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Primary Structure of a Protein C Activator from *Agkistrodon contortrix contortrix* Venom†

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ABSTRACT: The amino acid sequence of a protease, protein C activator, from *Agkistrodon contortrix contortrix* venom was determined. Peptide fragments obtained by chemical or enzymatic cleavage of the S-carboxymethylated protein were purified by gel filtration and reverse-phase high-performance liquid chromatography. The present study demonstrates that protein C activator from *A. contortrix contortrix* venom is a trypsin-type serine protease that is composed of 231 residues with a molecular weight of 25 095 for the polypeptide portion of the molecule. By analogy to the mammalian serine proteases, the catalytic triad in venom protein C activator consists of His-40, Asp-85, and Ser-177. The protein also contains three N-linked glycosylation sites at Asn-21, Asn-78, and Asn-129. The amino acid sequence of protein C activator exhibits a high degree of sequence identity with other snake venom proteases: 73% with batroxobin, 68% with flavoxobin, and 55% with Russell's viper venom factor V activator.

Protein C is a two-chain vitamin K dependent protein that circulates in mammalian blood as a precursor to a serine protease (Stenflo, 1976; Esmon et al., 1976; Kisiel et al., 1976; Kisiel, 1979). Human protein C is activated in vivo by the proteolytic release of a dodecapeptide from the amino-terminal end of its heavy chain by a complex of thrombin and thrombomodulin on the endothelial cell surface (Esmon & Owen, 1981). Activated protein C, in contrast to the activated vitamin K dependent coagulant factors, exhibits strong anticoagulant activity through its ability to degrade rapidly and specifically factors Va and VIIIa (Kisiel et al., 1977; Suzuki et al., 1983; Eaton et al., 1986).

Protein C is also activated in vitro by other nonphysiological activators, such as trypsin (Esmon et al., 1976; Kisiel et al., 1976) and the factor X activator from Russell's viper venom (Kisiel et al., 1976). Recently, the crude venom of the Southern Copperhead snake (*Agkistrodon contortrix contortrix*) has been shown to activate protein C rapidly (Stocker et al., 1986). A protease, protein C activator, has now been purified to homogeneity from Southern Copperhead venom in a number of laboratories (Stocker et al., 1986; Kisiel et al., 1987a; Exner & Vaasjoki, 1988; Orthner et al., 1988), and one of these preparations is commercially available under the trade name Protac. Protac has been used successfully in the development of clinically useful, sensitive functional assays for both protein C (Martinoli & Stocker, 1986; Frances & Seyfert, 1987; McCall et al., 1987) and protein S, a cofactor for the expression of activated protein C anticoagulant activity (Suzuki & Nishioka, 1988).

The molecular properties of the venom protein C activator, designated in this paper ACC-C,[1] have been described in detail

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[1] Abbreviations: ACC-C, a protease from *Agkistrodon contortrix contortrix* venom that activates protein C; BNPS-skatole, 2-(2′-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; CM, carboxymethyl; CNBr, cyanogen bromide; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; kDa, kilodaltons; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PMSF, phenylmethanesulfonyl fluoride; aPMSF, *p*-amidinophenylmethanesulfonyl fluoride; PPACK, D-Phe-Pro-Arg chloromethyl ketone; PTH, phenylthiohydantoin; RVV-V, a protease from Russell's viper venom that activates factor V; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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(Kisiel et al., 1987b; Exner & Vaasjoki, 1988; Orthner et al., 1988). ACC-C is a single-chain glycoprotein with an apparent molecular weight of 37 000–40 000 as determined by SDS–PAGE and analytical ultracentrifugation. The results of inhibition studies indicated that ACC-C is a serine protease (Kisiel et al., 1987b; Orthner et al., 1988). In addition, we recently reported that the NH_2-terminal sequence of ACC-C revealed extensive similarity with that found in the amino terminus of several pancreatic and coagulation serine proteases (Kisiel et al., 1987b). We now report the complete amino acid sequence of ACC-C and demonstrate that its structure is typical of serine proteases. The amino acid sequence of ACC-C exhibits a high degree of sequence identity with other snake venom serine proteases that activate coagulation factors.

