# Characterization of a Protein C Activator from the Venom of *Agkistrodon contortrix contortrix*†

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*Received July 15, 1987; Revised Manuscript Received December 11, 1987*

ABSTRACT: An enzyme capable of activating protein C has been purified 60-fold from the venom of the Southern copperhead snake (*Agkistrodon contortrix*) by ion-exchange and gel filtration chromatography. The purified enzyme consists of a single polypeptide with an apparent molecular weight of 37 000. The isoelectric point of the protein C activator was determined to be 6.3 when measured by chromatofocusing. The enzyme was inhibited by *p*-nitrophenyl *p*-guanidinobenzoate, phenylmethanesulfonyl fluoride, and D-Phe-Pro-Arg-$CH_2Cl$ but was not affected by cysteine-directed reagents or by metal chelators. These results suggest that the enzyme is a serine protease. Protein C activator was capable of hydrolyzing the thrombin substrate tosyl-Gly-Pro-Arg-*p*-nitroanilide (TGPRpNA), and steady-state kinetic studies determined that the $K_m$ for amidolysis of this substrate was 1.1 mM while the $V_{max}$ was 66 $s^{-1}$. The activator demonstrated considerable substrate specificity since the amidolysis of D-Phe-Pip-Arg-pNA, D-Ile-Pro-Arg-pNA, Bz-Ile-Glu-Gly-Arg-pNA, D-Val-Leu-Arg-pNA, and pyrGlu-Pro-Arg-pNA was less than 10% of that of TGPRpNA when measured under identical conditions using 1.0 mM substrate concentrations. The enzyme appears to be thrombin-like in its preference for arginyl as compared to lysyl chloromethyl ketones as well as by its inhibition by benzamidine and *p*-aminobenzamidine. However, the substrate specificity of the activator is distinguished from $\alpha$-thrombin in that it does not clot fibrinogen and does not react with antithrombin III or hirudin. The purified enzyme is an extremely effective activator of protein C, and gel studies demonstrated that activation of protein C was associated with cleavage of the heavy chain. Steady-state kinetic parameters revealed an apparent $K_m$ for protein C of 0.57 $\mu$M and an apparent $V_{max}$ of 0.02 $s^{-1}$. It was found that the activation of protein C was inhibited by $CaCl_2$ and by NaCl. A $K_{i,app}$ of 99 $\mu$M and 120 mM was measured for the inhibition by $Ca^{2+}$ and NaCl, respectively. $Ca^{2+}$ and NaCl had no effect on the amidolysis of TGPRpNA catalyzed by the activator or on the inhibition of the enzyme by D-Phe-Pro-Arg-$CH_2Cl$, suggesting that the effect of $Ca^{2+}$ and NaCl likely results from their interaction with protein C.

Protein C is a trace plasma glycoprotein (4 $\mu$g/mL plasma) which plays a critical role in the regulation of hemostasis. The importance of protein C has been directly demonstrated by identifying individuals with heterozygous or homozygous deficiency of the molecule who have histories of venous thromboembolism or suffer severe thrombotic events and early death, respectively [see review by Clouse and Comp (1986)].

Human protein C is a 62 000 molecular weight protein consisting of two disulfide-linked subunits (Kisiel, 1979). It is structurally related to the vitamin K dependent coagulation proteins (Beckmann et al., 1985; Foster et al., 1985). Limited proteolysis of a dodecapeptide at the N-terminus of the heavy chain results in conversion of the zymogen protein C to the serine protease activated protein C (Kisiel et al., 1977). This reaction, which occurs in the microvasculature on the surface of endothelial cells, is catalyzed by a 1:1 stoichiometric complex of the enzyme thrombin with the cell-surface protein thrombomodulin (Esmon & Owen, 1981; Esmon et al., 1982). Activated protein C functions as an anticoagulant by proteolytic degradation of factors Va and VIIIa (Walker et al., 1979; Tracy et al., 1983; Suzuki et al., 1983; Fulcher et al., 1984; Eaton et al., 1986). In addition, activated protein C promotes fibrinolysis, which has been attributed to its ability to neutralize plasminogen activator inhibitor (Sakata et al., 1985; Taylor & Lockhart, 1985; van Hinsbergh et al., 1985).

There is considerable interest in developing an easier and more precise method for determining the functional levels of protein C in a variety of clinical disorders. This requires the identification of protein C activators which may have more desirable properties in terms of availability, efficiency, and especially specificity than the physiological activator thrombin/thrombomodulin. There are three recent reports on the purification and properties of a protein C activator (PCA)[1] from the venom of the Southern copperhead snake (*Agkistrodon contortrix contortrix*). The purification of a 37 000-dalton nonezymatic PCA (Martinoli & Stocker, 1986) as well as of an $M_r$ 20 000 protease (Klein & Walker, 1986) has been described. More recently, Kisiel et al. (1987) have reported the isolation of an $M_r$ 37 000 protease. For the most part, the properties of these enzymes have not been characterized, and the relationship between activators remains unknown. The purpose of this investigation was to purify and further char-

† This work was supported in part by Grant HL30200 from the National Heart, Lung, and Blood Institute.

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[1] Abbreviations: PCA, protein C activator; pNA, *p*-nitroanilide; EDTA, ethylenediaminetetraacetic acid; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PEG, poly(ethylene glycol) 8000; PMSF, phenylmethanesulfonyl fluoride; PPACK, D-Phe-Pro-Arg-$CH_2Cl$; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TGPRpNA, tosyl-Gly-Pro-Arg-*p*-nitroanilide; TLCK, tosyl-Lys-$CH_2Cl$; Tris, tris(hydroxymethyl)aminomethane; SBTI, soybean trypsin inhibitor; TM, thrombomodulin; BSA, bovine serum albumin.

