothrombin, and protein C can be subsequently activated by factor XIa, Russell's viper venom X coagulant protein, crude taipan snake venom, and thrombin, respectively. The N-terminal sequence of the peptides resulting from the ARHγ digest of porcine factor IX shows that at least three bonds are hydrolyzed: (1) at position 152, seven residues from the Arg145–Ala146 factor XIa cleavage site; (2) at position 167 within the factor IX activation peptide; and (3) at position 177, three residues from the Arg180–Val181 factor XIa cleavage site. The degradation of factor IX by ARHγ is not affected by several serine protease inhibitors. ARHγ catalyzes the degradation of both the heavy and light chains of porcine factor VIII which results in the inability of thrombin to activate factor VIII. ARHγ also catalyzes the degradation of porcine antithrombin III which abolishes its ability to inhibit thrombin. These findings may have relevance to studies of hemostatic derangements following envenomation by this snake. Additionally, several novel coagulation factor derivatives have been generated for structure–function studies.

Many snake venoms contain enzymes that are capable of catalyzing the hydrolysis of specific bonds in coagulation proteins. This proteolytic degradation of coagulation proteins usually occurs at or near physiologic cleavage sites. In the Viperidae family, viperine vipers (e.g., Russell's viper and *Echis carinatus*) contain venoms that are known for their effect on coagulation zymogens and procofactors such as prothrombin, factor X, and factor V, whereas the crotalin vipers (e.g., rattlesnakes and the *Akistrodon* genus) are known for their fibrinogenolytic effects (Reid, 1984). Additionally, venoms from several families have been identified that cleave and inactivate antithrombin III (AT-III)[1] (Kress & Catanese, 1980).

The Malayan pit viper (*Akistrodon rhodostoma*) can cause serious hemorrhage and occasionally death after biting human beings. The predominant effect of its venom has been attributed to an enzyme that catalyzes the removal of fibrinopeptide A from fibrinogen with subsequent clot formation (Ewart et al., 1970). The enzyme, named ancrod, has been isolated (Esnouf, 1967). Because of its fibrinogenolytic properties in vivo, it is currently used as an anticoagulant in the prevention and treatment of venous thrombosis.

We now show that, in addition to its effects on fibrinogen, crude Malayan pit viper venom contains both an amidolytic activity toward H-D-phenylalanyl-L-piperyl-L-arginyl-*p*-nitroanilide (S2238) and a proteolytic activity toward several coagulation proteins. Two enzymes, distinct from ancrod, are described which are separately responsible for these activities.

Experimental Procedures

Materials. Crude venoms, bovine factor VII and factor X deficient plasma, ovalbumin, BAEE, lysozyme, avidin, myoglobin, and CH-Sepharose 4B were purchased from Sigma Chemical Co. (St. Louis, MO). Guanidine hydrochloride was purchased from Heico Division, Whittaker Corp. (Delaware

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SDS–PAGRE, SDS–polyacrylamide gel radioelectrophoresis; ARH, *Akistrodon rhodostoma* hydrolase; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DFP, diisopropyl phosphorofluoridate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; QAE, quaternary aminoethyl; RVV-XCP, Russell's viper venom factor X coagulant protein; AT-III, antithrombin III; TBS, Tris-buffered saline (0.15 M NaCl/0.02 M Tris-HCl, pH 7.4); TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; S2238, H-D-phenylalanyl-L-piperyl-L-arginyl-*p*-nitroanilide; MES, 2-(*N*-morpholino)ethanesulfonic acid; HPLC, high-performance liquid chromatography; kDa, kilodalton(s); piperyl, piperidinylcarbonyl.

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Water Gap, PA). [^{3}H]DFP and EN^{3}HANCE were purchased from NEN Research Products, Du Pont, Inc. (Boston, MA). NPGB was purchased from United States Biochemical Corp. (Cleveland, OH). Activated partial thromboplastin was purchased from General Diagnostics (Morris Plains, NJ). Factor IX, factor VIII, and factor XI deficient plasmas were purchased from George King Biomedical, Inc. (Overland Park, KS). QAE-cellulose was purchased from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin, malate dehydrogenase, and β-lactoglobulin were purchased from Serva (Westbury, NY). S2238 was purchased from Helena Laboratories (Beaumont, TX). Porcine factor VIII concentrate was purchased from Porton Products (Encino, CA). Ancrod was from Connaught Laboratories (Willowdale, Ontario).

Coagulation Proteins. Porcine factors IX, IXaβ, and X, prothrombin, thrombin, and human factor XIa were prepared as described previously (Lollar et al., 1984). Porcine factor VIII was isolated from commercial concentrate by anti-factor VIII affinity chromatography (Fass et al., 1982). Human fibrinogen was purified from fresh frozen plasma (Straughn & Wagner, 1966). The factor X activator (RVV-XCP) from crude Russell's viper venom was isolated exactly as described (Kisiel et al., 1976) and was coupled to CH-Sepharose 4B before use according to instructions provided by the supplier. Porcine AT-III was prepared from the supernatant of barium citrate adsorbed plasma by heparin–Sepharose chromatography (Owen, 1975). It was greater than 95% pure as judged by SDS–PAGE.

The isolation of porcine protein C follows the same procedure as for other porcine vitamin K dependent proteins through chromatography on QAE-Sephadex (Lollar et al., 1984). Protein C emerges from this column as a shoulder prior to the prothrombin peak. Fractions containing protein C activity were pooled, dialyzed against 0.05 M imidazole hydrochloride, 1 mM $CaCl_2$, and 1 mM benzamidine, pH 6.0, and applied to a column (1.5 × 30 cm) of heparin–Sepharose equilibrated in the same buffer. Approximately 60 mg of protein was applied to the column from a preparation derived from 10 L of porcine plasma. The composition of this mixture was approximately 50% protein C and 50% prothrombin. Proteins were eluted with a 0–0.4 M NaCl gradient in the same buffer. Protein C eluted as the last peak from the column and was approximately 90% pure as judged by SDS–PAGE. The fractions containing protein C activity were pooled, dialyzed against 0.01 M MES, 0.01 M Tris, 0.15 M NaCl, 2.5 mM Ca^{2+}, and 1 mM benzamidine, pH 6.0, and applied to a dextran sulfate–agarose column (1.5 × 30 cm) equilibrated in the same buffer. Protein C was eluted with a 0.15–0.75 M NaCl gradient (Kisiel, 1979) and emerged at approximately 0.4 M NaCl. Analysis of the preparation by SDS–PAGE revealed that greater than 95% of the protein consisted of a doublet with apparent molecular weights of 51 000 and 53 000. Reduction with β-mercaptoethanol revealed a light chain with molecular weight 21 000 and two heavy chains with molecular weights of 37 000 and 39 000. Approximately 2 mg of protein C was obtained per liter of starting plasma. Protein C was stored at 4 °C in the eluting buffer.

Venom-degraded factor IX was prepared by reacting 14 μM factor IX with 0.24 μM ARHγ (defined under Results) in TBS and 5 mM $CaCl_2$ for 24 h. The reaction volume was 17 mL. The ARHγ-degraded factor IX then was isolated by heparin–Sepharose chromatography using the conditions described previously for the preparation of porcine factor IXaβ. In a parallel experiment, a mixture of factor IX and factor IXaβ resulting from partial activation by factor XIa was chromatographed on a separate column of heparin–Sepharose under identical conditions. The elution position of factor IX is ahead of factor IXaβ. The elution position of ARHγ-degraded factor IX was identical with factor IXaβ. ARHγ-degraded factor IX was either used immediately or stored in aliquots at −80 °C in TBS.