Experimental Procedures

ACC-C was purified from crude *A. contortrix contortrix* venom as described previously (Kisiel et al., 1987a). TPCK-treated bovine trypsin was obtained from Worthington and further purified on benzamidine–agarose (Fujikawa & McMullen, 1983). *Staphylococcus aureus* V8 protease was obtained from Miles. Guanidine hydrochloride (Ultrapure) was obtained from Schwarz/Mann. BNPS-skatole and cyanogen bromide were purchased from Pierce, and acetonitrile and methanol (HPLC grade) were products of J. T. Baker. All sequenator reagents and solvents were obtained from Applied Biosystems.

Reduction of S-carboxymethylation of ACC-C was performed by a modified procedure of Crestfield et al. (1963), where β-mercaptoethanol and urea were replaced with dithioerythritol and 7 M guanidine hydrochloride. CM-ACC-C (3 mg) was digested overnight at room temperature in 0.5 mL of 70% formic acid containing 2% CNBr, and the resulting peptides were initially separated by gel filtration on a Sephadex G-50 superfine column (1.5 × 90 cm) equilibrated with 4 M guanidine hydrochloride. Purity of the peptides was tested by sequence analysis after desalting on the C_3 reverse-phase column. The Asn–Gly bond of CM-ACC-C was cleaved with hydroxylamine according to the method of Steinman et al. (1974), and the resulting peptides were separated by a Sephadex G-50 superfine column (1.5 × 90 cm) equilibrated with 2.5% HCOOH.

Digestions with trypsin and *S. aureus* V8 enzyme were performed by incubating peptides in 0.1 M NH_4HCO_3 for 6 h at 37 °C with 1% (w/w) of the enzymes. The cleavage of tryptophanyl bonds was performed by the method of Omenn et al. (1970), and the acid cleavage of a peptide was carried out by heating for 4 h at 110 °C in 2% HCOOH in vacuo. The resulting peptides were separated by HPLC using an Altex Ultrapore C_3 reverse-phase separation column (0.46 × 7.5 cm) or a Bondapak C_{18} column (4.9 × 300 mm) connected to a Waters HPLC system. A gradient system composed of 0.1% trifluoroacetic acid (solvent A) and 0.08% trifluoroacetic acid in 80% acetonitrile (solvent B) was employed for the elution of peptides from both the C_3 and C_{18} columns at a flow rate of 1.5 mL/min. The effluents were monitored at 214 nm, and peptides were collected manually.

Automated sequence analyses were performed with an Applied Biosystems 477A protein sequencer connected to an on-line PTH analyzer (Model 120A). Repetitive yields of 90–95% were routinely obtained. The amino acid compositions of peptide hydrolysates (6 N HCl, 110 °C for 24 h) were determined by a Waters Picotag System according to the method of Bidlingmeyer et al. (1984).

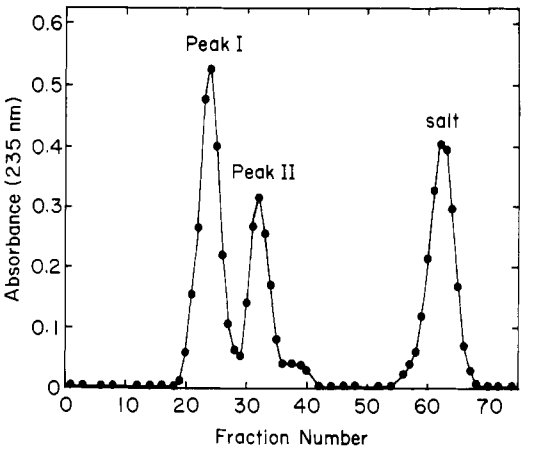

FIGURE 1: Separation of the fragments produced by CNBr digestion. The CNBr digest of CM-ACC-C was applied to a Sephadex G-50 column (1.5 × 90 cm) equibrated with 4 M guanidine hydrochloride and eluted as described under Experimental Procedures. Two-milliliter fractions were collected, and the absorbance at 235 nm was determined.

