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Role of Meizothrombin and Meizothrombin-(des F1) in the Conversion of Prothrombin to Thrombin by the *Echis carinatus* Venom Coagulant†

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ABSTRACT: The venom of the saw scaled viper, *Echis carinatus*, contains a protein capable of converting prothrombin to an active enzyme. This prothrombin activator (ECV-P) has been purified 40-fold by affinity chromatography on wheat germ agglutinin–Sepharose 4B followed by ion-exchange chromatography on DEAE-Sephacel. ECV-P was homogeneous as assessed by dodecyl sulfate–polyacrylamide gel electrophoresis. Sialic acid, neutral sugars, and hexosamines were present at 4.2, 7.2, and 5.8 wt %, respectively. Amino acid analysis of reduced and carboxymethylated ECV-P revealed an unusually high cysteine content of 9 mol %. ECV-P is inhibited by chelators such as ethylenediaminetetraacetic acid and *o*-phenanthroline but is active after treatment with Chelex. However, atomic absorption studies revealed that neither Ca, Co, Cr, Cu, Fe, Mn, Ni, nor Zn ion is present at more than 0.01 mol/mol of active protein after Chelex treatment. Gel electrophoretic studies in the presence and absence of inhibitors were performed to determine the initial products of the reaction of ECV-P with human prothrombin. In the presence of diisopropyl fluorophosphate, or benzamidine, the major product is meizothrombin-(des F1). However, when dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was utilized as an inhibitor of any thrombin-like enzymes which are formed by ECV-P, the initial product was meizothrombin; meizothrombin-(des F1) appeared as a second product. By removal of ECV-P after the production of meizothrombin and addition of additional prothrombin, it was demonstrated that meizothrombin can convert prothrombin to prethrombin-1, prethrombin-2, and thrombin. Similarly, meizothrombin-(des F1), produced from the reaction of ECV-P with prethrombin-1, can also convert prothrombin to these products. These results demonstrate that unlike thrombin, meizothrombin and meizothrombin-(des F1) can convert prothrombin to an active enzyme.

The activation of prothrombin is the last step of the series of zymogen to enzyme conversions which are involved in the coagulation cascade. During normal blood clotting, this step is catalyzed by the coagulation enzyme factor Xa, with Ca^{2+}, phospholipid, and factor Va acting as cofactors (Suttie & Jackson, 1977). Prothrombin can also be converted to an active enzyme by specific proteases obtained from the venoms of the Taipan snake, *O. scutellatus* (Denson et al., 1971; Owen & Jackson, 1973), the boomslang, *D. typus* (Mackay et al., 1969; Guillin et al., 1978), the tiger snake, *N. scutellatus scutellatus* (Jobin & Esnouf, 1966), and the saw scaled viper, *Echis carinatus* (Schieck et al., 1972; Franza et al., 1975; Kornalik & Blomback, 1975; Morita et al., 1976). In the course of our studies on prothrombin activation, we became interested in using the venom coagulant enzyme of *E. carinatus* (ECV-P)[1] as a model system for the study of prothrombin

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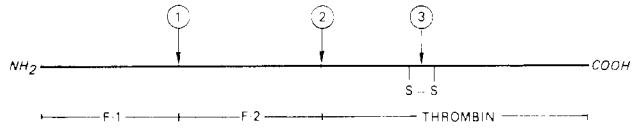

FIGURE 1: Schematic representation of prothrombin. Arrows 1 and 2 indicate bonds cleaved to release fragments 1 and 2, respectively. Cleavage at arrow 3 is required for the formation of enzymatic activity.

activation in the absence of phospholipid, Ca^{2+}, and other cofactors. Due to the conflicting reports of whether one (Morita et al., 1976) or two bonds (Franza et al., 1975; Kornalik & Blomback, 1975) are cleaved by ECV-P and the tedious purification procedure (Morita & Iwanaga, 1978), we decided to develop a simple purification procedure for preparing homogeneous ECV-P and to use this preparation to investigate the mechanism by which this enzyme activates prothrombin.

This report presents evidence that the initial product of the reaction of ECV-P with prothrombin is meizothrombin. An unexpected finding was that either meizothrombin or meizothrombin-(des F1) can also convert prothrombin to thrombin. While this work was in progress, Briët et al. (1981) presented evidence that ECV-P converts prothrombin to meizothrombin in the presence of dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA).

Terminology. The nomenclature used for prothrombin and its activation fragments, intermediates, and products is that recommended by the International Committee on Thrombosis and Haemostasis (Jackson, 1977). Figure 1 is a schematic diagram illustrating prothrombin and the various proteolytic cleavage sites. Fragment 1 is the peptide that arises from the amino-terminal region by cleavage of the Arg–Ser bond designated by arrow 1. Prethrombin-1 is the remainder of the molecule. Similarly, fragment 2 and prethrombin-2 are the products resulting from cleavage at the Arg–Thr bond of prethrombin-1 (arrow 2). Fragment 1·2 results from cleavage of prothrombin at point 2 only. Cleavage at point 3, an Arg–Ile bond, is required for thrombin activity and results in a two-chain molecule. Thrombin is formed from cleavages at bonds 2 and 3, meizothrombin is formed when cleavage occurs at bond 3 only, and meizothrombin-(des F1) results from cleavage at bonds 1 and 3. The light chain of thrombin (amino-terminal end) is the A chain, and the heavy chain (carboxyl-terminal end) is the B chain. In addition, fragment 1 can be cleaved to a two-chain fragment which is reducible to subfragment α and subfragment β (Franza et al., 1975).