Isolation of Venom Proteins. Crude, lyophilized Malayan pit viper venom (500 mg), lot 92F-0311, was dissolved in 0.05 M NaCl, 0.01 M histidine, 5 mM $CaCl_2$, and 1 mM benzamidine, pH 6.0, to a final concentration of 170 mg/mL. The solution was applied to a column (1.5 × 75 cm) of G-75 Sephadex equilibrated in the same buffer at room temperature. The flow rate was controlled at 30 mL/h by a pump. Fractions (2 mL) were collected and assayed for fibrinogen clotting activity, amidase activity toward S2238, and proteolytic activity. Proteolytic activity was monitored by adding samples to factor IX (0.2 mg/mL) in TBS and 5 mM $CaCl_2$ for 1 h at 22 °C followed by SDS–PAGE. The elution volumes of the proteolytic and amidase activity were identical and were greater than the fibrinogen clotting activity. Fractions containing the activities were pooled and dialyzed against 0.01 M Tris–phosphate, pH 8.5 (molarity refers to Tris). Fractions containing fibrinogen clotting activity also were pooled separately and dialyzed against the same buffer. Both samples were applied at a flow rate of 50 mL/h at room temperature to separate columns (2.5 × 30 cm) of QAE-cellulose equilibrated in dialysis buffer. The columns were washed with 0.01 M Tris–phosphate, pH 7.0, at a flow rate of 200 mL/h until the absorbance at 280 nm had leveled, and then the proteins were eluted with a 0.01 M Tris–phosphate (pH 7.0)/0.2 M Tris–phosphate, pH 6.0, gradient. Fractions containing fibrinogen clotting activity were pooled and stored at 4 °C for further analysis.

Proteolytic and amidase activities again emerged together from the QAE-cellulose column. Fractions that contained the activities were pooled and concentrated by dialysis into 80% saturated ammonium sulfate at 4 °C. The precipitate was collected by centrifugation at 20000*g* for 30 min. The pellet was dissolved in a minimal volume of 0.025 M Tris–borate, pH 8.3, and dialyzed against the same buffer at 4 °C. The enzyme that contained amidase activity, designated ARHβ, could be inhibited by DFP. Therefore, ARHβ was identified in the QAE-cellulose pool by SDS–PAGE using [^{3}H]DFP as follows. The inhibitory reaction was carried out in TBS at 22 °C with 0.2 mg/mL of the QAE-cellulose pool and 10 μM [^{3}H]DFP (50 000 dpm/mL). 2-Propanol was used in a separate control reaction. Greater than 95% of the amidase activity was inhibited at 16 h. Then the sample was prepared for SDS–PAGRE (see below).

The enzyme possessing proteolytic activity, designated ARHγ, was isolated by preparative electrophoresis. Glycerol was added to a final concentration of 5% (v/v) along with a trace of bromophenol blue to the QAE-cellulose pool. The resulting solution, approximately 2 mL, 10 mg/mL, was layered over 60 mL (6-cm height) of 7.5% polyacrylamide in 0.05 M Tris–borate, pH 8.3, in a custom-made preparative electrophoresis apparatus (Nesheim, 1978) containing the same buffer in the upper and lower reservoirs. Electrophoresis was carried out at 4 °C at 10 W. After the dye had passed through the gel, 3-mL fractions were collected at 50 mL/h. Fractions were assayed for amidase activity (Figure 1A) and proteolytic activity. The fractions were also analyzed by SDS–PAGE (Figure 1B).

Fractions containing no visible ARHβ by SDS–PAGE of at least 20 μg of material were pooled, were dialyzed either

against TBS or against 0.05 M Tris–borate, pH 8.3, and were considered suitable for physical, chemical, and functional characterization. Aliquots of some of the material were stored at −80 °C, and the enzymatic activity remained after thawing. Alternatively, ARHγ retained enzymatic activity after storage in TBS at 4 °C for at least 1 month.

Assays. Clotting assays for factors VIII, IX, X, and XIa, thrombin, and thrombin-like activity of venom as well as BAEE esterase activity of factor IXa have been described previously (Lollar et al., 1984). In factor XIa assays, a concentration of 1 unit/mL was arbitrarily assigned to the amount of material required to shorten the clotting time of factor XI deficient plasma to 70 s. U.S. standard thrombin, lot J (Bureau of Biologics, Food and Drug Administration, Bethesda, MD), was used as the standard in thrombin assays.

Factor IXa was assayed by using factor IX deficient plasma (Fujikawa & Davie, 1974). A standard curve was constructed using factor IXaβ by plotting the log of the clotting time against the log of the factor IXaβ concentration. One unit per milliliter was arbitrarily assigned to a factor IXaβ concentration of 0.1 μM. Factor Xa was assayed in a manner similar to the assay of factor X except Russell's viper venom was omitted. Purified factor Xa was used as a standard. Activated protein C was assayed by using S2238 (0.4 mM) as substrate (Kisiel et al., 1977) or by coagulation assay (Comp & Esmon, 1979). Chromogenic substrate assays of the Malayan pit viper venom were done at room temperature in TBS using S2238 (0.2 mM) as substrate. An instrument with 450/415-nm filters was employed. Readings are reported as the difference in absorbance (A_d) between 415 and 450 nm. Active-site titrations with NPGB were done to establish the concentration of active sites in factor IXaβ, factor Xa, and venom preparations (Chase & Shaw, 1970).

Sedimentation Analysis. Sedimentation analysis was done by using a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. Carriage travel and the absorbance at 280 nm were converted from analog output to a digital signal using an ISAAC (Cyborg Corp., Newton, MA) analog-to-digital conversion system. Reduction of data from sedimentation equilibrium and velocity runs was done with software developed by Dr. M. N. Blackburn (Peterson & Blackburn, 1985).

Molecular weight determination was made by analysis at sedimentation equilibrium under conditions of meniscus depletion (Yphantis, 1961). Runs were made on samples that had been extensively dialyzed against either 6.0 M guanidine hydrochloride or TBS/5 mM $CaCl_2$. All absorbance values from the meniscus to the bottom of the cell were included in the analysis. The data were fit by nonlinear least-squares analysis using the Marquardt algorithm (Bevington, 1969) to the equation:

$$A = A(r_0)\exp[\sigma/2(r^2 - r_0^2)] + A_b$$

where A is the observed digitized absorbance plus base line, $A(r_0)$ the absorbance at some reference radius, r_0, in the cell, r the radial distance, A_b the digital base line, and σ the effective reduced molecular weight. Three parameters, σ, $A(r_0)$, and A_b, were determined in the fitting procedure. Estimated standard deviations associated with σ were less than 2% of the value of σ in all runs. The molecular weight was calculated by using σ, the partial specific volume ($\bar{v}$) of ARHγ, the solution density, the rotor speed, and temperature as described (Yphantis, 1961). The partial specific volume was calculated from the amino acid and carbohydrate composition (Cohn & Edsall, 1943; Gibbons, 1972) without correction for solvent–protein interaction. Solution density was measured by using a pycnometer. Intrarun standard deviations were estimated by propagation of error analysis (Bevington, 1969) in which the standard deviation of σ was determined by least-squares analysis and those of $\bar{v}$ and the solution density were estimated to be 0.01 mL/g and 0.001 g/mL, respectively.