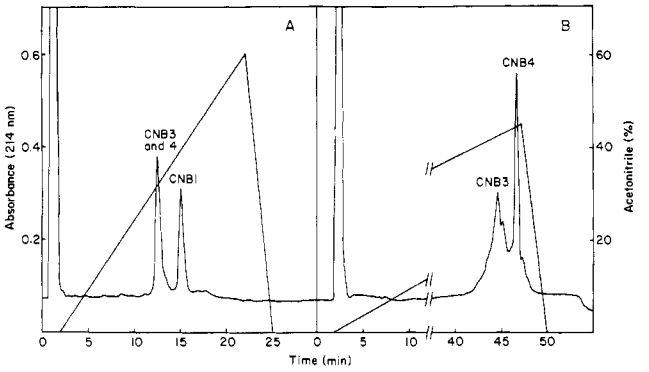

FIGURE 2: Separation of the peptides in peak II by HPLC. (A) Peak II from the gel filtration column (Figure 1) was applied directly to the C_3 column, and the peptides were eluted by a gradient of acetonitrile as described under Experimental Procedures. (B) The first peak from the C_3 column (A) was dried in a speed vac concentrator and applied to a C_{18} column, and peptides were eluted as described under Experimental Procedures.

Results

Cleavage of Methionyl Bonds of Intact ACC-C by Cyanogen Bromide. CM-ACC-C (3 mg) was treated by cyanogen bromide, and the digest was first applied to a gel filtration column of Sephadex G-50 equilibrated with 4 M guanidine hydrochloride. Two major peptide peaks, peak I and peak II, were separated (Figure 1), and the homogeneity of each peak was tested by sequence analysis. Peak I contained a homogeneous peptide termed CNB5. Peak II, which gave a multiple sequence, was further purified by HPLC using a C_3 reverse-phase column as described under Experimental Procedures, and two peaks were separated (Figure 2A). The first peak was, however, further separated by a C_{18} reverse-phase column (Figure 2B) and two homogeneous peptides, CNB3 and CNB4, were obtained. The second peak from the C_3 reverse-phase column was found to be homogeneous and termed CNB1 (residues 1–46). No significant sequence was obtained in the salt peak or in the shoulder at the descending edge of peak II.

The sequence of the first 40 residues of CNB1 was unequivocally determined except for residue 21, where no PTH amino acid was detected. This peptide originated from the amino-terminal end of the molecule, since this sequence was identical with that previously obtained by sequence analysis of the intact protein (Kisiel et al., 1987b). Sequence analysis