Materials and Methods

Fresh frozen human cryosupernatant was supplied by the Baltimore Regional Blood Service, American Red Cross. Crude lyophilized *Echis carinatus* venom was obtained from the Miami Serpentarium. Wheat germ agglutinin affinity columns were purchased from Vector Laboratories and DEAE-Sephacel was from Pharmacia Chemicals. Ultrapure Tris and urea were purchased from Schwarz/Mann. Polyacrylamide gel electrophoresis reagents and molecular weight markers were purchased from Bio-Rad. Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222), D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), and Bz-Phe-Val-Arg-*p*-nitroanilide (S-2160) were purchased from Kabi Diagnostica. Iodoacetamide and 2-mercaptoethanol were from Aldrich Chemical Co. Dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) was synthesized as described by Nesheim et al. (1979).

Preparation of Prothrombin, Meizothrombin, and Meizothrombin-(des F1). Prothrombin was purified from fresh frozen human cryosupernatant by the method of Di Scipio et al. (1977).

Meizothrombin was prepared by incubating ECV-P (3.3 μM) with prothrombin (22 μM) and DAPA (0.36 mM) in 0.55 mL Tris-HCl, pH 7.5, for 15 min at room temperature. The reaction was quenched by the addition of 1 mL of wheat germ agglutinin gel slurry which binds ECV-P. The bound ECV-P was removed by filtration. Immediately prior to use, the excess DAPA was removed by gel filtration through Sephadex G-50. Meizothrombin-(des F1) was prepared in the same way except prethrombin-1 (11 μM) was substituted for prothrombin. In all experiments using meizothrombin and meizothrombin-(des F1) as enzymes, control incubations were run in the absence of prothrombin or prethrombin-1 to ensure that there was no enzymically active ECV-P contamination. Competition between prothrombin and ECV-P for wheat germ agglutinin was ruled out by control experiments which indicated that prothrombin has no detectable affinity for the wheat germ agglutinin under the conditions used for removal of the ECV-P from the reaction mixture.

Electrophoretic Analysis. Protein samples were electrophoresed in the reduced or unreduced state according to the procedure of Weber & Osborn (1969) or Laemmli (1970). Bovine serum albumin, ovalbumin, chymotrypsin, and ribonuclease were used as molecular weight markers. Prothrombin, prethrombin-1, prothrombin fragment 1, and thrombin were also used as standards to identify the products of prothrombin activation. The protein bands were visualized by staining with Coomassie Blue (Weber & Osborn, 1969) or PAS (Segrest & Jackson, 1972).

The samples were prepared by removing aliquots of the reaction mixtures at the specified time intervals and adding them to 0.1 volume of 10% $NaDodSO_4$. The mixture was immediately placed in a boiling water bath for 5 min. For the reduced samples, 1% β-mercaptoethanol was then added. An aliquot containing about 30 μg of protein was applied to 10% acrylamide gels. Molecular weights of the activated products were determined by comparison with protein standards.

High-Performance Liquid Chromatography. Gel filtration analysis of prothrombin and its derivatives was performed on a TSK type SW 3000 gel permeation column. The column was equilibrated, and the proteins were eluted with 0.1 M Tris-HCl, pH 7.2, containing 0.15 M NaCl. The products were detected at 254 nm by using a Waters Model 440 wavelength absorbance detector.

The various proteins were identified by comparison with authentic samples or $NaDodSO_4$–polyacrylamide gel electrophoresis of duplicate samples (Kosow et al., 1981).

Spectrophotometric Assays. The activity of ECV-P was determined by its ability to activate prothrombin. The steady-state rate of activation of human prothrombin was determined by measuring the acceleration of the rate of hydrolysis of S-2238. The spectrophotometric data were fitted to the equation $A_{410} = at^2/2$ (Kosow et al., 1973; Kosow, 1975) by a linear regression program on a PDP 11/34A computer. The absorbance data were converted to the concentration of

[1] Abbreviations: ECV-P, the prothrombin activator protein from *E. carinatus* venom; RVV-X, the factor X activator from *V. russelli* venom; S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; S-2238, D-Phe-pipecolyl-Arg-*p*-nitroanilide; S-2160, Bz-Phe-Val-Arg-*p*-nitroanilide; PAS, periodic acid–Schiff; DFP, diisopropyl fluorophosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; NPGB, *p*-nitrophenyl guanidinobenzoate; DAPA, dansylarginine *N*,*N*-(3-ethyl-1,5-pentanediyl)amide; Bz, benzoyl; EDTA, ethylenediaminetetraacetic acid.

thrombin formed per unit time by the equation thrombin $(M)/t = a/(\epsilon k/2)$ using a value of 9400 for the molar absorbtion coefficient (ϵ) of *p*-nitroanilide at 410 nm and an experimentally determined first order rate constant (k) of 52/s for α-thrombin hydrolysis of S-2238 at our standard conditions. All assays were performed in a Cary 118B spectrophotometer with the cell compartment maintained at 30 °C. The standard reaction mixture contained the following: triethanolamine hydrochloride, pH 7.5, 50 mM; S-2238, 0.1 mM; prothrombin, 116 nM; ECV-P, 0.3–1.5 nM, in a final volume of 0.6 mL.