The sedimentation coefficient of ARHγ was determined by the method of second moments (Cantor & Schimmel, 1982) in TBS, 20 °C. The same result was obtained at starting concentrations of 0.05 and 0.2 mg/mL. The frictional coefficient, f, of ARHγ and that of an equivalent anhydrous sphere, f_{min}, then were calculated as described (Cantor & Schimmel, 1982).

Chemical Analysis. Amino acid composition with the exception of half-cystine and tryptophan was determined on 24-h hydrolysates (110 °C with gaseous HCl) using precolumn derivatization with phenyl isothiocyanate and reversed-phase HPLC for quantitation (Bidlingmeyer et al., 1984). Half-cystine composition was determined after performic acid oxidation (Lee & Dreschert, 1979). Tryptophan determinations were made after methanesulfonic acid hydrolysis (Simpson et al., 1976). Neutral sugars were measured by the phenol–sulfuric acid assay (Dubois et al., 1956). Mannose was used as the standard. Sialic acid determination was measured by the thiobarbituric acid assay of Warren (1959). Hexosamines were determined from 10-h 4 N HCl hydrolysates (Termine et al., 1980) using ion-exchange HPLC and postcolumn derivatization with *o*-phthaldehyde for quantitation (Klapper, 1982).

Sequence Analysis. Automated N-terminal sequence analyses were performed on an Applied Biosystems 470A sequencer (Hewick et al., 1981); phenylthiohydantoin–amino acids were identified by reversed-phase HPLC (Tarr, 1981). Porcine factor IX was mixed with ARHγ at a ratio of 40:1 (w/w) in TBS/5 mM $CaCl_2$ for 48 h at 22 °C. Sequence analysis of the entire digest revealed the presence of approximately equimolar amounts of four amino acids in each cycle. The four peptides were partially resolved by using reverse-phase HPLC on a RPSC–Ultrasphere column (Beckman Instruments, Berkeley, CA) developed with an aqueous TFA/acetonitrile gradient. Three peaks were obtained. One of the peaks yielded the sequence from a single peptide, designated peptide 3 or P3. The second peak contained two peptides, designated P1 and P2. The N-terminal sequence of single-chain factor IX was readily identified as one of the sequences (P1), allowing the determination of the other N-terminal sequence (P2). Finally, sequencing of various fractions of the third peak revealed one sample that contained three peptides, two of which were identified as P1 and P2. This allowed the determination of the N-terminal sequence of the remaining peptide, P4.

Extinction Coefficients. The following published values were used for extinction coefficients ($E_{1cm}^{0.1\%}$): porcine thrombin, 1.99; porcine factor X, 0.84; porcine factor IXa, 1.52 (Lollar et al., 1984); human factor IX, 1.49 (Fujikawa et al., 1974). The extinction coefficients at 280 nm of porcine protein C, porcine factor IX, porcine prothrombin, and ARHγ were determined by measurement of samples at 205 and 280 nm (van Iersel et al., 1985). Malate dehydrogenase, bovine serum albumin, ovalbumin, β-lactoglobulin, avidin, myoglobin, and lysozyme were used as standards. The following values ($E_{1cm}^{0.1\%}$) were obtained: ARHγ, 1.26; porcine factor IX, 1.35; porcine protein C, 1.55; porcine prothrombin, 1.69.

Electrophoresis. Discontinuous SDS–PAGE was done by using the buffer system of Laemmli (1970). Samples containing 1% (w/v) SDS with or without 1–2% (v/v) β-mer-

captoethanol were heated at 100 °C for 2–5 min. Gradient polyacrylamide gels were made at concentrations given in the figure legends. For all gels, the ratio of *N,N'*-methylenebis-(acrylamide) to acrylamide was 2.7 to 100 (w/w). Polymerization was catalyzed by 0.067% (w/v) ammonium persulfate. Autoradiography of [^{3}H]DIP–ARHβ was done after SDS–PAGE. Sensitivity was increased by using EN^{3}HANCE according to directions supplied by the manufacturer. Proteins were stained with 0.1% (w/v) Coomassie blue R-250 with the exception of experiments involving factor VIII in which silver staining was employed (Morrissey, 1981). Molecular weight standards used were myosin (200 000), phosphorylase *b* (97 000), bovine serum albumin (68 000), ovalbumin (43 000), chymotrypsinogen (26 000), β-lactoglobulin (18 000), and lysozyme (14 000) (Bethesda Research Laboratories, Gaithersburg, MD).

Results

Identification of Venom Enzymes. The rationale for the inception of this study was that several activators of coagulation proteins have been found in snake venoms and that these enzymes have been useful in the preparation, assay, and structure–function studies of coagulation factors. Because of our interest in factor IX, 25 commercially available crude venoms were somewhat arbitrarily selected and tested for their ability to convert factor IX to factor IXaβ. Porcine factor IX (1 mg/mL) was mixed with crude venom (0.05 mg/mL) in TBS/5 mM $CaCl_2$ at room temperature for 1 h. Samples were analyzed by SDS–PAGE. Only the venom of Malayan pit viper resulted in a product that was electrophoretically indistinguishable from purified factor IXaβ. However, clotting assay of this product for factor IXa revealed no activity. Additionally, the crude Malayan pit viper venom was found to have activity toward the chromogenic substrate S2238.

The crude venom was then fractionated to isolate the proteolytic activity of interest. Gel permeation chromatography (Experimental Procedures) of crude venom revealed that thrombin-like activity eluted before the amidase activity and that the latter activity coeluted with proteolytic activity (not shown).

The proteolytic activity was further purified by QAE-cellulose chromatography (Experimental Procedures) and again coeluted with the amidase activity (not shown). Analysis of the pooled protein peak by SDS–PAGE revealed a major band (apparent M_r 26 000) and a faint minor band (apparent M_r 25 000), both with an electrophoretic mobility greater than ancrod. The amidase activity could be completely inhibited by DFP or benzamidine, indicating that the enzyme responsible for the activity is a serine protease. However, active-site titration of the preparation with NPGB yielded only about 0.1 mol of active site per mole of protein (assuming an average molecular weight of 26 000 and an extinction coefficient of 1 mL mg^{-1} cm^{-1}). Furthermore, the amidase activity could be inhibited by [^{3}H]DFP, and subsequent SDS–PAGE revealed that the labeled protein was associated with the minor band. We therefore designate this serine protease ARHβ since it is the second hydrolase in the Malayan pit viper venom to be identified functionally and electrophoretically.

The thrombin-like activity also was purified further by QAE-cellulose chromatography and is designated ARHα. A major band and a minor band were evident on SDS–PAGE analysis of the pool of the activity. The major band comigrated with commercial ancrod (data not shown). Addition of ancrod to factor X, prothrombin, and factor IX under the conditions used for the crude venom (see above) resulted in no apparent proteolysis.