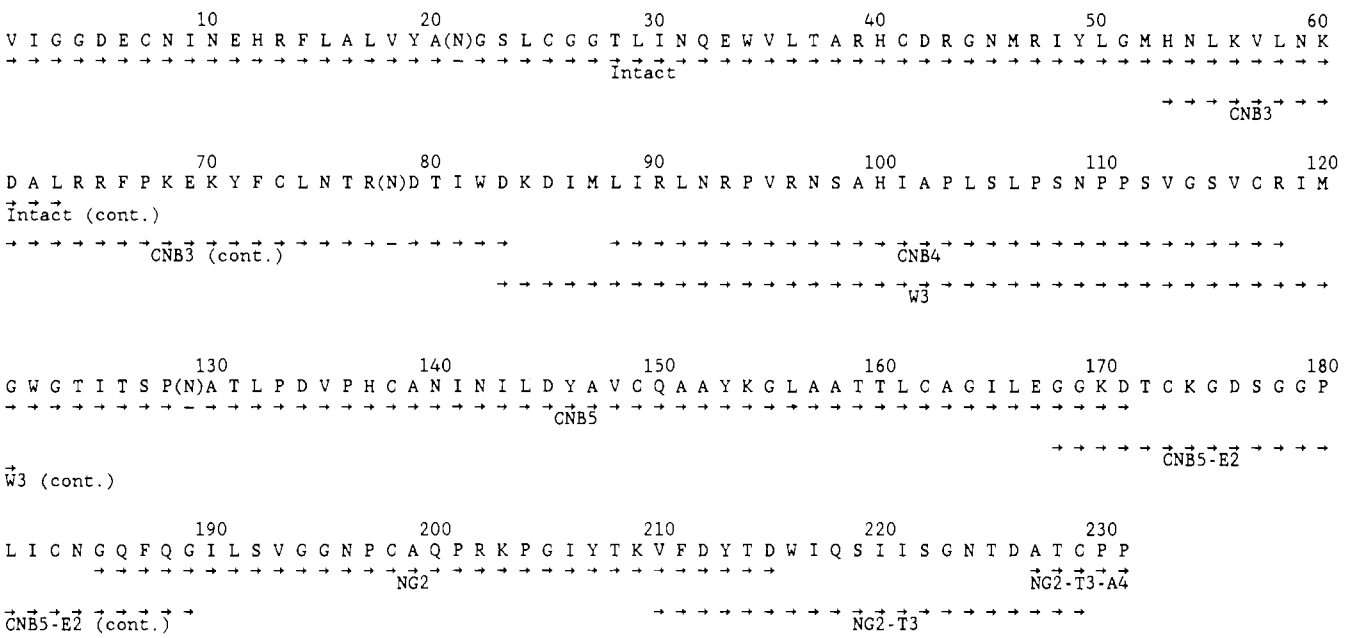

FIGURE 3: Summary of the peptides used for sequence determination of ACC-C. The residues marked with an arrow were identified by sequence analysis. The Asn residues in parentheses were tentatively assigned as the attachment sites for N-linked carbohydrate chains. The designations used for the peptides are as follows: CNB for cyanogen bromide; NG for Asn–Gly cleavage; T for trypsin, W for tryptophanyl cleavage; A for acid cleavage; E for *S. aureus* V8 protease.

of CNB3 and CNB4 identified residues from 53 to 83 and from 88 to 118, respectively, with the exception of residue 78. The sequence analysis of CNB5 identified 50 amino acid residues starting from residue 121 to 171, except for a blank at residue 129 (Figure 3).

ACC-C contains four methionine residues in the sequence, and the isolation of five cyanogen bromide fragments was expected. Except for a small fragment, CNB2 (residues 47–52), which was probably eluted in the salt peak in the gel filtration column, all four fragments were separated, and a total of 165 of 231 residues were identified. The amino acid compositions in the hydrolysates of four cyanogen bromide fragments are in good agreement with those obtained from the sequences (Table I). The remaining sequences were determined from the peptides produced by the following chemical and enzymatic digestions.

Cleavage at Tryptophanyl Bonds. CM-ACC-C was treated with BNPS-skatole according to the method of Omenn et al. (1970) to cleave tryptophanyl bonds, and the resulting peptides were separated by the C_3 reverse-phase column. Eight peaks were obtained, and each was subjected to sequence analysis. The entire sequence of one of the peptides, W3, was determined. The sequence of this peptide filled the sequence gaps (residues 84–87 and 119–120) between the CNBr fragments.

S. aureus V8 Digest of CNB5. In order to extend the sequence of CNB5, it was digested with *S. aureus* V8 enzyme and two peptides were separated by the C_3 reverse-phase column. The sequence of one of these peptides, CNB5-E2, extended the sequence of CNB5 to residue 189.