The rate of activation of factor X was measured by the method of Morris et al. (1978).

Fluorometric Assay of Prothrombin Activation. Human prothrombin in 1.2 mL of 0.01 M Tris buffer, pH 7.5, containing 0.15 M NaCl was mixed with 40 μL of 5 mM DAPA in a fluorescence cuvette, and the activation was initiated by adding 200 μL of ECV-P (0.05 mg/mL). The sample was then irradiated at 350 nm while the fluorescence change was monitored at 530 nm using a Perkin-Elmer fluorometer (Model 44B).

Other Analyses. Amino acid analysis was performed as previously described (Bolotin et al., 1977). *N*-Acetylneuraminic acid was determined by the procedure of Aminoff (1961), hexose by the method of Dubois et al. (1956), and hexosamine by the method of Boas (1953). Prothrombin protein concentration was determined by absorbance at 280 nm using an $A_{280}^{1\%}$ of 13.8 (Kisiel & Hanahan, 1973), and ECV-P protein concentration was determined by the method of Lowry et al. (1951) for the analytical procedures or by absorbance assuming an $A_{280}^{1\%}$ of 10 (Morita & Iwanaga, 1978).

Results

Purification of ECV-P. ECV-P was purified by the following procedure. All operations were performed at 4 °C.

Approximately 1 g of crude venom was dissolved in 25 mL of 0.02 M Mes–Tris buffer, pH 5.9, 2×10^{-3} M benzamidine, and 5 mM DFP. The sample was centrifuged at 16000*g* for 1 h and the supernatant applied to a 2.5 × 10 cm Sepharose 4B–wheat germ agglutinin column which was previously equilibrated with 0.02 M Mes–Tris, pH 5.9, containing 2×10^{-3} M benzamidine (buffer A). Elution of the proteins was achieved by washing the column sequentially with 85 mL of buffer A, 120 mL of 1.3 M NaCl in buffer A, and 120 mL of 1.3 M NaCl plus 0.2 M *N*-acetylglucosamine in buffer A (Figure 2A). ECV-P was eluted with the latter solution. This procedure gives a 20-fold purification with an overall recovery of about 80%. The column can be regenerated with an aqueous solution of 1% $NaDodSO_4$.

The fractions which contained ECV-P activity were pooled and dialyzed against 0.05 M Tris-HCl, pH 8.0, and applied to a DEAE-Sephacel column (1 × 45 cm). The ECV-P was eluted with a linear NaCl gradient formed by 300 mL of 0.05 M Tris-HCl, pH 8.0, and 300 mL of 0.05 M Tris, pH 8.0, containing 0.5 M NaCl. An overall purification of 40-fold with a recovery of about 17% was achieved with this procedure (Figure 2B). The fractions which contained ECV-P activity were concentrated to about 1 mg/mL by ultrafiltration and stored at −70 °C. The material was judged pure by virtue of a single band upon $NaDodSO_4$–polyacrylamide gel electrophoresis on 6% gels. Additionally, a single band was observed when stained with PAS. Table I summarizes the purification procedure.

Physical and Chemical Properties. The molecular weight of the purified ECV-P was estimated to be 55 000 by $NaDodSO_4$–polyacrylamide gel electrophoresis (inset of Figure 2B). ECV-P was assumed to be a glycoprotein since the gels also gave a single PAS positive band in the same position as the Coomassie Blue positive protein band. These results are the same as those reported by Morita & Iwanaga (1978), indicating that our preparation of ECV-P is the *E. carinatus* procoagulant.

Table I: Purification of ECV-P

preparation	total protein (mg)	total act. (units)[a]	sp act. (units/mg)	recovery (%)
crude venom	1000	10	0.01	
wheat germ agglutinin	40	8.0	0.2	80
DEAE-Sephacel	4.2	1.7	0.4	17

[a] One unit of ECV-P catalyzes the formation of 1 μmol of thrombin per min under the conditions described under Materials and Methods.

Table II: Amino Acid and Carbohydrate Composition of ECV-P

amino acid	mol %[a]	amino acid	mol %[a]
Lys	4.8	Ala	5.4
His	2.0	Cys[b]	8.6
Arg	4.6	Val	3.8
Asp	15.9	Met	1.7
Thr	6.9	Ile	8.8
Ser	6.2	Leu	6.9
Glu	7.4	Tyr	3.4
Pro	4.4	Phe	2.6
Gly	6.5		

carbohydrate	wt %
hexose	7.2
N-acetylhexosamine	5.8
N-acetylneuraminic acid	4.2

[a] Average values of 24-, 48-, and 72-h hydrolysates corrected for destruction of hydoxyamino acids and incomplete hydrolysis as previously described (Bolotin et al., 1977). [b] Cysteine was determined as carboxymethylcysteine.