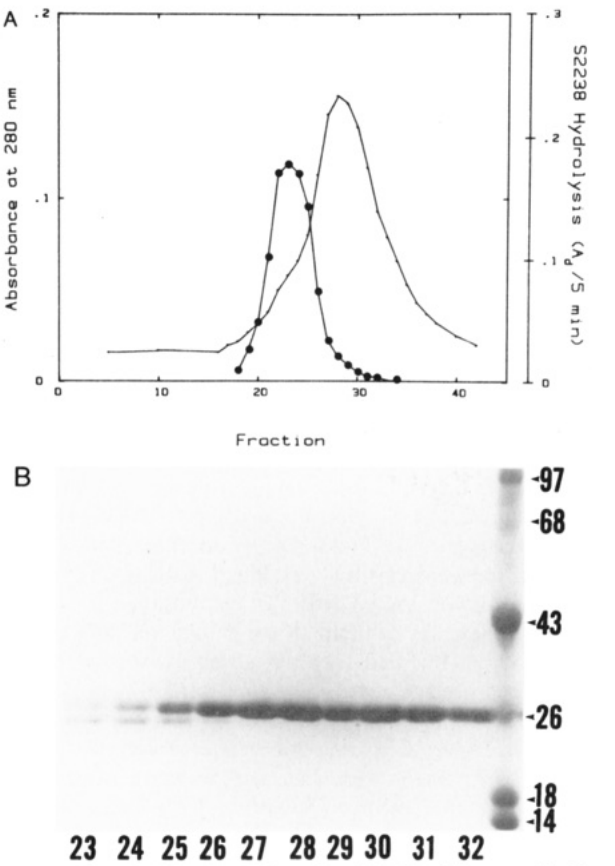

FIGURE 1: Preparative electrophoresis of QAE-cellulose pool. The method is outlined under Experimental Procedures. (A) Electrophoretogram: fractions (3 mL) were monitored for absorbance (dots) and activity toward S2238 (circles). (B) Purity of ARHγ. Aliquots (50 μL) from the fractions shown in Figure 1A were analyzed by 10–18% SDS–PAGE. Fractions 28–33 were pooled for further analysis of ARHγ.

Table I: Molecular Weight of ARHγ

conditions	rotor speed (rpm)	M_r[a]
native[b]	24 000	26 000 ± 700
	30 000	24 500 ± 670
denatured[c]	30 000	26 300 ± 1 000
	40 000	25 500 ± 940
		av 25 600 ± 800[d]

[a] Errors refer to intrarun sample standard deviation from propagation of error analysis. [b] TBS/5 mM $CaCl_2$. [c] 6.0 M guanidine hydrochloride. [d] Interrun sample standard deviation.

To further differentiate ARHβ from the proteolytic activity, pooled fractions from QAE-cellulose chromatography were further fractionated by preparative PAGE (Figure 1A). The amidase activity migrated as a shoulder in front of the main protein peak. The peak of proteolytic activity was associated with the major band of protein (not shown). Fractions from preparative PAGE that contained no detectable ARHβ by Coomassie blue staining were pooled (Figure 1B). The protein corresponding to this electrophoretic band is designated ARHγ. When ARHγ (10 μg/mL) was preincubated for 1 h at 20 °C with 10 mM benzamidine, 10 mM DFP, 10 mM tosyllysyl chloromethyl ketone, 0.01 mM dansylglutamylglycylarginyl chloromethyl ketone, or 0.5 μM soybean trypsin inhibitor, no inhibition of proteolysis of factor IX was seen.

Characterization of ARHγ. The molecular weight of ARHγ was determined by sedimentation equilibrium. Table I summarizes results of data obtained under denaturing and nondenaturing conditions at different rotor speeds. There is no significant effect due to rotor speed which suggests that

Table II: Physical Properties of ARHγ

property	value	property	value
M_r	25 600	f/f_{min}	1.39
$s^0_{20,w}$ (S)	2.6	$E^{0.1\%}_{1cm}$	1.26
$\bar{v}$ (mL/g)	0.69		

Table III: Amino Acid and Carbohydrate Composition of ARHγ

	mol of amino acid/mol of protein (M_r 25 600)[a]
Ala	5.5
Arg	4.9
Asx	19.2
Cys	7.2
Glx	15.8
Gly	8.1
His	9.1
Ile	11.1
Leu	9.2
Lys	9.8
Met	3.2
Phe	6.3
Pro	4.1
Ser	12.9
Thr	4.8
Trp	2.1
Tyr	6.8
Val	7.5
neutral sugar	21% (w/w)
glucosamine	5% (w/w)
galactosamine	0
sialic acids	8% (w/w)

[a] Mean of two determinations.

the preparation sediments as a single species. The protein does not self-associate to any significant extent at concentrations used for functional analyses in this study since the results are very similar under denaturing conditions. By averaging the results in Table I, a molecular weight of 25 600 is obtained. Additionally, since there is no effect of reduction of sulfhydryl groups on the mobility of ARHγ during SDS–PAGE, it follows that ARHγ is a single-chain protein.

The sedimentation coefficient of ARHγ is 2.6 S (Table II). The frictional ratio, obtained from this determination combined with the molecular weight and the partial specific volume, is 1.39. This value is higher than a tabulation of frictional ratios for 20 nearly spherical proteins (Squire & Himmel, 1979). This indicates that ARHγ has a nonglobular structure. Other data obtained from this protein are given in Tables II and III. ARHγ is a heavily glycosylated protein containing 34% neutral and amino sugars by weight. This in part explains its low partial specific volume relative to most proteins. Additionally, ARHγ is relatively rich in hydrophilic residues (Asx and Glx).

Degradation of Factor IX by ARHγ. The proteolytic alterations of porcine factor IX that are catalyzed by ARHγ were examined and interpreted with the aid of knowledge that exists regarding the proteolysis of human and bovine factor IX. Terminology from human factor IX sequence will be used throughout (Kurachi & Davie, 1982; McMullen et al., 1983). The activation of single-chain human factor IX by factor XIa occurs as the result of the hydrolysis of an Arg–Ala bond at position 145 and an Arg–Val bond at position 180 (DiScipio et al., 1978). Similar cleavages of bovine factor IX occur (Fujikawa et al., 1974). As a result, an M_r 45 000 two-chain protein is formed, and an M_r 10 000 activation peptide is released. Depending on the order of the two bond cleavages, two intermediates are possible, and both have been observed (Braunstein et al., 1981; Lindquist et al., 1978). The intermediate formed after a single bond cleavage at position 145 is termed factor IXα. The intermediate formed after a single bond cleavage at position 180 is called factor IXaα. Factor IXaα can be obtained in stable form by the action of Russell's viper venom factor X coagulant protein (RVV-XCP), which catalyzes the hydrolysis of the bond at position 180 much more rapidly than the bond at position 145 (DiScipio et al., 1978).

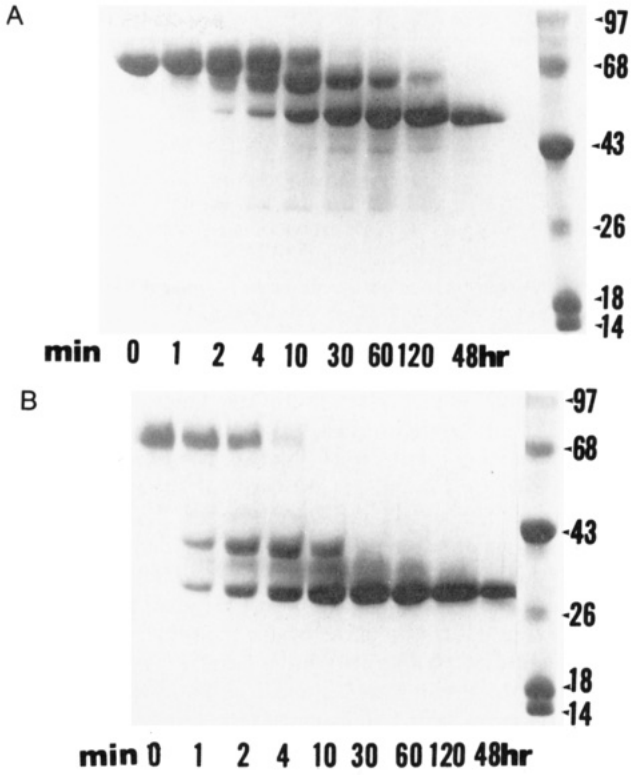

FIGURE 2: Degradation of factor IX by ARHγ. Factor IX, 0.2 mg/mL, was reacted with 10 μg/mL ARHγ in TBS/5 mM $CaCl_2$, 20 °C, for the indicated times, and 10-μg samples were analyzed by 10–18% SDS–PAGE. (A) Nonreduced; (B) reduced.