Cleavage of Asn–Gly Bonds of Intact ACC-C. One Asn–Gly bond was found at residue 184–185 in the sequence of CNB5-E2. Cleavage of this bond would produce a peptide that covers the undetermined carboxy-terminal portion of the molecule. CM-ACC-C was treated with hydroxylamine and, as expected, two peptides were separated by gel filtration. The second peak, NG2, gave the sequence starting from Gly-185 and extended the sequence of CNB5-E2 to residue 215.

To complete the sequence of NG2, this peptide was further digested by trypsin. Three tryptic peptides were separated by the C_3 column, and one of the peptides, NG2-T3, did not contain Lys or Arg in its composition, indicating that this peptide originated from the carboxy-terminal end. The sequence of this peptide extended the sequence of NG2 to residue 229. However, this sequence was incomplete, since the sequence did not contain two Pro residues that are present from the compositional analysis. NG2-T3 was then subjected to acid cleavage, and 17 peaks were separated by the C_{18} column. An examination of the amino acid composition data showed that only one peak, NG2-T3-A4, contained Pro. The sequence of this peptide extended the sequence of NG2-T3 to residue 231. The amino acid compositions of NG2 and its subfragments are shown in Table I. The composition found in the sequence from 210 to 231 agreed with that obtained in the hydrolysate of NG2-T3 and NG2-T3-A4, indicating the Pro residue 231 was the carboxy terminus.

The amino acids at positions 21, 78, and 129 were not identified by sequence analysis. These residues are very likely carbohydrate-linked Asn residues, since they are followed by a -X-Thr/Ser sequence, which is the consensus signal sequence for the attachment of carbohydrate to asparagine. The reasonably high yields for Thr and Ser residues in the entire sequence indicate the absence of O-linked carbohydrate chains in ACC-C. ACC-C contains 16% carbohydrate (Kisiel et al., 1987b), corresponding to 6–6.5 kDa. Assuming carbohydrates are equally distributed in three chains attached to these residues, then each chain is composed of approximately 2–2.2 kDa. This is in good agreement with the size of the carbohydrate chains determined in various coagulation factors (Mizuochi et al., 1979, 1980, 1983).

ACC-C is composed of 231 amino acid residues with the following composition: Asp_{13}, Asn_{18}, Thr_{14}, Ser_{11}, Glu_5, Gln_6, Pro_{15}, Gly_{23}, Ala_{16}, Val_{11}, Met_4, Ile_{19}, Leu_{21}, Tyr_7, Phe_5, Lys_{10}, His_5, Arg_{12}, $Cys/2_{12}$, and Trp_4. The molecular weight for the polypeptide portion of ACC-C is calculated to be 25 095, and the molecular weight of ACC-C is approximately 31 000 with the addition of the three carbohydrate chains. This number differs considerably from the previously reported molecular weight of 37 000–40 000 (Kisiel et al., 1987b; Stocker et al., 1987; Exner & Vaasjoki, 1988; Orthner et al., 1988) estimated by SDS–PAGE. In all likelihood, this difference is due to