Table III: Inhibitors of ECV-P

inhibitor	inhibition[a] (%)
5 mM pyridine-2,6-dicarboxylic acid	100
10 mM *o*-phenanthroline	100
5 mM EDTA	100
5 mM EDTA–5 mM $CaCl_2$	0
50 mM $CaCl_2$	0
50 mM $MnCl_2$	100
2 mM β-mercaptoethanol	100
10 mM L-cysteine	100
17 mM ascorbic acid	100
1 μM iodoacetamide	57
100 μM iodoacetamide	75

[a] The activity of ECV-P was measured as described in the text.

The amino acid and carbohydrate compositions of ECV-P are shown in Table II. Our values for the amino acid composition are very similar to those reported by Morita & Iwanaga (1978) except for Met (1.7% vs. 2.9%), Ile (8.8% vs. 4.6%), Arg (4.6% vs. 6.8%), and Cys (8.6% vs. 3.8%). The unusually high value for Cys is surprising in view of the fact that the protein migrates the same before and after reduction on $NaDodSO_4$–polyacrylamide gels. The total carbohydrate composition was determined to be 16.6% with about an equal distribution of hexose, hexosamine, and sialic acid. The carbohydrate composition of ECV-P has not been previously reported.

The activity of ECV-P is inhibited by EDTA and other chelating agents (Morita & Iwanaga, 1978; Table III). We,

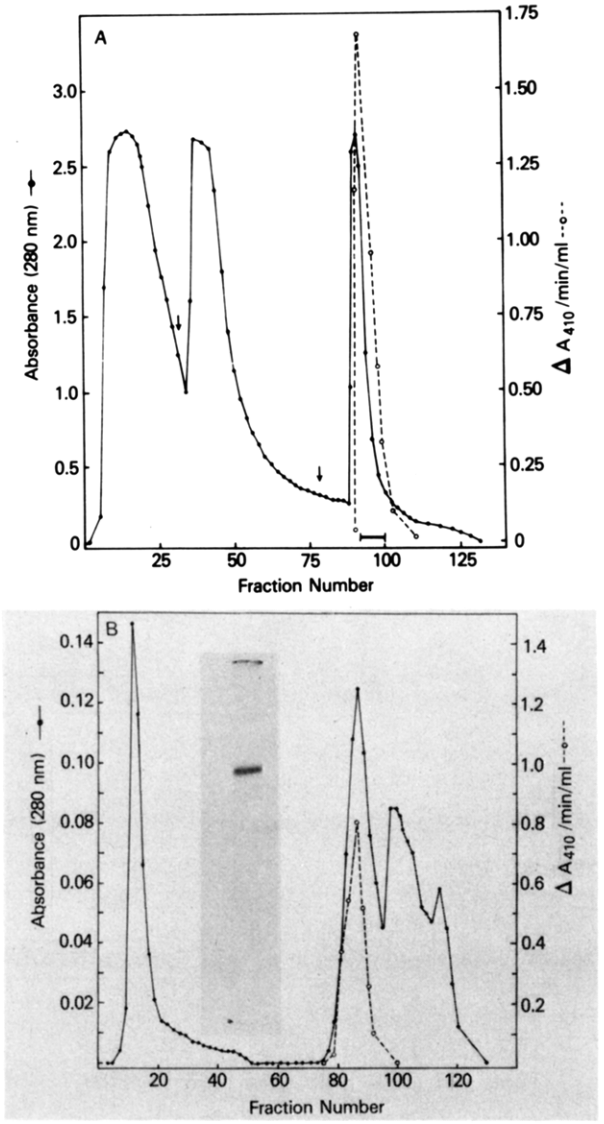

FIGURE 2: (A) Affinity chromatography on a column of wheat germ agglutinin–Sepharose (2.5 × 10 cm). Elution of the protein was achieved as described in the text. The arrows mark where the eluting solution was changed. The solid horizontal line under the activity peak indicates which tubes were pooled for use in the next step. (B) Ion-exchange chromatography on DEAE-Sephacel (1 × 45 cm). ECV-P was eluted as described in the text. The inset shows the pattern of the pooled active fractions upon electrophoresis on a 6% polyacrylamide gel in the presence of $NaDodSO_4$.

therefore, attempted to determine whether or not ECV-P contained a tightly bound metal ion by atomic absorption on a mutielement atomic absorption spectrometer equipped with a continuum source and an echelle monochromator modified for wavelength modulation (Harnley & O'Haver, 1977; Harnley et al., 1979). The ECV-P was prepared for atomic absorption spectroscopy by passing a solution of protein (2 mg/mL) through a mixed-bed Chelex G-25 (Bio-Rad, Na^+) column (0.9 × 10 cm) previously equilibrated with 50 mM Tris, pH 8.0, and 0.15 M NaCl. Virtually no change in the specific activity of the protein was observed during this procedure, and the protein recovery was quantitative. Atomic absorption was performed with a 10-μL sample containing 0.8 mg of protein/mL. These studies revealed that neither Ca, Co, Cr, Cu, Fe, Mn, Ni, Mo, nor Zn ion is present at more than 0.01 mol/mol. Thus, if ECV-P is a metalloenzyme, the metal ion is not one of the ions usually found in such proteins. $NaDodSO_4$ gels performed after Chelex treatment indicated no breakdown of the protein had occurred, and interestingly,