A time course of the proteolysis of porcine factor IX by ARHγ is shown in Figure 2A,B. Near complete conversion to an end product that is indistinguishable from factor IXaβ occurs within 2 h. This product is stable for at least 48 h. However, an intermediate is present in the unreduced gel between substrate and final product. This is inconsistent with cleavages at only positions 145 and 180. If it is assumed that ARHγ induces cleavages that are at or near the factor XIa cleavage sites, then the only explanation for the intermediate present in the unreduced gel is a third cleavage in the region of the activation peptide.

Support for this hypothesis was obtained from studies in which porcine factor IX was reacted with RVV-XCP and then further reacted with ARHγ (not shown). After a 1-h incubation with RVV-XCP, a product was formed that has been identified as factor IXaα by other investigators. In a reduced SDS–PAGE gel, this was evident from the appearance of two bands corresponding to the heavy chain and the light chain/activation peptide of factor IX. Subsequently, ARHγ was added to RVV-XCP-treated factor IX, and the reaction was allowed to proceed. An intermediate that moved between factors IX and IXaβ was seen again in the unreduced gel. In the reduced gel, an intermediate between the heavy chain and light chain/activation peptide was seen. After 1 h, nearly all of the product appeared to be factor IXaβ. These findings indicate that ARHγ catalyzes cleavages at or near the Arg145–Ala146 factor XIa cleavage site and additionally at a site within the activation peptide itself.

To further characterize the cleavages induced by ARHγ, porcine factor IX was incubated with ARHγ for 48 h followed

Table IV: Sequence Data from ARHγ Digest of Porcine Factor IX[a]

Peptide 1		YNSGKL--FVRG
Homologous sequence	(B)[b]	1-YNSGKLγγFVRG
	(H)	YNSGKLγγFVQG
		Peptide 2 Peptide 3
		MDYE-STEVEP LTES-QTSDD
Homologous sequence	(B)	147-AETIFSNTNYENSSEAEIIWDNVTQSNQSFDEFSR
		146-AEAVFPDVDYVNPTEAETILDNITQGTQSFNDFTR
Peptide 4		FIRIVGGENAKP
Homologous sequence	(B)	179-FSRVVGGNDANR
	(H)	178-FTRVVGGEDAKP

[a] Single-letter notation for amino acids is employed (IUPAC–IUB Commission on Biochemical Nomenclature, 1968). γ, γ-carboxyglutamic acid. [b] B, bovine; H, human.

by N-terminal sequence analysis of the digest. Four peptides (P1, P2, etc.) were identified and partially sequenced as described under Experimental Procedures (Table IV). The sequence obtained from P1 is identical with the amino-terminal sequence of the human and bovine factor IXaβ light chain. Of the 10 residues identified in P2, 5 are identical with the corresponding sequence from positions 153–163 in the human factor IX activation peptide. Two additional identical sites are found in this region in the bovine molecule. Additionally, the unidentified residue at position 5 in P2 is a glycosylation site in bovine and human factor IX. Thus, one cleavage site is seven amino acids C-terminal to the factor XIa cleavage site at position 145. The nine residues sequenced from P3 align with positions 168–177 in the bovine and human factor IX activation peptides with five sites of identity. Residue 5 in P3 could not be identified which is also consistent with a glycosylation site at Asn-172 in the molecule. The 12 residues sequenced in P4 align with positions 178–189 in the human factor IX activation peptide/heavy chain, revealing that another ARHγ cleavage site is three residues N-terminal from the factor XIa cleavage site at position 180. In summary, three cleavages in porcine factor IX that are catalyzed by ARHγ have been identified. Placement of the cleavages is shown schematically in Figure 3.

Functional Properties of ARHγ-Degraded Factor IX. The isolation of ARHγ-degraded factor IX on a preparative scale was done by heparin–Sepharose chromatography (Experimental Procedures). The product was assayed for coagulant activity, BAEE activity, and "active sites" by NPGB titration (Table V) and was not found to have detectable clotting activity or active sites.

Since the product, presumably des(153–178)-factor IX, conceivably can be converted to factor IXaβ by cleavages at positions 145 and 180 by factor XIa or factor VIIa, the kinetics of activation of the purified ARHγ-degraded factor IX by factor XIa were measured. A comparison to the activation of nondegraded factor IX under identical conditions is shown

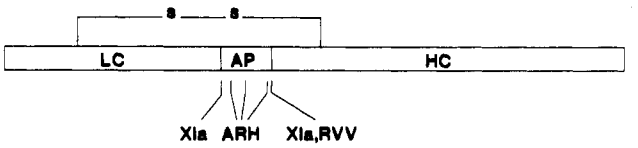

FIGURE 3: ARHγ cleavage sites in factor IX. The sites are shown to scale within the framework of the polypeptide backbone. LC, light chain; AP, activation peptide; HC, heavy chain. Also shown are factor XIa and RVV-XCP cleavage sites.

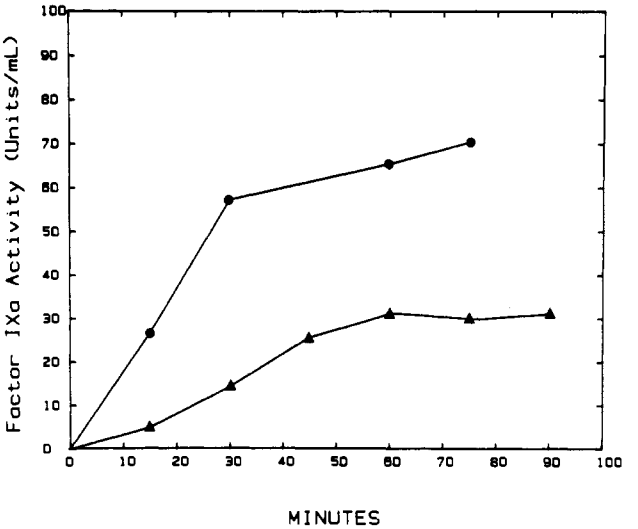

FIGURE 4: Activation of ARHγ-degraded factor IX by factor XIa. Factor IX, 0.2 mg/mL (circles), or ARHγ-degraded factor IX, 0.2 mg/mL (triangles), was reacted with 2 units/mL factor XIa in TBS/5 mM $CaCl_2$ at 37 °C for the indicated times and then assayed for factor IXa activity as outlined under Experimental Procedures.

in Figure 4. Although the rate and perhaps the extent of activation of the degraded protein are less than those of the native protein, it is clear that significant activation of the ARHγ-degraded factor IX can be achieved.