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acterize the protein C activator(s) from this venom.

## Materials and Methods

*Materials.* Venom from the Southern copperhead *Agkistrodon contortrix contortrix* was purchased from the Miami Serpentarium, Salt Lake City, UT. The chromatographic media SP-Sephadex C-50, Sephacryl S-200 (superfine), Sephadex G-100, and the chromatofocusing gel PBE 94 and Polybuffer 74 were from Pharmacia, Inc., Piscataway, NJ. The chromogenic substrates, all of which were *p*-nitroanilide (pNA) derivatives, were obtained as follows: tosyl-Gly-Pro-Arg-pNA (TGPRpNA) was from Boehringer Mannheim, Indianapolis, IN; H-D-Val-Leu-Arg-pNA-2HCl (S-2266), H-D-Phe-Pip-Arg-pNA (S-2238), D-Ile-Pro-Arg-pNA (S-2288), pyrGlu-Pro-Arg-pNA (S-2366), and Bz-Ile-Glu-Gly-Arg-pNA (S-2222) were purchased from Helena Laboratories, Beaumont, TX. The concentration of each chromogenic substrate was determined by measurement of its absorbance at 316 nm using a molar extinction coefficient of 13 000 $M^{-1}$ $cm^{-1}$. Hirudin was obtained from Pentapharm Ltd., Basel. Soybean trypsin inhibitor (type I-S), heparin (porcine intestinal mucosa, grade 1), NPGB, PMSF, TLCK-HCl, iodoacetamide, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) were from Sigma Chemical Co., St. Louis, MO. *p*-Aminobenzamidine dihydrochloride and benzamidine hydrochloride were from Aldrich Chemical Co., Milwaukee, WI. PPACK-2HCl was from Calbiochem, La Jolla, CA. Ultrapure guanidine hydrochloride was from Schwarz/Mann, Inc., Spring Valley, NY. Ultrapure NaCl was from Alfa/Ventron Corp., Danvers, MA, and was used in the experiments which examined its effect on protein C activation.

*Proteins.* Human protein C was purchased from American Diagnostica Inc., Greenwich, CT. Human $\alpha$-thrombin was purified as previously described and had a specific activity of 2400 units/mg of protein (Orthner & Kosow, 1981). Antithrombin III was produced from human plasma for the American Red Cross by the method of Wickerhauser and Williams (1979). These proteins were greater than 95% homogeneous as judged by SDS–PAGE. Protein concentrations were determined spectrophotometrically by using the following molecular weights and absorption coefficients: protein C, $M_r$ 62 000 and $E^{1\%}_{280nm}$ = 14.5 (Kisiel, 1979); $\alpha$-thrombin, $M_r$ 36 500 and $E^{1\%}_{280nm}$ = 18.3 (Fenton et al., 1977); PCA, $M_r$ 37 000 and $E^{1\%}_{280nm}$ = 10.0 (see Results); antithrombin III, $M_r$ 58 000 and $E^{1\%}_{280nm}$ = 6.5 (Nordenman et al., 1977). The amount of active PCA or $\alpha$-thrombin was determined by using the active-site titrant NPGB according to the method of Chase and Shaw (1967). The active-site concentration of purified PCA used in these studies was 0.86 mol/mol of protein, while the active-site concentration of $\alpha$-thrombin was 0.80 mol of active site/mol.

*SDS–PAGE.* Samples were electrophoresed on slabs consisting of a 13% acrylamide running gel [acrylamide:bis(acrylamide) ratio of 42:1] and a 4% stacking gel using the buffer system of Laemmli (1970) and stained with Coomassie brilliant blue. Molecular weights were estimated from a standard curve of log molecular weight vs relative migration distance using the protein standards bovine serum albumin (68 000), ovalbumin (43 000), chymotrypsinogen A (25 700), and cytochrome *c* (12 500).

*High-Pressure Gel Permeation Chromatography.* Analyses were performed on a Beckman Model 341 isocratic liquid chromatograph interfaced to a Spectra-Physics SP4270 integrator. Separations were run at 25 °C on a 7.5 × 300 mm TSK3000SW column (Beckman Instruments, Inc., San Ramon, CA) using 0.05 M Tris-HCl/0.15 M NaCl, pH 7.4, buffer at a flow rate of 1.0 mL/min. The eluant was monitored for absorbance at 280 nm, and 200-$\mu$L fractions were analyzed for the ability to hydrolyze TGPRpNA as well as the ability to activate protein C. The molecular weight of PCA was estimated from a standard curve of log molecular weight vs retention time using the protein standards IgG (158 000), ovalbumin (43 000), and myoglobin (17 000).

*Chromatofocusing.* This was carried out at 25 °C using a 0.9 × 15 cm column containing PBE 94 polybuffer exchanger equilibrated in 0.025 M imidazole hydrochloride, pH 7.5. After application of PCA equilibrated in this same buffer, elution was performed with Polybuffer 74 (1:8 dilution), pH 4.0, at a flow rate of 1 mL/min. Fractions (2 mL) were monitored for pH, absorbance at 280 nm, amidolysis of TGPRpNA, and activation of protein C.

*Chromogenic Assays.* The amidolytic activity of PCA was measured on a Cary 219 spectrophotometer in 1-cm path-length cuvettes thermostated to 30 °C. Assays were performed in 0.05 M Tris-HCl/0.15 M NaCl, pH 7.5, containing 1 mg/mL PEG (TSP buffer) in a total volume of 0.60 mL. The reactions were started by the addition of PCA to a final concentration of 8.5 nM, and the formation of *p*-nitroaniline was monitored continuously at 410 nm. A molar extinction coefficient of 9600 $M^{-1}$ $cm^{-1}$ was used to determine concentrations of *p*-nitroaniline. Kinetic parameters were determined by unweighted linear least-squares analysis of double-reciprocal plots of the initial velocity as a function of substrate concentration.