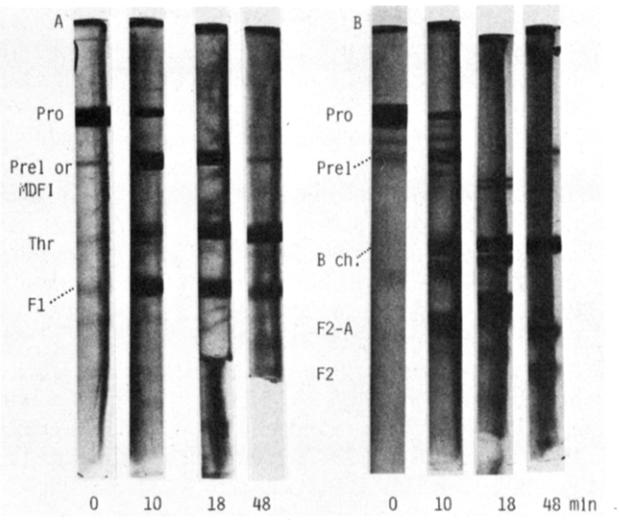

FIGURE 3: Time course of prothrombin activation by ECV-P in the absence of inhibitors. Prothrombin (1.7 mg) and ECV-P (19.4 μg) were incubated in 1.5 mL of 10 mM Tris, pH 7.5, containing 0.15 M NaCl. $NaDodSO_4$ gel electrophoresis in the absence (A) and presence (B) of 1% β-mercaptoethanol on 10% acrylamide gels was performed by the procedure of Laemmli (1970). The abbreviations used are the following: Pro, prothrombin; MT, meizothrombin; Prel, prethrombin-1; MDFI, meizothrombin-(des F1); Pre2, prethrombin-2; F 1·2, fragment 1·2; Thr, thrombin; F1, fragment 1; F2, fragment 2.

kinetic studies showed that the enzyme was still inhibited by 5 mM EDTA.

Enzymatic Properties. In agreement with the results of Morita & Iwanaga (1978), we could not detect the activation of factor X or the hydrolysis of S-2222, S-2238, S-2160, or tosylarginine methyl ester by ECV-P. Plots of the reciprocal of the rate of activation of prothrombin vs. the reciprocal of the prothrombin concentration gave a value of 0.45 μM for the K_m of prothrombin and a k_{cat} of 20/min.

As shown in Table III, ECV-P is inhibited by the chelators EDTA, *o*-phenanthroline, and pyridine-2,6-dicarboxylic acid. The inhibition by EDTA and *o*-phenanthroline could not be reversed by the subsequent addition of up to 50 mM Ca^{2+}; however, premixing equimolar amounts of Ca^{2+} and EDTA before the addition of ECV-P prevented the inhibition. Although these data are indicative of ECV-P being a metalloprotein, we could find no metal ion in the ECV-P (see previous section). The enzyme is also inhibited by the reducing agents β-mercaptoethanol, cysteine, and ascorbate, as well as by the alkylating agent iodoacetamide. In contrast to these results, Morita & Iwanaga (1978) found no inhibition with 10 mM iodoacetate.

Activation of Human Prothrombin by ECV-P, Meizothrombin, and Meizothrombin-(des F1). The conversion of prothrombin to split products was studied in the presence and absence of proteolytic enzyme inhibitors in order to deduce the temporal sequence of events. Figure 3 shows the $NaDodSO_4$–polyacrylamide gel electrophoresis pattern obtained for reduced and nonreduced reaction mixtures incubated in the absence of inhibitors. After 10 min most of the prothrombin has been converted to prethrombin-1 and meizothrombin-(des F1) as indicated by the appearance of bands corresponding to prethrombin-1 and the B chain on the reduced gels. The lack of a fragment 1 band and the appearance of a band corresponding to a molecular weight of 19 000 on the reduced gels demonstrate that fragment 1 has been converted to a two-chain fragment consisting of a subfragment α and subfragment β as reported by Franza et al. (1975). By 18 min only trace amounts of prethrombin-1 remain, as es-

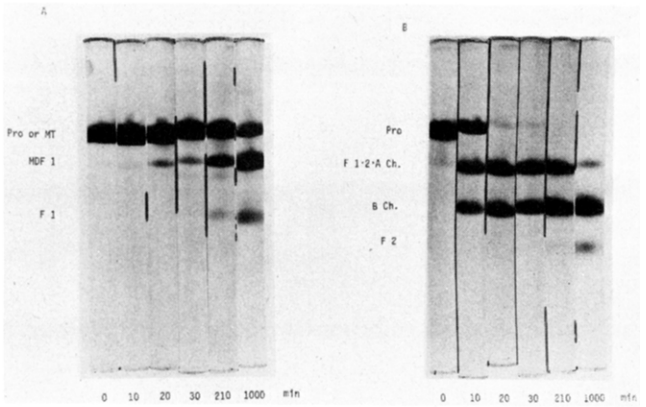

FIGURE 4: Time course of prothrombin activation by ECV-P in the presence of DAPA. Prothrombin (1.4 mg) and ECV-P (10 μg) were incubated in 1.8 mL of 10 mM Tris, pH 7.5, containing 0.15 M NaCl in the presence of 0.125 mM DAPA. The electrophoresis was performed in the absence (A) or presence (B) of 1% β-mercaptoethanol by the procedure of Weber & Osborn (1969).