Degradation of Prothrombin by ARHγ. The addition of ARHγ to porcine prothrombin results in the degradation of prothrombin to several products moving in the region between the M_r 26000 and 43000 standards (Figure 5). The products were chromatographed on SP-Sephadex and eluted with 0.6 M NaCl/0.02 M Tris-HCl, pH 7.4, since thrombin is known to bind tightly to cation-exchange resins. The eluate had no detectable amidase activity or fibrinogen clotting activity (Table V). Treatment of the eluate with taipan venom, which converts prothrombin to α-thrombin (Owen & Jackson, 1973), resulted in an increase in fibrinogen clotting activity to 1000 units/mg compared to a value of 3000 units/mg for porcine thrombin (Table V). This indicates that ARHγ-degraded prothrombin also contains an accessible activation site(s).

Table V: Functional Properties of ARHγ-Degraded Proteins

	% activity[a]				
	after ARHγ (10 μg/mL)		after activator		
	chromogenic substrate	coagulant[b]	chromogenic substrate	coagulant[b]	activator
factor IX (0.2 mg/mL)	<5[c]	<5	ND	40	factor XIa (2 units/mL, 60 min)
factor X (0.1 mg/mL)	ND[e]	<5	ND	100	RVV-XCP (10 μg/mL, 60 min)
prothrombin (0.2 mg/mL)	<5[d]	<5	ND	33	taipan venom (10 μg/mL, 60 min)
antithrombin III (0.2 mg/mL)	ND	<5	NA[f]	NA	NA
protein C (0.1 mg/mL)	<5[d]	<5	96[d]	<10	thrombin (5 units/mL, 3 h)
factor VIII (0.5 unit/mL)	ND	<5	ND	<10	thrombin (1 unit/mL, 2 min)

[a] All activities are expressed relative to an equivalent mass of the purified active forms of the protein. All reactions were in TBS/5 mM $CaCl_2$, except protein C activation in which calcium was omitted. ARHγ was added for 60 min except for the experiment with factor VIII in which a 10-min reaction time was used. [b] By clotting assay for respective factors. [c] Activity toward BAEE and NPGB. [d] Activity toward S2238. [e] ND, not determined. [f] NA, not applicable.

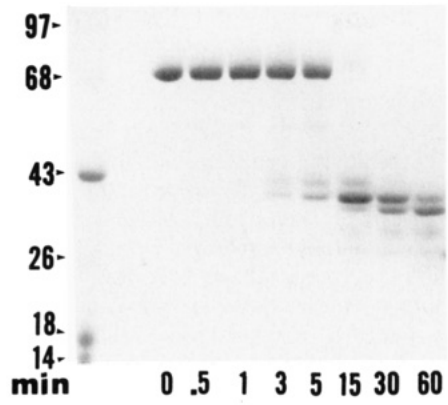

FIGURE 5: Degradation of prothrombin by ARHγ. Prothrombin, 0.2 mg/mL, was reacted with 10 μg/mL ARHγ in TBS/5 mM $CaCl_2$ for the indicated times, and the nonreduced products were analyzed by 10–18% SDS–PAGE. Standards are shown in the first lane.

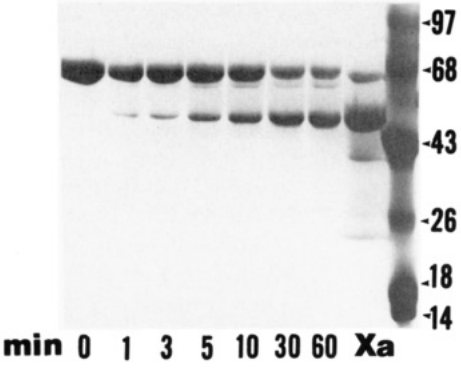

FIGURE 6: Degradation of factor X by ARHγ. Factor X, 0.2 mg/mL, was reacted with 10 μg/mL ARHγ in TBS/5 mM $CaCl_2$ at 20 °C for the times indicated, and 10 μg of the nonreduced products was analyzed by 10–18% SDS–PAGE. The lane marked "Xa" is a factor X preparation that has been passed once over RVV-XCP–Sepharose.

Degradation of Factor X, Protein C, Factor VIII, and AT-III by ARHγ. The addition of ARHγ to porcine factor X results in its degradation to a product that is indistinguishable from factor Xa when analyzed by SDS–PAGE (Figure 6). Factor Xa assays during the time course of this experiment yielded no measurable activity. However, addition of RVV-XCP to the reaction mixture resulted in activation of the degraded factor X to 100% of the value obtained for factor Xa (Table V).

Porcine protein C preparations consist of a doublet when analyzed by SDS–PAGE under nonreducing conditions (Figure 7), which also has been described in human protein C preparations (Kisiel, 1979). Examination of the products formed after degradation of protein C by ARHγ under reducing conditions reveals an increase in mobility of both the heavy and light chains (Figure 7B), indicating that at least two cleavages are made. No enzymatic activity was detectable during the time course shown in Figure 7 (Table V). Addition of thrombin to ARHγ-treated protein C resulted in the generation of full amidolytic activity, but no anticoagulant activity could be detected.

The porcine factor VIII used in this study consists of two heavy polypeptide chains with apparent molecular weights of 166 000 and 82 000 that have identical N-terminal sequences and a single light-chain polypeptide with an apparent molecular weight of 76 000 (Fass et al., 1982; Toole et al., 1984) (Figure 8). The preparation has no measurable cofactor activity in the activation of factor X by factor IXa. Addition of thrombin to factor VIII results in the generation of cofactor activity in a process that is associated with the proteolysis of

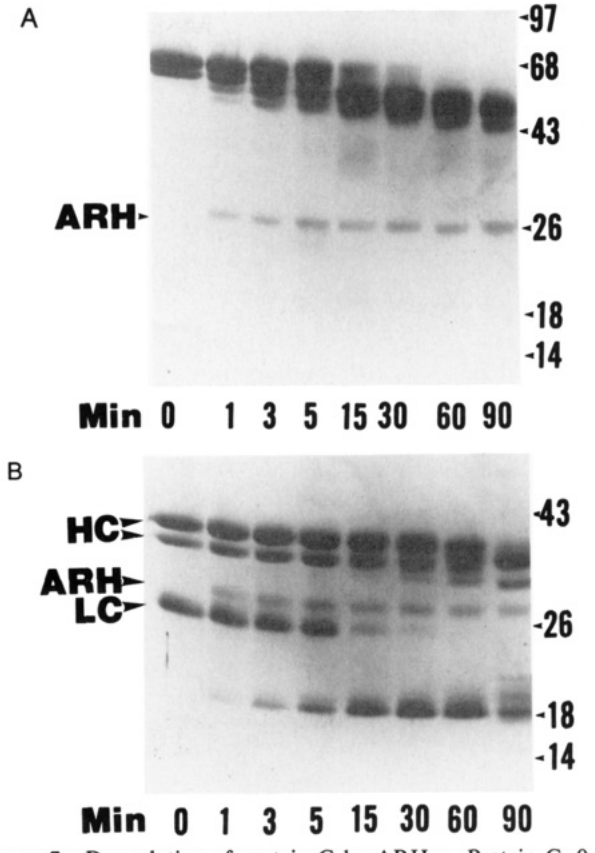

FIGURE 7: Degradation of protein C by ARHγ. Protein C, 0.1 mg/mL, was reacted with 10 μg/mL ARHγ in TBS, 20 °C, for the indicated times, and 10 μg of the products was analyzed by 10–18% SDS–PAGE. (A) Nonreduced; (B) reduced; HC, heavy chains; LC, light chain; ARH, ARHγ.