Table I: Amino Acid Compositions of Selected Peptides from ACC-C

	fragment, residue no.						
	CNB1, 1–46	CNB3, 53–87	CNB4, 88–120	CNB5, 121–231	NG2, 185–231	NG2–T3, 204–231	NG2–T3–A4, 227–231
Asx	5.9 (7)	7.3 (8)	3.3 (3)	12.4 (13)	4.8 (5)	3.6 (4)	
Thr	1.9 (2)	1.6 (2)		8.9 (10)	3.8 (4)	2.8 (3)	1.0 (1)
Ser	1.1 (1)		3.7 (5)	5.2 (5)	2.8 (3)	2.0 (2)	
Glx	3.8 (4)	1.0 (1)		6.5 (6)	4.3 (6)	1.2 (1)	
Pro		1.1 (1)	4.6 (5)	9.1 (9)	5.4 (5)	2.6 (2)	2.2 (2)
Gly	6.4 (6)		1.0 (1)	15.0 (15)	6.6 (6)	1.4 (1)	
Ala	3.3 (3)	1.0 (1)	2.2 (2)	9.6 (10)	2.4 (2)	1.3 (1)	1.0 (1)
Cys[a]	2.3 (3)	0.5 (1)	0.8 (1)	3.8 (7)	1.0 (2)	0.5 (1)	0.8 (1)
Val	2.9 (3)	1.0 (1)	3.0 (3)	4.9 (4)	2.3 (2)	1.1 (1)	
Met[b]	0.4 (1)	0.4 (1)	0.4 (1)	0.4 (1)			
Ile	3.0 (3)	1.8 (2)	3.2 (3)	9.2 (10)	5.1 (5)	2.6 (3)	
Leu	5.4 (5)	3.8 (4)	4.3 (4)	7.9 (7)	1.4 (1)		
Tyr	0.8 (1)	0.6 (1)		3.1 (4)	1.1 (2)	0.9 (1)	
Phe	1.0 (1)	1.9 (2)		2.1 (2)	1.9 (2)	0.9 (1)	
His	2.0 (2)	0.9 (1)	1.0 (1)	1.2 (1)			
Lys		4.1 (5)		4.7 (5)	2.1 (2)		
Arg	2.9 (3)	2.8 (3)	4.3 (4).	1.3 (1)	1.1 (1)		
Trp[c]	(1)	(1)		(2)	(1)	(1)	

[a] Measured as CM–Cys. [b] Measured as homoserine. [c] Not determined.

abnormal migration of glycoproteins on SDS–PAGE (Segrest & Jackson, 1972).

Discussion

Recently, the amino acid sequences of three snake venom serine proteases have been established: batroxobin (Itoh et al., 1988), flavoxobin (Shieh et al., 1988), and RVV-V (Tokunaga et al., 1988). Batroxobin and flavoxobin have thrombin-like activities, which convert soluble fibrinogen to fibrin clots. RVV-V is an activator of coagulation factor V and has no apparent thrombin-like activity. The sequence of ACC-C is compared with these venom proteases, as well as bovine thrombin (Figure 4). All the venom proteases are composed of virtually the same number of residues and are aligned with each other by the insertion of a few gaps. Thrombin, which is about 20 residues longer than those venom proteins, requires more gaps for the alignment. ACC-C has considerably higher similarity to flavoxobin (68% identity), batroxobin (73% identity), and RVV-V (55% identity) than to thrombin (30% identity).

With the sequence homology, it is evident that ACC-C is a typical serine protease. In this regard, ACC-C contains the three catalytic residues, His-40, Asp-85, and Ser-1778, that form the charge-relay system in serine proteases. These residues are analogous to the catalytic triad composed of His-57, Asp-102, and Ser-195 in chymotrypsin (Sigler et al., 1968). ACC-C contains an aspartic acid residue at position 171 located at the bottom of the substrate binding pocket characteristic for the trypsin-type enzymes that cleave arginyl and lysyl bonds. Like many other trypsin-type serine proteases, ACC-C also contains two highly conserved glycine residues at positions 194 and 205 that presumably result in an open binding pocket, which can accommodate large amino acid side chains. In total, ACC-C contains 27 of the 29 invariant amino acids found in eukaryotic serine proteases and bacterial trypsin (Young et al., 1978).

The two exceptions occur at residues 13 (Arg instead of Pro) and 39 (Arg instead of Ala). To our knowledge, the substitution of Arg for Pro at position 13 appears to be the first reported instance for serine proteases with an amino acid other than Pro at this position. The Arg-39 adjacent to the active-site His residue is also highly unusual among serine proteases. With the exception of crayfish trypsin (Titani et al., 1983), all serine proteases sequenced to date contain an invariant Ala at this position. Interestingly, Orthner et al. (1988) reported a second-order rate constant of 2.6×10^3 M^{-1} min^{-1} for the PPACK inactivation of ACC-C. This rate is markedly lower than the rate of 6.9×10^8 M^{-1} min^{-1} previously reported for the inactivation of thrombin by this inhibitor (Kettner & Shaw, 1981). This inhibitor inactivates thrombin by the alkylation of the active-site His residue. Whether the decreased rate observed for the alkylation of ACC-C is caused by the substitution of Arg-39 for Ala is speculative.