*Protein C Activation Assays.* The initial rate of protein C activation was measured in plastic microtiter plates (Immulon II, Dynatech Laboratories, Inc., Alexandria, VA) thermostated to 30 °C on a warm plate. Protein C was equilibrated in TSP buffer (except when noted otherwise) in a total volume of 190 $\mu$L. The reaction was initiated by the addition of 10 $\mu$L of PCA to a final concentration of 6.4 nM. At exactly 1.0 and 6.0 min, the reaction was stopped by the addition of 5 $\mu$L of 50 mg/mL soybean trypsin inhibitor, which was found to inhibit PCA. After the addition of S-2266 to a final concentration of 1.15 mM and buffer to a total volume of 300 $\mu$L, the plate was read on an MR600 reader (Dynatech) at 410 nm at 1.0-min intervals for 10 min. *p*-Nitroaniline solutions of known absorbance were used to calibrate the plate reader to identical absorbances as obtained on the spectrophotometer when using 1-cm path-length cuvettes. The rate of amidolysis was calculated from the absorbance vs time data by linear least-squares analysis, and the concentration of activated protein C was calculated on the basis of its specific activity as measured under identical conditions. The specific activity of activated protein C determined under these conditions was 0.11 $min^{-1}$ $\mu g^{-1}$. In all cases, initial velocity conditions were used in which less than 10% of the protein C was converted to activated protein C. In experiments which examined the effect of metal ions on the activation reaction, following the addition of soybean trypsin inhibitor, an appropriate amount of metal was added to equalize its concentration during the assay of activated protein C.

*Inhibitor Studies.* The effects of various inhibitors of different classes of proteases were examined. Concentrated stock solutions of inhibitors were prepared fresh before use as follows: NPGB and PMSF were dissolved in *N*,*N*-dimethylformamide; *p*-aminobenzamidine, iodoacetamide, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) were dissolved in 0.30 M Tris-HCl, pH 7.5; all other compounds were dissolved in water. Inhibition mixtures contained 63.5 nM PCA in the absence or presence of the indicated con-

Table I: Purification of Protein C Activator (PCA)[a]

| step | volume (mL) | protein (mg/mL) | total protein (mg) | PCA act. (units/mL) | total act. (units) | sp act. (units/mg) | x-fold purification | yield (%) |
|---|---|---|---|---|---|---|---|---|
| venom | 26 | 22.3 | 580 | 2144 | 55744 | 96 | 1 | 100 |
| SP-Sephadex | 36 | 1.1 | 39 | 1239 | 44604 | 1144 | 12 | 80 |
| Sephacryl S-200 | 30 | 0.5 | 16 | 1288 | 38626 | 2414 | 25 | 69 |
| Sephadex G-100 | 34 | 1.5 | 5 | 9064 | 30816 | 6163 | 64 | 55 |

[a] Protein was determined by the method of Lowry (1951), using human serum albumin as a standard. PCA was monitored by the protein C activation assay as described under Results.

centrations of inhibitors in TSP buffer in a total volume of 580 μL. Following 60 min at 25 °C, 20 μL of 6 mM TGPRpNA was added, and the initial rate of amidolysis was measured.

The kinetic constants for the inactivation of PCA by PPACK were determined by measuring the time course of inactivation at varying concentrtions of inhibitor. Incubation mixtures contained 460 nM PCA in TSP buffer in a total volume of 480 μL. The reaction was started by the addition of 20 μL of varying concentrations of PPACK to final concentrations of 3–15 μm. Aliquots (20 μL) were removed at various times, and residual activity was measured by chromogenic assay. The data were fit to the model

$$E + I \overset{K_i}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_2} E{-}I \qquad (1)$$

where E is PCA, I is PPACK, E·I is a reversible complex, $K_i$ is the dissociation constant of E·I, $k_2$ is the first-order alkylation rate constant, and E–I is the inactive alkylated PCA. Kinetic constants were determined by using eq 2 (Kitz & Wilson, 1962) where $k_{app}$ is the apparent pseudo-first-order rate constant obtained from a plot of log (percent activity remaining) vs time. Kinetic constants were obtained from a double-reciprocal plot of $k_{app}$ as a function of PPACK concentration. The best-fit line was determined by linear regression analysis using the method of least squares.

$$1/k_{app} = K_i/k_2(1/I) + 1/k_2 \qquad (2)$$

## Results

*Purification of PCA.* A protein C activator has been purified to homogeneity from crude venom by a three-step chromatographic procedure. The process was monitored by a protein C activation assay which was performed as follows: protein C was incubated in a microtiter plate at 30 °C at a final concentration of 2.5 units/mL (Martinoli & Stocker, 1986) in TSP buffer containing 5.5 mM EDTA in a 200-μL total volume. The activation was started by the addition of 10 μL of PCA-containing sample and allowed to proceed for exactly 6.0 min, at which time the reaction was stopped by the addition of 5 μL of 50 mg/mL soybean trypsin inhibitor. Following the addition of S-2266 to 1.15 mM and antithrombin III and heparin to 83 nM and 0.1 unit/mL, respectively, and adjustment of the volume to 300 μL, the plate was read at selected intervals and the rate of amidolysis calculated. Each sample was assayed in both the absence and presence of protein C in order to correct for any endogenous amidolytic activity that was not dependent upon protein C. In this assay, the amount of activated protein C generated, as measured by its amidolytic activity, was directly proportional to the concentration of PCA. One unit of activity was defined as the amount of PCA which resulted in activated protein C having a rate of 1 milliabsorbance unit per minute.