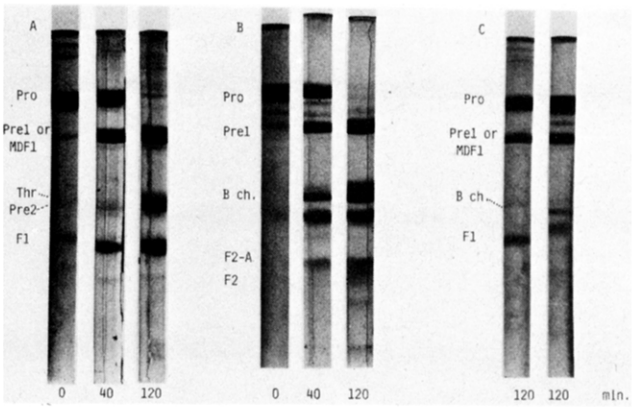

FIGURE 5: Time course of prothrombin activation by meizothrombin. Meizothrombin (0.114 mg) after removal of ECV-P by wheat germ agglutinin was incubated with prothrombin (1.72 mg) as described in Figure 3. Electrophoresis was performed by the procedure of Laemmli (1970) in the absence (A) or presence (B) of 1% β-mercaptoethanol. The same amount of ECV-P was treated with wheat germ agglutinin and reacted with prothrombin in order to ensure that the reaction is not due to ECV-P contamination of the meizothrombin. Electrophoresis was performed (C) in the absence (left) and presence of 1% β-mercaptoethanol (right).

sentially all of the band corresponding to prethrombin-1 plus meizothrombin-(des F1) is reducible. By 48 min all of the meizothrombin-(des F1) has been converted to thrombin. The identity of thrombin and the B chain on these and other gels was confirmed by coelectrophoresing one sample from each experiment with an authentic sample of thrombin.

When no inhibitors of thrombin are present, one cannot determine which bond cleavages are catalyzed by ECV-P and which cleavages are catalyzed by the proteolytically active products. We, therefore, investigated the ECV-P catalyzed proteolysis of prothrombin in the presence of benzamidine, DFP, or DAPA.

When either 25 mM benzamidine or 10 mM DFP was added to the reaction mixture, qualitatively similar results were obtained, indicating that these inhibitors are inefffective in eliminating side products.

When ECV-P is incubated with prothrombin in the presence of 10^{-4} M DAPA, meizothrombin accumulates (Figure 4). Meizothrombin-(des F1) appears only after long incubation times. Thus, ECV-P can cleave the Arg–Ile bond to form meizothrombin very rapidly. In a slower reaction, meizothrombin is converted to meizothrombin-(des F1) by either ECV-P or autolysis. Similar results have been reported by Briët et al. (1981).

When no inhibitors were present, significant thrombin formation was observed by 18 min (Figure 3). When DAPA, an inhibitor of thrombin-like proteases, was present, no bond cleavage between prothrombin fragment 2 and the A chain was observed. From these observations we deduced that either meizothrombin or meizothrombin-(des F1) or both were catalyzing the formation of thrombin from meizothrombin-(des F1). Meizothrombin and meizothrombin-(des F1) were prepared as described under Materials and Methods to test this hypothesis. When meizothrombin was incubated with prothrombin, both prethrombin-1 and meizothrombin-(des F1) are formed (Figure 5). The appearance of the B chain at 40 min before significant amounts of thrombin are formed indicates the formation of meizothrombin-(des F1). A control incubation in which ECV-P was incubated in the absence of prothrombin and adsorbed with wheat germ agglutinin and the supernatant incubated with prothrombin served as a control (Figure 5C). After 120 min some prethrombin-1 was formed, and minor bands corresponding to prethrombin-2 and the B chain are detectable. From these results we can conclude that over 90% of the B chain is produced by meizothrombin and only a trace amount of ECV-P, if any, is contaminating our

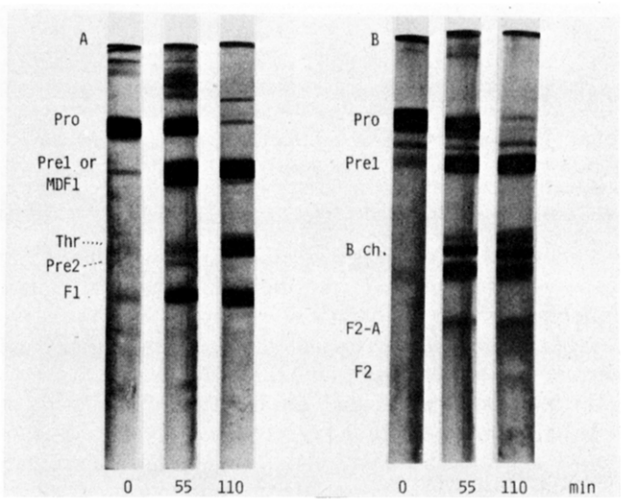

FIGURE 6: Time course of prothrombin activation by meizothrombin-(des F1). Meizothrombin-(des F1) (48.3 μg) was incubated with 1.72 mg of prothrombin as described in Figure 3. Electrophoresis was performed in the absence (A) or presence (B) of 1% β-mercaptoethanol.

reaction mixture. The kinetic experiments described below give further evidence for this. Similar results are obtained when meizothrombin-(des F1) was incubated with prothrombin (Figure 6).