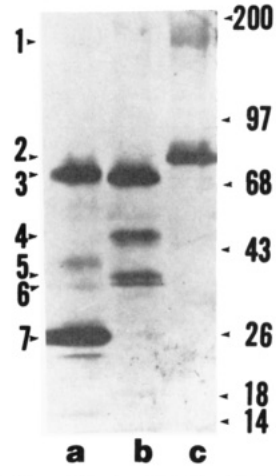

FIGURE 8: Degradation of factor VIII by ARHγ. Factor VIII, 10 units/mL, was reacted with (a) 10 μg/mL ARHγ, (b) 0.1 μg/mL thrombin, or (c) buffer control for 1 h in TBS/5 mM $CaCl_2$, and the products (~0.3 μg) were identified by silver staining after 5–15% SDS–PAGE under nonreducing conditions. Bands identified are as follows: (1) 166-kDa heavy chain; (2) 82-kDa heavy chain (faint) and 76-kDa light chain; (3) 69-kDa degraded light chain; (4) 43-kDa heavy-chain fragment; (5) 35-kDa heavy-chain fragment; (6) thrombin; (7) ARHγ.

all polypeptide chains (Fass et al., 1982; Lollar et al., 1985). Addition of ARHγ to porcine factor VIII results in the proteolysis of all polypeptide chains to products that are different from those due to thrombin. Degradation of factor VIII by ARHγ resulted in a complete loss of measurable activation by thrombin (Table V).

Reaction of AT-III with ARHγ also results in proteolytic degradation when the products are examined by SDS–PAGE

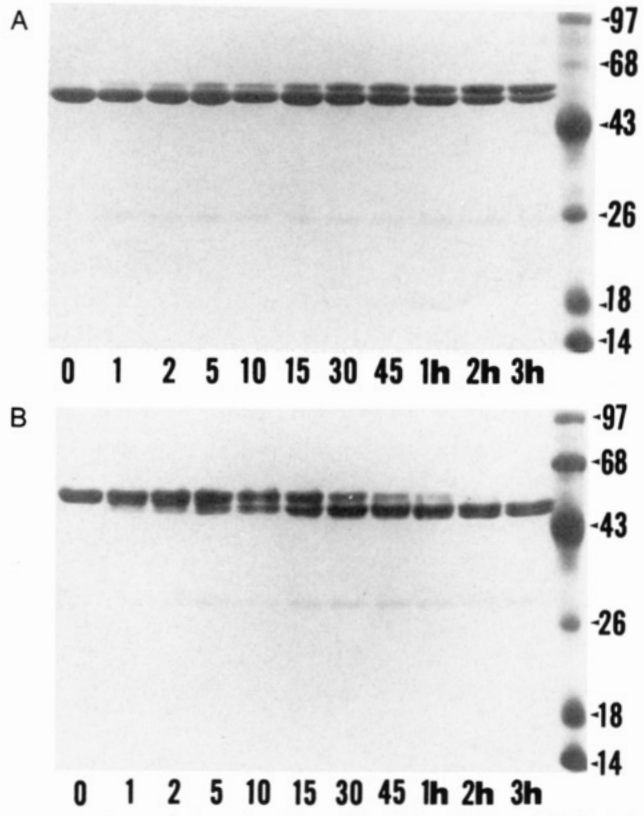

FIGURE 9: Degradation of antithrombin III by ARHγ. AT-III, 0.3 mg/mL, was reacted with ARHγ in TBS/5 mM $CaCl_2$ for the indicated times. The products (10 μg) were identified after 10–18% SDS–PAGE: (A) nonreduced; (B) reduced.

under reducing conditions (Figure 9B). The reaction results in the formation of two bands, one with a slightly smaller apparent molecular weight than AT-III and the other that migrates ahead of the 14000 molecular weight standard. When the same samples are examined by SDS–PAGE under nonreducing conditions (Figure 9A), a product is formed that appears slightly larger than AT-III. This indicates that ARHγ cleaves AT-III to a two-chain, disulfide-linked species. The resulting degraded AT-III was unable to inhibit porcine thrombin (Table V).

Lack of Effect of ARHγ on Fibrinogen and Plasma Clotting Assays. Fibrinogen (1.25 mg/mL) in TBS/5 mM $CaCl_2$ at 37 °C did not clot in 240 min after the addition of ARHγ to a final concentration of 5 μg/mL. In contrast, addition of this amount of ARHα or commercial ancrod to an identical solution resulted in a clot in 12–15 s. Additionally, no cleavages were evident following SDS–PAGE of fibrinogen that had been exposed to ARHγ.

Since ARHγ catalyzes the hydrolysis of several clotting factors, its effect on plasma clotting assays was studied. Citrated pig plasma was incubated with 5 μg/mL ARHγ or buffer control at 37 °C for up to 1 h. At 15-min intervals, samples were taken for prothrombin time (Sigma) and activated partial thromboplastin time (General Diagnostics) determinations. No effect was seen in either assay. This indicates that ARHγ either is inhibited by some factor in plasma or catalyzes cleavages that are functionally silent in plasma clotting assays.

Discussion

In addition to the fibrinogenolytic enzyme, ancrod, the venom of the Malayan pit viper contains at least two other hydrolases. The three enzymes are designated *Akistrodon rhodostoma* hydrolases α, β, and γ in order of the identification of their different substrate specificities with an electrophoretic band. Ancrod, or ARHα, is separated from the other two enzymes by gel permeation chromatography. It has no activity toward factors IX or X, or prothrombin. The second enzyme, ARHβ, has activity toward S2238 that is inhibited by DFP and benzamidine. It has no detectable fibrinogen clotting activity. It appears to be a minor component in the crude venom compared to ancrod. Inhibition of its activity by [^{3}H]DFP followed by SDS–PAGE and autoradiography shows that the radioactive band is associated with the minor, fast-moving band in Figure 1. The third hydrolase, ARHγ, is fractionated by following its ability to catalyze the degradation of factor IX, and fractions that are greater than 95% homogeneous are obtained following preparative electrophoresis of the QAE-cellulose pool (Figure 1). It appears to be a major component of the crude venom by SDS–PAGE (not shown). ARHγ has no detectable activity toward fibrinogen or S2238 and is not inhibited by a variety of serine protease inhibitors. Additionally, it does not affect the prothrombin time or activated partial thromboplastin time after prolonged incubation with normal citrated plasma.

Although ARHγ was initially identified following a screen of several snake venoms for potential factor IX activating activity, we subsequently found that the purified enzyme catalyzed the proteolytic degradation of every coagulation protein we had in the laboratory with the exception of fibrinogen. In the case of the vitamin K dependent proteins that have been examined (prothrombin, protein C, and factors IX and X), products are generated that are electrophoretically similar to or indistinguishable from the corresponding activated enzymes. The enzyme appears to catalyze a rather limited and specific proteolysis since a single major electrophoretic species of degraded factor IX can be generated within 2 h followed by no apparent additional proteolysis after an additional 46-h incubation (Figure 2A). The products have no detectable enzymatic activity, although in the case of prothrombin and factors IX and X, the products can be further proteolytically degraded to a species with at least partial coagulant activity.