The first step of the purification procedure was essentially the same as that described by Klein and Walker (1986) except for the inclusion of a protease inhibitor in the buffer. Lyophilized venom (500 mg) was dissolved in 25 mL of 0.02 M imidazole hydrochloride/0.10 M NaCl, pH 6.5, containing 10 mM *p*-aminobenzamidine, dialyzed vs this same buffer, and applied to an SP-Sephadex C-50 column (1.4 × 42 cm) at 25 °C. The column was rinsed with the above buffer until unbound protein was no longer detected in the eluate which was conveniently monitored by high-pressure gel permeation chromatography. The column was then developed with a linear gradient of 0.10–0.60 M NaCl (300-mL total volume) in this same buffer, collecting 2.7-mL fractions. This procedure resulted in the separation of four protein peaks which were determined by Lowry assay (Lowry et al., 1951). Protein C activator activity was found associated only with the first protein peak. SDS–PAGE analysis showed that fractions having the highest activity consisted of a major protein band of estimated $M_r$ 20 000 with lesser amounts of several higher molecular weight species. Peak 1 was pooled and concentrated by ultrafiltration in an Amicon cell fitted with a PM10 membrane (Amicon Corp., Danvers, MA) and applied to a column of S-200 Sephacryl (2.5 × 85 cm) equilibrated in 0.05 M Tris-HCl/0.15 M NaCl, pH 7.5, containing 6.0 M urea at 4 °C. Four protein peaks were partially separated, and the protein C activation activity coincided with the second peak. The presence of urea was required to affect the separation at this step. In its absence, a single protein peak was obtained, presumably due to protein–protein interactions of contaminants. The peak 2 fractions were pooled and concentrated as described above and chromatographed on a G-100 Sephadex column (2.0 × 97 cm) at 4 °C in 0.05 M Tris-HCl/0.15 M NaCl, pH 7.5, at a flow rate of 0.5 mL/min. The purification procedure is summarized in Table I and resulted in an overall 60-fold purification at a 50% yield. The specific activity of purified PCA was 159 absorbance units $min^{-1}$ (mg of protein)$^{-1}$ using 0.2 mM TGPRpNA in the chromogenic assay. The

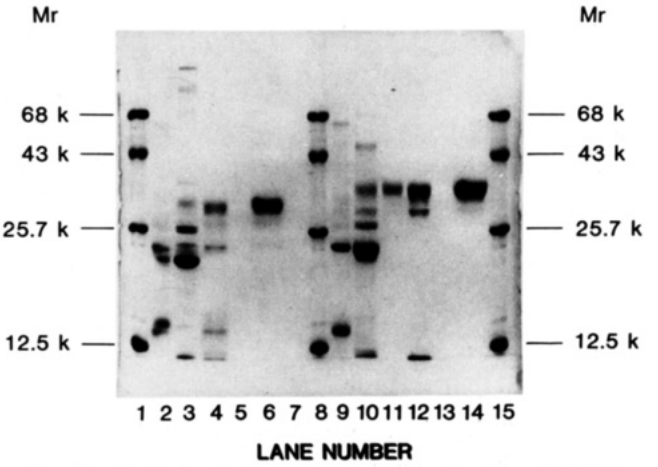


FIGURE 1: Analysis of samples from the purification of PCA by SDS–PAGE. Lanes 1, 8, and 15, reduced protein standards; lanes 2 and 9, venom; lanes 3 and 10, SP-Sephadex pool 40–55; lane 11, S-200 Sephacryl pool 69–73; lanes 6 and 14, G-100 final product. Lanes 5, 7, and 13, empty; lanes 2–6, nonreduced; lanes 9–14, reduced.

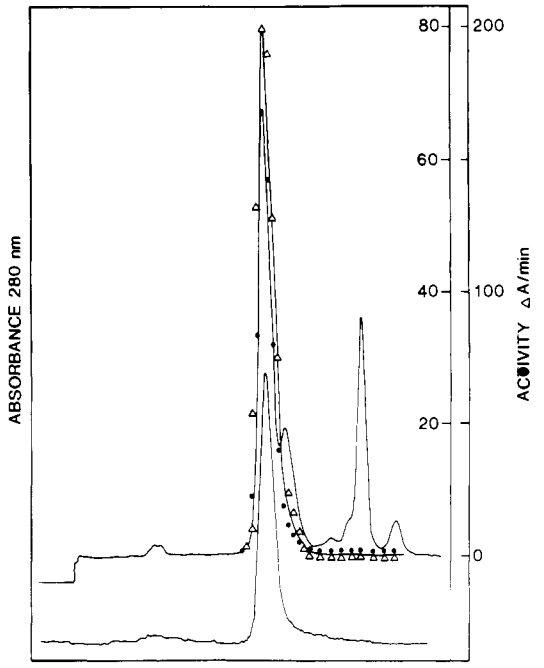


FIGURE 2: High-pressure gel permeation chromatography of samples from the purification of PCA. (Upper tracing) Pool of peak 2 fractions from the S-200 Sephacryl chromatography. (Lower tracing) Purified PCA (—), absorbance at 280 nm; (●) protein C activator activity; (△) amidolytic activity on TGPRpNA.