The reaction of prothrombin with ECV-P, meizothrombin, and meizothrombin-(des F1) was also followed by analysis of the reaction mixtures using gel permeation high-performance liquid chromatography (HPLC). Figure 7 shows a typical time course analysis of the reaction between meizothrombin-(des F1) and prothrombin. This technique is limited by the fact that although prothrombin can be separated from its products, meizothrombin, meizothrombin-(des F1), and prethrombin-1 have similar elution times (Kosow et al., 1981). However, the rate of utilization of prothrombin can easily be determined by measuring the area under the prothrombin peak. In this experiment all the prothrombin was utilized by 110 min and a large peak of a mixture of meizothrombin, meizothrombin-(des F1), and prethrombin-1 accumulated. A kinetic study of prothrombin utilization by meizothrombin and meizothrombin-(des F1) determined by measuring pro-

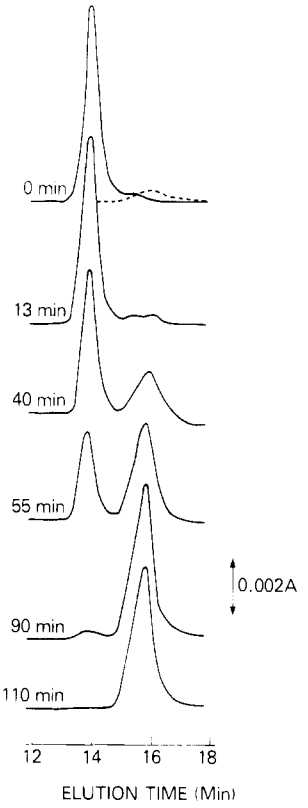

FIGURE 7: Time course of prothrombin activation by meizothrombin-(des F1) analyzed by gel permeation HPLC. Reaction conditions are the same as described for Figure 6. Analysis was performed as described in the text.

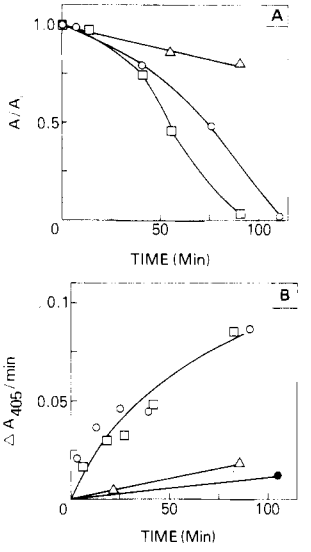

FIGURE 8: (A) Rate of utilization of prothrombin by meizothrombin and meizothrombin-(des F1). Meizothrombin and meizothrombin-(des F1) were prepared by incubation of prothrombin or prethrombin-1, respectively, with ECV-P as described in the text. After removal of the ECV-P by wheat germ agglutinin gel and removal of excess DAPA by gel filtration, 114 μg of meizothrombin (□), 48.3 μg of meizothrombin-(des F1) (○), or an equivalent amount of a gel treatment control incubation mixture in which ECV-P was incubated alone prior to wheat germ agglutinin (△) was mixed with 1.72 mg of prothrombin in 1.5 mL of 10 mM Tris, pH 7.5, containing 0.15 M NaCl. At the times indicated, 5-μL aliquots were analyzed by HPLC as described in the text. A/A_0 is the ratio of observed height of prothrombin peak to that at zero time. (B) The esterase activity of duplicate samples of the reaction mixtures was measured by incubation of 1 μL of reaction mixture with 0.62 mL of 0.1 mM S-2238 in 50 mM triethanolamine hydrochloride, pH 7.5. A reaction mixture which contained prothrombin alone (●) was also assayed for esterase activity.

thrombin peak heights is shown in Figure 8A. Since there is always some DAPA in the meizothrombin and meizothrombin-(des F1) preparations, the rates are very slow. However, the results clearly demonstrate that both of these enzymes can convert prothrombin to other forms.

The reaction was also analyzed by incubating aliquots of the reaction mixture with the chromogenic substrate S-2238 in order to demonstrate that both meizothrombin and meizothrombin-(des F1) can activate prothrombin to an active enzyme(s). The data illustrated in Figure 8B give clear evidence that prothrombin activation is occurring. The initial rate of active site formation is 20 times more rapid than that of the no enzyme control (●) and 10 times more rapid than that of the control designed to test for ECV-P contamination (△).

Discussion

Previously described procedures for the purification of ECV-P have either not yielded a homogeneous product (Franza et al., 1975) or required many steps (Schieck et al., 1972; Morita & Iwanaga, 1978). The procedure described here takes advantage of the fact that ECV-P is a glycoprotein and will therefore be held up on a Sepharose 4B wheat germ agglutinin column. Whereas the procedure of Morita & Iwanaga (1978) requires five column steps and gives only an 8% recovery, our procedure requires but two column steps and gives a 17% recovery. The amino acid composition of our preparation of ECV-P is similar to that reported by Morita & Iwanaga (1978). In addition, we have performed a carbohydrate analysis on the protein (Table II). We have also analyzed ECV-P for metals since the enzyme is inhibited by metal ion chelators (Morita & Iwanaga, 1978; Table III), and the inhibition by EDTA can be prevented if the EDTA is first mixed with equimolar $CaCl_2$ (Table III). To our surprise, neither Ca, Co, Cr, Cu, Fe, Mn, Zn, Ni, nor Mo ion could be detected in amounts greater than 0.01 mol/mol. Also, adding Ca^{2+} to the enzyme after EDTA did not reverse the inhibition due to the chelator. Thus, it is not clear whether the metal chelators inhibit by reacting with a protein-bound metal ion or by reacting with an effector site on the ECV-P.