Inasmuch as both ACC-C and thrombin activate protein C by limited proteolysis, it was of interest to compare the sequences of these two proteins. When the sequences were arranged so as to maximize identities, ACC-C was found to be 30% identical with bovine thrombin (Figure 4). Of particular interest is the presence in ACC-C of a positively charged segment at the carboxy-terminal side of the active-site His residue between Arg-43 and His-100. In this region of ACC-C, 14 basic residues (Arg_7, Lys_5, and His_2) greatly outnumber four acidic residues (Asp_3, Glu_1). Thrombin also has an Arg/Lys-rich segment in the corresponding region, which is involved in fibrin(ogen) recognition, heparin binding, and mitogenic activity (Fenton & Bing, 1986). Hirudin, a specific anionic peptide inhibitor of thrombin, binds to this region and neutralizes thrombin coagulant and mitogenic activity (Fenton & Bing, 1986). The function of this region in ACC-C is unknown, but like the corresponding region in thrombin, may be important in substrate recognition.

ACC-C contains 12 Cys/2 that probably form six intrachain disulfide bonds. By analogy to the disulfide pairings in bovine trypsin (Kaufman, 1965), the six disulfide bridges in ACC-C would appear to be Cys_7–Cys_{162}, Cys_{25}–Cys_{42}, Cys_{72}–Cys_{229}, Cys_{117}–Cys_{182}, Cys_{149}–Cys_{162}, and Cys_{173}–Cys_{198}. Among these six disulfide bridges, the Cys_{72}–Cys_{229} pairing appears to be unique to the snake venom serine proteases as it has only been reported in batroxobin (Itoh et al., 1987), flavoxobin (Shieh et al., 1988), and RVV-V (Tokunaga et al., 1988).

To date, five laboratories have isolated and characterized a protein C activator from Southern Copperhead venom (Klein & Walker, 1986; Kisiel et al., 1987a,b; Stocker et al., 1987; Exner & Vaasjoki, 1988; Orthner et al., 1988). The preparation of protein C activator described by Stocker and coworkers is commercially available under the tradename Protac. With the exception of the protein C activator described by Klein and Walker (1986), all preparations of venom protein C activator appear to be similar in molecular weight and in