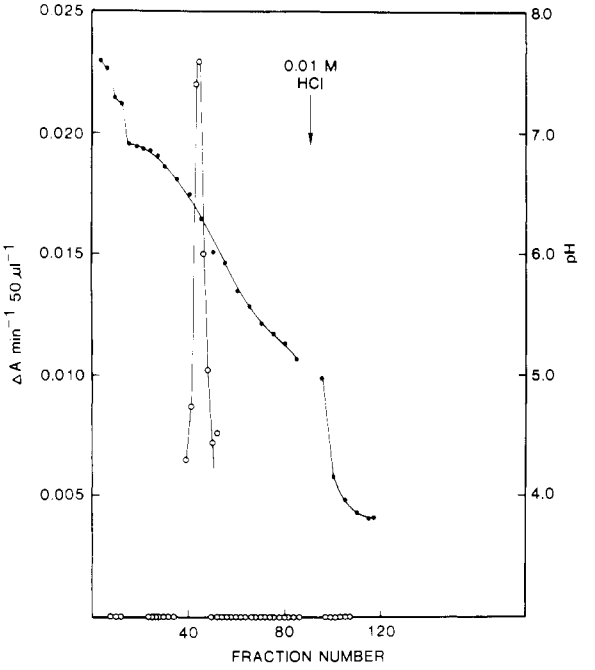


FIGURE 3: Chromatofocusing of purified PCA. (●) pH profile; (○) PCA activity on TGPRpNA.

protein was stored at 4 °C in 0.05 M Tris-HCl/0.15 M NaCl, pH 7.5, and no loss in activity was detectable after 3.5 months.

*Characterization of PCA.* Figure 1 shows the SDS–PAGE analysis of samples from the various steps of the purification. Lanes 3 and 10 show the material following the SP-Sephadex step, which consisted of a major component of estimated molecular weight of 20 000 with several minor components. Lanes 6 and 14 are the purified protein C activator which appeared as a single band, which was not reducible, and which had an estimated molecular weight of 37 000 in the presence of β-mercaptoethanol. The samples were also analyzed by high-pressure gel permeation chromatography in Tris–saline buffer, pH 7.5. As shown in Figure 2 (upper trace), the heterogeneity of the material resulting from the second chromatographic step was evident. While protein C activator activity as well as amidolytic activity on TGPRpNA coeluted with the major protein peak, several other peaks could be resolved. As seen in the lower trace, purified protein C activator resulting from the final step eluted as a single protein peak with an estimated molecular weight of 39 000. The elution behavior using gel filtration indicated that the activator did not self-associate under these nondenaturing conditions. Figure 3 shows the analysis of PCA by chromatofocusing. PCA eluted as a single peak having an apparent p*I* of 6.3. Again, both the protein C activation and amidolytic activities coeluted with the protein peak. These results, utilizing separation techniques based upon both size and charge, demonstrated the purity of the isolated protein as well as strongly suggested that the $M_r$ 37 000 protein was responsible for its activity.

*Effect of Protease Inhibitors on PCA.* Table II shows the effects of inhibitors of various classes of proteases on the activity of PCA. NPGB, a specific reagent for the active-site serine residue of serine proteases, completely inhibited the amidolytic activity of PCA. In addition, the protein C activation activity was also completely inhibited by NPGB. The active-site concentration of PCA could be readily titrated with NPGB (Chase & Shaw, 1967) and was 86% ± 5% of the protein concentration calculated from the absorbance at 280 nm assuming an absorption coefficient of 10 as well as determined chemically (Lowry et al., 1951). PCA was also inhibited by the serine protease inhibitors PMSF and PPACK. PCA exhibited a thrombin-like specificity as seen by its relative sensitivity to arginyl as compared to lysyl chloromethyl ketones and its inhibition by benzamidines. PCA was inhibited by soybean trypsin inhibitor, while antithrombin III/heparin or hirudin had little or no effect. This is in contrast to control experiments with α-thrombin, which was completely inhibited at these concentrations of antithrombin III/heparin or hirudin. In addition, PCA activity was not affected by cysteine-directed reagents or by metal chelators which confirmed that it was not a thiol or a metalloprotease.

Table II: Effect of Protease Inhibitors on Protein C Activator

| protease class | inhibitor | concn | % activity ($\bar{x}_2$ ± SD) |
|---|---|---|---|
| serine | NPGB | $1.0 \times 10^{-3}$ (M) | 0 |
| | PMSF | $1.0 \times 10^{-3}$ (M) | 18.2 ± 2.1 |
| | TLCK | $1.0 \times 10^{-3}$ (M) | 109.8 ± 3.7 |
| | | $5.0 \times 10^{-3}$ (M) | 88.8 ± 8.9 |
| | PPACK | $5.0 \times 10^{-5}$ (M) | 0 |
| | benzamidine | $1.0 \times 10^{-2}$ (M) | 68.5 ± 5.9 |
| | *p*-aminobenzamidine | $1.0 \times 10^{-2}$ (M) | 14.1 ± 1.6 |
| | soybean trypsin inhibitor | 500 μg/mL | 0 |
| | antithrombin III | $6 \times 10^{-8}$ (M) | 89.1 ± 6.5 |
| | heparin | 1 unit/mL | |
| | hirudin | 25 μg/mL | 96.8 ± 0.5 |
| thiol | iodoacetamide | $1.0 \times 10^{-3}$ (M) | 98.6 ± 6.1 |
| | *N*-ethylmaleimide | $1.0 \times 10^{-3}$ (M) | 97.2 ± 2.1 |
| | 5,5′-dithiobis(2-nitrobenzoic acid) | $1.0 \times 10^{-3}$ (M) | 97.9 ± 5.0 |
| metallo | EDTA | $1.0 \times 10^{-2}$ (M) | 103.4 ± 3.0 |
| | *o*-phenanthroline | $4.0 \times 10^{-3}$ (M) | 91.7 ± 11.2 |

*Kinetic Studies.* Steady-state kinetic studies were performed to determine the kinetic parameters of PCA hydrolysis of various substrates. A double-reciprocal plot of initial velocity vs TGPRpNA concentration was linear ($r^2 = 0.99$). From these data, it was calculated that the $K_m$ for amidolysis of TGPRpNA was 1.1 mM and the $V_{max}$ was 66 $s^{-1}$. PCA demonstrated considerable substrate specificity since the initial