Two different reaction paths have been proposed for the activation of prothrombin by ECV-P. Morita et al. (1976) have proposed that ECV-P first cleaves the Arg–Ile bond linking the A and B chains of prothrombin (arrow 3, Figure 1) to form meizothrombin, which then autocatalytically cleaves itself (at arrow 1, Figure 1) to produce fragment 1 and meizothrombin-(des F1). Conversely, Kornalik & Blomback (1975) and Franza et al. (1975) have proposed that ECV-P cannot only cleave fragment 1 from prothrombin but that this is the first step in the activation scheme. The ECV-P was presumed to then cleave the Arg–Ile bond in prethrombin-1 to form meizothrombin-(des F1). We have been able to obtain results similar to those reported by all of the above laboratories when we used the same inhibitors that were used in the respective studies. In the presence of either no inhibitor, benzamidine, or DFP, we obtained evidence for the initial formation of prethrombin-1 and meizothrombin-(des F1). These results were obtained by Kornalik & Blomback (1975) in the absence of inhibitors, by Franza et al. (1975) in the presence of DFP, and by Morita et al. (1976) in the presence of DFP plus benzamidine. Only in the presence of either hirudin or NPGB were Morita et al. (1976) able to detect meizothrombin. We were also able to detect small amounts of meizothrombin when NPGB was present.

Recently, Nesheim et al. (1979) have shown that DAPA is an effective reversible inhibitor of thrombin with a K_i of 10^{-7}

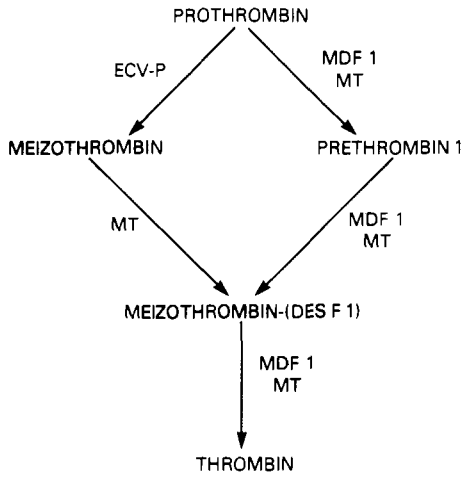

FIGURE 9: Activation of prothrombin with ECV-P, meizothrombin, and meizothrombin-(des F1). The names of the substrates and products are written in full while the enzymes are abbreviated as follows: ECV-P, the prothrombin activator derived from *E. carinatus* venom; MT, meizothrombin; MDF1, meizothrombin-(des F1).

M. When DAPA is utilized as an inhibitor, meizothrombin is the major product of the reaction between ECV-P and prothrombin while small but detectable amounts of meizothrombin-(des F1) are also formed (Figure 4).

The formation of meizothrombin-(des F1) was decreased when EDTA was added to inhibit ECV-P after all the prothrombin was converted.[2] These results indicate that the initial product is meizothrombin and that either ECV-P can also cleave meizothrombin to meizothrombin-(des F1) or EDTA can prevent autolysis of meizothrombin to meizothrombin-(des F1). Further studies are needed to determine the mechanism of EDTA inhibition. Since meizothrombin does not accumulate in the absence of thrombin inhibitors, it follows that meizothrombin can autolyze to meizothrombin-(des F1).

In order to determine whether meizothrombin or meizothrombin-(des F1) was capable of cleaving the bond between fragment 2 and prethrombin-2, we incubated catalytic amounts of these enzymes with prothrombin. To our surprise, both meizothrombin and meizothrombin-(des F1) converted prothrombin to a mixture of prethrombin-1, and the proteolytically active enzymes meizothrombin-(des F1) and thrombin (Figures 5 and 6). That active enzymes were formed was demonstrated by the increase of S-2238 amidolytic activity during the course of these reactions (Figure 8B). This result was unexpected since thrombin is not capable of converting prothrombin to an active enzyme.

The results of this study are summarized in Figure 9. Prothrombin is converted to meizothrombin by ECV-P. Meizothrombin-(des F1) is produced from meizothrombin by autolysis. Meizothrombin-(des F1) can also be formed by the action of either meizothrombin or meizothrombin-(des F1) on prothrombin with the intermediate formation of prethrombin-1. The final reaction is thrombin production from meizothrombin-(des F1) which is catalyzed by both meizothrombin and meizothrombin-(des F1). Novoa & Seegers (1980) claim that a likely sequence for thrombin formation by factor Xa also begins with meizothrombin production.

Acknowledgments

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[2] M.-J. Rhee, S. Morris, and D. P. Kosow, unpublished observations.

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