N-Terminal sequencing of the products generated by the action of ARHγ on factor IX suggests a reason for these observations. With reference to the human factor IX molecule, cleavages are made at position 152, seven residues C-terminal to the Arg145–Ala146 factor XIa cleavage site, and at position 178, three residues N-terminal to the Arg180–Val181 cleavage site. The product is presumably des(153–177)-factor IX. A cleavage analogous to the Arg180–Val181 cleavage, resulting in the release of an activation peptide, has been found during the activation steps of all serine proteases studied to date. In chymotrypsin, this results in the formation of an internal salt bridge between Ile-16 and Asp-194 made possible by hydrolysis of the Arg15–Ile16 bond. Therefore, at least one reason that ARHγ-degraded factor IX lacks enzymatic activity apparently is due to the three-residue tail that prevents formation of the critical salt bridge. Since ARHγ-degraded factor IX can be activated by factor XIa (Figure 4), it is probable that the Arg181–Val182 cleavage site can be recognized by factor XIa despite the fact that residues are missing in the substrate N-terminal to P_3.[2]

The similarity of the effect of ARHγ on factor IX, factor X, prothrombin, and protein C suggests that cleavages are made in the activation peptide. This suggests a common

[2] The nomenclature of Schecter and Berger (1967) is used to designate amino acid residues with respect to the scissile bond.

structural determinant despite the fact that there is little amino acid sequence homology in this region between these proteins. The function of the activation peptide region is not understood. One possibility is that it confers substrate specificity. It appears that ARHγ can form degraded proteins that are missing substantial amounts of the activation peptide. Therefore, the study of the kinetic parameters involved in the activation of these degraded proteins may provide insight into the substrate specificity conferred upon these proteins by virtue of their structure N-terminal to the activation site.

In addition to cleavages at positions 152 and 178, the degradation of factor IX by ARHγ involves a third cleavage in the region of the activation peptide. This is suggested strongly by the appearance of an intermediate seen after SDS–PAGE of nonreduced samples obtained during the proteolysis of factors IX and IXα (Figure 2). The intermediate would result from the removal of a peptide smaller than the activation peptide generated by factor XIa. The final products then result from a third bond cleavage with subsequent removal of a second small peptide. These results are supported by sequence analysis (Table IV). Peptide 3 aligns with residues 168–177 in human factor IX. Since the same intermediate is seen in nonreduced gels of the time course of factor IX and factor IXα proteolysis, we conclude that the major pathway for the order of bond cleavages is position 178, then position 167, and then position 152.

Factor VIII must be activated proteolytically in order to function as a procoagulant. Thrombin is the most potent known activator. Pretreatment of factor VIII with ARHγ results in proteolysis of all polypeptide chains (Figure 8) and is associated with the loss of the ability of thrombin to activate factor VIII. ARHγ also degrades AT-III in a process that is associated with loss of activity. The cleavage(s) is (are) apparently within one of the disulfide-bonded regions of the molecule because a reduction in mobility during SDS–PAGE only occurs in reduced samples. It is interesting to note that several venoms have been found that cleave and inactivate AT-III (Kress & Catanese, 1980). A protein from *Crotalus adamanteus* has been isolated, and the three cleavage sites in the AT-III molecule have been identified (Kress & Catanese, 1981). The inactivating cleavage occurs nine residues N-terminal to the reactive-site Arg384–Ser385 bond. The same protein makes a similar cleavage in α_1-antitrypsin which, like AT-III, is a member of a large family of proteins known as serpins (Carrell & Travis, 1985). It has been proposed that venoms, toxins, and endogenous proteases inactivate serpins by making cleavages N-terminal to the reactive site of the proteins and that the inactivation process is accompanied by a conformational change from a "stressed" state to a "relaxed" state (Carrell & Owen, 1985). This would increase the effective hydrodynamic radius of the molecule and may explain the paradoxical decrease in electrophoretic mobility of nonreduced AT-III after cleavage by ARHγ (Figure 9A).

Bites by the Malayan pit viper are noteworthy because they result in a prolonged hypofibrinogenemia in man without producing a significant bleeding diathesis (Reid et al., 1963). This observation led to clinical trials of ancrod as an anticoagulant. Ancrod has been found to be effective in both prophylaxis and treatment of deep vein thrombosis (Tibbutt et al., 1974; Barrie et al., 1974). Analysis of commercial ancrod in our laboratory by SDS–PAGE shows no evidence of ARHβ or ARHγ, indicating that the pharmacological effect of the preparation is not due to previously unsuspected contaminants, assuming the material used in the clinical trials was also free of ARHβ and ARHγ. However, the crude venom appears to contain substantial amounts of ARHγ which has the ability to catalyze modifications in numerous coagulation proteins. It is conceivable that this enzyme is responsible for some of the clinical effects following bites by this snake.

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Mapping of the Adenosine 5′-Triphosphate Binding Site of Type II Calmodulin-Dependent Protein Kinase†

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ABSTRACT: The specificity of the ATP-binding site of the type II calmodulin-dependent protein kinase was probed with 25 analogues of ATP modified at various positions of the molecule. The analogues were compared by their ability to compete with ATP in the protein kinase reaction. The result of this comparison indicates that the enzyme is most sensitive to modifications at, or replacement of, the purine moiety. Changes at the triphosphate chain are much better tolerated, although the enzyme exhibited a selective sensitivity to changes in the conformation of this group. The smallest contribution to the specificity of ATP binding appears to be made by the ribose ring. The K_i values obtained for a subset of these analogues were compared to those previously reported for phosphorylase *b* kinase and the cyclic nucleotide dependent protein kinases [Flockhart, D. A., Freist, W., Hoppe, J., Lincoln, T. M., & Corbin, J. D. (1984) *Eur. J. Biochem. 140*, 289–295]. A striking similarity in the responses of these protein kinases to modifications of the ATP molecule suggests that the type II calmodulin-dependent protein kinase is related to these enzymes. Support for this conclusion was provided, recently, through comparisons of the deduced primary structures of the α and β subunits of the type II calmodulin-dependent protein kinase with the protein sequences of the catalytic subunits of phosphorylase *b* kinase and cAMP-dependent protein kinase [Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N., & Kelly, P. T. (1987) *Science (Washington, D.C.) 237*, 293–297; Bennett, M. K., & Kennedy, M. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1794–1798], which indicated areas of extensive homology.

Type II calmodulin-dependent protein kinase (CaM kinase II)[1] is a multifunctional protein kinase that has been isolated from rabbit liver (Ahmad et al., 1982; Payne et al., 1983), rabbit skeletal muscle (Woodgett et al., 1983), rat brain (Bennett et al., 1983; Kennedy et al., 1983), and several other tissues [reviewed by Nairn et al. (1985)]. The enzyme is most prominent in the brain (Bennett et al., 1983; Nairn et al., 1985) where it is thought to be involved in regulating synaptic activity (Browning et al., 1985; Nairn et al., 1985) and in prolonging the effects triggered by the transient Ca^{2+} signal (Miller & Kennedy, 1986; Lai et al., 1986; Schworer et al., 1986).

The enzyme isolated from rat brain has a native molecular weight of approximately 630 000 and is composed of α (M_r

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[1] Abbreviations: CaM kinase II, type II calmodulin-dependent protein kinase; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ApCpp, adenosine 5′-(α,β-methylenetriphosphate); AppCp, adenosine 5′-(β,γ-methylenetriphosphate); AppNp, adenosine 5′-(β,γ-imidotriphosphate); 2-Cl-ATP, 2-chloroadenosine 5′-triphosphate; etheno-ATP, 1,N^6-ethenoadenosine 5′-triphosphate.

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