Table III: Kinetic Constants of Protein C Activation by Various Enzymes

| enzyme | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | species | conditions |
|---|---|---|---|---|---|
| thrombin[a] | 1.8 | 0.009 | $5.0 \times 10^3$ | bovine | 50 mM Tris/0.10 M NaCl, pH 7.4, 37 °C |
| | 9.6 | 0.007 | $7.3 \times 10^2$ | bovine | as above + 1.0 mM $CaCl_2$ |
| thrombin[b] | 1.2 | 0.043 | $3.6 \times 10^4$ | bovine | 20 mM Tris/0.10 M NaCl, pH 7.5, 37 °C, with 10 mg/mL BSA + 0.1 mM EDTA |
| | ~60 | 0.020 | $3.3 \times 10^2$ | bovine | as above + 3 mM $CaCl_2$ |
| thrombin/TM[b] | >100 | ~80 | $\sim 8.0 \times 10^5$ | bovine | as above + 0.1 mM EDTA |
| | 8 | 4.2 | $5.3 \times 10^5$ | bovine | as above + 3 mM $CaCl_2$ |
| thrombin/TM[c] | 9.8 | 0.17 | $1.7 \times 10^4$ | human | 20 mM Tris/0.15 M NaCl, pH 7.4, 37 °C, with 5 mg/mL BSA + 2.5 mM $CaCl_2$ |
| PCA[d] | 0.08 | 0.27 | $3.4 \times 10^6$ | human | 50 mM Tris/1 mg/mL PEG, pH 7.5, 30 °C |
| | 0.6 | 0.02 | $3.3 \times 10^4$ | human | as above + 0.15 M $NaCl_2$ |
| | 1.3 | 0.002 | $1.5 \times 10^3$ | human | as above + 2 mM $CaCl_2$ |

[a] Amphlett et al. (1981). [b] Esmon et al. (1983). [c] Salem et al. (1984). [d] C. L. Orthner (unpublished results).

rate of amidolysis of D-Phe-Pip-Arg-pNA, D-Ile-Pro-Arg-pNA, Bz-Ile-Glu-Gly-Arg-pNA, D-Val-Leu-Arg-pNA, and pyr-Glu-Pro-Arg-pNA was less than 10% that of TGPRpNA when measured under identical conditions using 1.0 mM substrate concentrations. A chromogenic assay utilizing TGPRpNA was therefore routinely used as a convenient measure of PCA activity.

The kinetic parameters of PCA hydrolysis of protein C were also determined. A double-reciprocal plot of initial velocity as a function of protein C concentration was linear, from which it was calculated that the $K_m$ for protein C was 0.57 $\mu$M and the $V_{max}$ was 0.02 $s^{-1}$. These parameters are apparent values since they were determined in the presence of 0.15 M NaCl, which was found to inhibit the reaction using protein C as the substrate but not TGPRpNA (see below).

Quantitative information regarding the inhibition of PCA by PPACK was obtained by measuring the time course of inactivation of PCA at varying concentrations of PPACK. Semilog plots of percent activity versus time at PPACK concentrations of 3, 4, 6.7, and 15 $\mu$M were linear and of increasing negative slope. From a replot of these data (see Materials and Methods), it was determined that PPACK inactivation of PCA was characterized by a $K_i$ of 34 $\mu$M and an alkylation rate constant of 0.09 $min^{-1}$.

*Effect of Metals on Protein C Activation by PCA.* It was found that micromolar concentrations of $CaCl_2$ inhibited the activation of protein C by PCA. A plot of percent inhibition at increasing concentrations of $CaCl_2$ resulted in a hyperbolic inhibition curve (Figure 4A). A double-reciprocal plot of the data was linear (Figure 4B) with a $K_{i,app}$ for Ca(II) of 99 $\mu$M and a maximum inhibition of 100%. The effect of other divalent metal ions was also examined. Protein C was preincubated for 10 min in TSP buffer containing various metals at 1.0 mM and the initial rate of activation measured following the addition of PCA (5.8 nM). Comparable results were found with Ca(II) and Mn(II), with 94% and 97% inhibition, respectively, while Mg(II) and Ba(II) were less effective and caused 45% and 28% inhibition, respectively. In control experiments, it was found that none of these metals affected the assay of activated protein C under these conditions.

In order to determine whether metals were affecting the enzyme of the reaction, the effect of metals on PCA activity was examined. None of these metals, tested at a concentration of 1.0 mM, had an effect on the amidolysis of TGPRpNA (0.2 mM) by PCA. Likewise, up to 1.0 mM Ca(II) was tested and found to have no effect on the time course of inhibition of PCA by PPACK (4 $\mu$M). Thus, no evidence was found for an effect of Ca(II) or other divalent metals on PCA, even when measured at low substrate and inhibitor concentrations where a $K_m$ as well as a $V_{max}$ effect would be observed. Therefore,

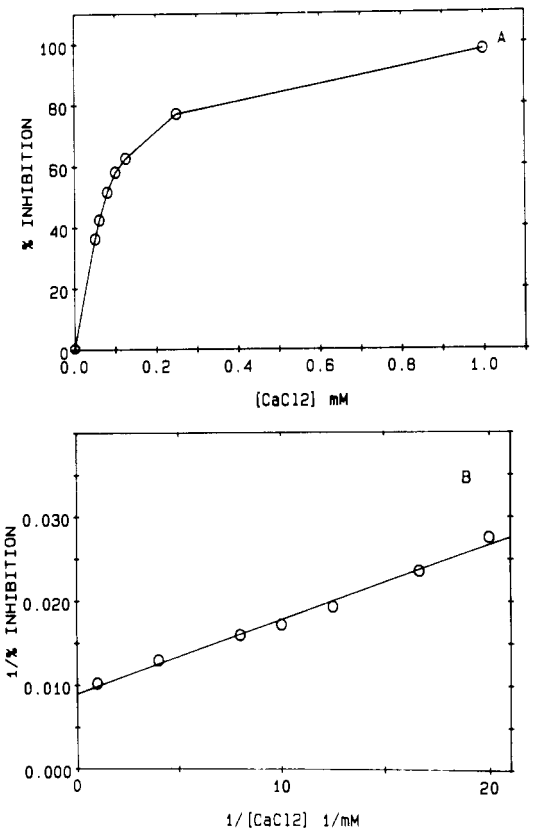


FIGURE 4: Inhibition of PCA activation of protein C by $CaCl_2$. (A) Initial rate at varying concentrations of $CaCl_2$. Percent inhibition represents $v_i$ in the absence of $CaCl_2$ minus $v_i$ in the presence of $CaCl_2$. (B) Double-reciprocal plot of percent inhibition at varying concentrations of $CaCl_2$. The equation of the best-fit line is $y = (8.85 \times 10^{-4})x + (8.98 \times 10^{-3})$ ($r^2 = 0.99$).

divalent cations were likely exerting their effect on protein C. Inhibition of the protein C activation reaction by Ca(II) was found to be completely reversible. Thus, incubation of protein C with 0.5 mM $CaCl_2$ caused an 88% inhibition, while following the addition of a 5-fold molar excess of EDTA over Ca(II), the percent inhibition was reversed to 12% ± 3%.

It was found that PCA activation of protein C was also inhibited by NaCl. In contrast to the inhibition by Ca(II), the NaCl effect occurred in the millimolar concentration range and exhibited positive cooperativity. The results were not influenced by the effect of NaCl on the amidolytic activity of activated protein C because the concentration of metal was only varied during the activation reaction and equalized during the assay of activated protein C. As shown in Figure 5A, a plot of the percent inhibition at increasing concentrations of NaCl was sigmoidal. These data were replotted in the form

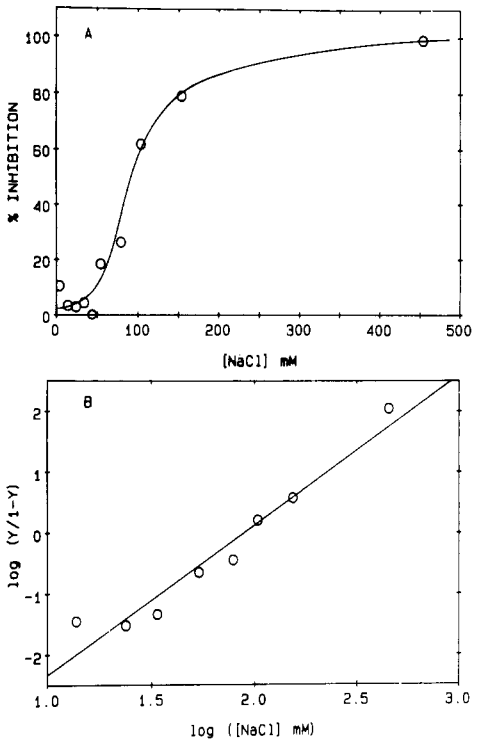


FIGURE 5: Inhibition of PCA activation of protein C by NaCl. (A) Percent inhibition at varying concentrations of NaCl. (B) Hill plot of the data where $Y$ is the fraction of maximum inhibition. The equation of the best-fit line is $y = 2.47x - 4.81$ ($r^2 = 0.94$).

of a Hill plot (Figure 5B) which indicated a Hill number of 2.5 and a $K_{0.5}$ of 88 mM NaCl.

Table III compares the kinetic parameters of protein C activation by PCA under varying ionic conditions to the published values for other enzymes. The second-order rate constant for protein C activation by PCA was found to be 4–200-fold greater than the values reported for protein C activation by the thrombin/thrombomodulin complex. This demonstrates that the catalytic efficiency of this enzyme compares favorably to that of other activators of protein C.

## Discussion

*Comparison of Protein C Activators from Southern Copperhead Venom.* Previously, two protein C activators from the venom of the Southern copperhead snake have been described. Protac (Martinoli & Stocker, 1986; Stocker et al., 1986) was found to be a protein having a molecular weight of 37 000 as measured by SDS–PAGE in the absence or presence of reducing agents. Its isoelectric point was found to be 3.0 as determined by isoelectric focusing. The activity of Protac was not inhibited by diisopropyl fluorophosphate, iodoacetamide, or EDTA, specific inhibitors of serine, sulfhydryl proteases, or metalloproteases, respectively. Incubation of Protac with protein C did not cause changes in its electrophoretic behavior as measured using crossed immunoelectrophoresis. These findings, taken together with data relating to activator dose and yield of activation product, suggested that Protac was not a protease but exerted its activating effect by forming a stoichiometric complex with protein C.

In addition, Klein and Walker (1986) reported the purification of a protein C activator, the properties of which were very different from Protac. This activator was also a protein but had a molecular weight of only 20 000 as measured by SDS–PAGE under nonreducing conditions and by gel filtration using Superose 6. The activity of the activator was inhibited by diisopropyl fluorophosphate, SBTI, and millimolar concentrations of EDTA. Antithrombin III had no effect on its activity. Activation of protein C using this enzyme resulted in cleavage of the heavy chain of protein C as monitored by SDS–PAGE.

*Characterization of PCA.* In this paper, we describe the purification and partial characterization of a protein C activator from this same venom which has properties which are clearly distinct from those previously reported. It was found to be a protein having a molecular weight of 37 000 estimated by SDS–PAGE in the absence or presence of reducing agents, ruling out the possibility that it was a dimer of the protein described by Klein and Walker (1986). The apparent molecular weight was 39 000 when estimated under nondenaturing conditions by high-pressure gel permeation chromatography, which agreed well with the estimate based on SDS–PAGE. The isoelectric point was 6.3 as determined by chromatofocusing, which is substantially higher than the isoelectric point of 3.0 reported for Protac. The activity of PCA was inhibited by the serine protease inhibitors NPGB, PMSF, PPACK, and SBTI. Activation of protein C resulted in proteolysis of its heavy chain as analyzed by SDS–PAGE. Unlike the activator described by Klein and Walker (1986), it was not inhibited by millimolar concentrations of EDTA.

The protein C activator described in the present study has several additional characteristics worth noting. It is an extremely stable enzyme and can be stored for several weeks in 6 M urea without loss of activity. The enzyme has no cofactor requirements in terms of metal ions since the activity of this enzyme is not altered by EDTA as well as of a variety of divalent metal ions.

Inhibition of PCA by several specific inhibitors established that the enzyme is a serine protease. It appears to be thrombin-like in its preference for arginyl as compared to lysyl chlormethyl ketones as well as by its inhibition by benzamidine and *p*-aminobenzamidine. However, PCA can be readily distinguished from $\alpha$-thrombin by its different substrate specificity. PCA does not clot fibrinogen. This was seen in experiments in which 20 $\mu$L of enzyme was added to 300 $\mu$L of citrated pooled human plasma and clotting detected by a fibrometer. Following the addition of PCA to a final concentration of 10 $\mu$g/mL, no clot was detected within 10 min. In contrast, the addition of $\alpha$-thrombin to a final concentration ranging from 0.1 to 2.0 $\mu$g/mL caused clot formation in 140–4 s. Furthermore, PCA is not inhibited by antithrombin III or hirudin under conditions which cause complete inhibition of $\alpha$-thrombin. These differences are also apparent when comparing the kinetic parameters of PCA amidolysis of chromogenic substrates or inactivation by peptidyl chloromethyl ketones. Thus, the $K_m$ of amidolysis of TGPRpNA by PCA is 1.1 mM as compared to the $K_m$ for $\alpha$-thrombin of 23 $\mu$M (Orthner & Kosow, 1980). The second-order rate constant of PPACK inactivation of PCA, as estimated by $k_2/K_i$, was $2.6 \times 10^3$ $M^{-1}$ $min^{-1}$, which is much lower than the value of $6.9 \times 10^8$ $M^{-1}$ $min^{-1}$ reported for the inactivation of $\alpha$-thrombin (Kettner & Shaw, 1981). Further work will be required for detailed mapping of the substrate specificity of PCA as compared to $\alpha$-thrombin.

*Inhibition of Protein C Activation by Metals.* PCA activation of protein C is inhibited by $CaCl_2$, while this metal has no effect on PCA activity itself. This is similar to what has been found for thrombin activation of protein C, which is also inhibited by $CaCl_2$ (Amphlett et al., 1981; Esmon et al., 1983), but differs from the thrombin/thrombomodulin activation which is stimulated by $CaCl_2$ (Esmon et al., 1982). A proteolytic derivative of bovine protein C, lacking residues 1–41

and therefore the γ-carboxyglutamyl residues, was found by equilibrium dialysis measurements to contain a single $Ca^{2+}$ binding site with a $K_i$ of 61 μM (Johnson et al., 1983). Binding of $Ca^{2+}$ to this site was associated with a conformational change in protein C which was observed using spectrophotometric techniques. This protein transition displayed a metal ion specificity, such that similar results were observed with $Mn^{2+}$ but not with $Mg^{2+}$. On the basis of the correlation between $Ca^{2+}$ binding, the protein transition, and the reaction rate, it was proposed that the effect of $Ca^{2+}$ on protein C activation was due to a metal ion induced conformational change in protein C. In the present studies, a similar $K_{i,app}$ (99 μM) of $Ca^{2+}$ inhibition of protein C activation by PCA as well as a similar metal ion specificity of this effect suggests the presence of a similar metal ion site on human protein C.

PCA activation of protein C was also inhibited by NaCl. As was the case for divalent metal ions, no evidence could be found for an effect of $Na^+$ on PCA interaction with small substrates or inhibitors. Previous studies, utilizing both steady-state kinetic and pre-steady-state kinetic techniques, have demonstrated a stimulatory effect of $Na^+$ and other monovalent cations on the activity of bovine activated protein C (Steiner et al., 1980; Steiner & Castellino, 1982, 1985a,b; Hill & Castellino, 1987). These results indicated that two sites or classes of sites for $Na^+$ are important for its kinetic effects. The dissociation constant for $Na^+$ was found to be 20 mM. In addition, $Na^+$ was found to cause an increase in the intrinsic fluorescence of activated protein C, with a $K_{m_{app}}$ of 120 mM NaCl, which provided physical evidence as well for a $Na^+$-induced conformational change in activated protein C (Steiner & Castillino, 1985a).

In the present study, $Na^+$ inhibition of PCA activation of protein C exhibited positive cooperativity with a Hill number of 2.5 and a $K_{0.5}$ of 90 mM NaCl. These results provide additional evidence for a $Na^+$-induced conformational change resulting from the binding of $Na^+$ to at least two to three sites on protein C. Analysis of the effect of $Na^+$ on the kinetic parameters of the PCA-catalyzed activation reaction showed that $Na^+$ decreased the affinity of the enzyme for protein C as well as the $k_{cat}$ value of the reaction. Thus, PCA should prove to be a useful enzyme in probing the metal ion induced conformations of protein C, in addition to its utility as a relatively specific and efficient activator of protein C.

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