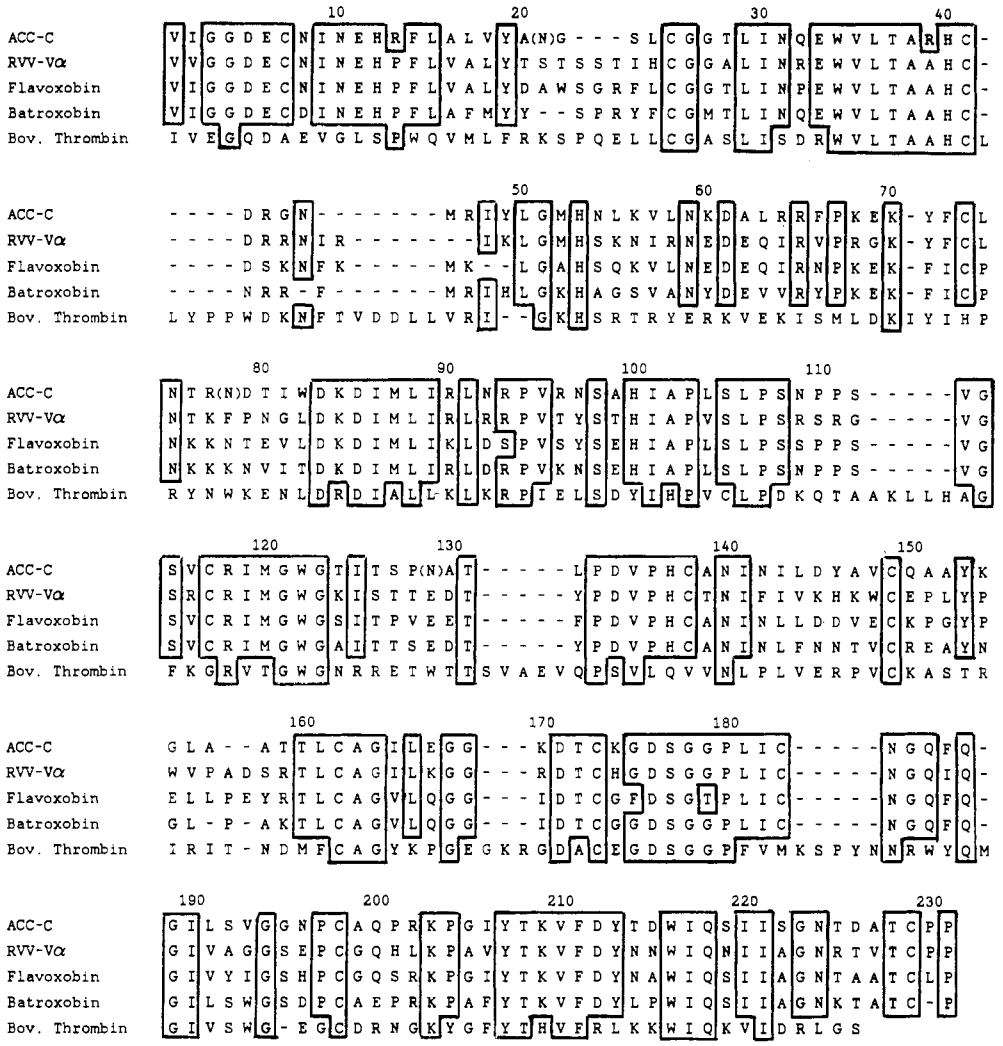

FIGURE 4: Alignment of the sequence of ACC-C with those of RVV-V, flavoxobin, batroxobin, and bovine thrombin A-chain. Gaps (–) were inserted to maximize the homology. The ACC-C numbering system is used. Four of five identical residues are boxed. The sequences were taken from the following references: RVV-Vα, Tokunaga et al. (1988); flavoxobin, Shieh et al. (1988); batroxobin, Itoh et al. (1987); thrombin, Magnusson et al. (1975).

their ability to activate protein C rapidly and specifically. Whether or not Protac is identical with the other three preparations described is, however, questionable, inasmuch as certain discrepancies are observed in other molecular properties of these enzymes. For example, Protac reportedly exhibits a p*I* of 3.0 (Stocker et al., 1987), whereas our laboratory reported that ACC-C resolved into three variant bands by isoelectric focusing with p*I*s ranging between 7.5 and 8.5 (Kisiel et al., 1987b). In addition, the preparation of venom protein C activator described by Orthner et al. (1988) exhibited a p*I* of 6.3, and Exner and Vassjoki (1988) reported that their protein C activator failed to bind to the PBE-94 chromatofocusing medium, suggesting the protein possessed a strong positive charge. When subjected to FPLC chromatofocusing [Mono P column equilibrated with 75 mM Tris–acetate (pH 9.3) and eluted with 10% Polybuffer 96 (pH 6.0)], our highly purified ACC-C preparation resolved into 14 distinct peaks that eluted from the column between pH 9.1 and 7.5 (W. Kisiel, unpublished results). Examination of the ACC-C sequence revealed that the molecule contains a predominance of basic (27 Arg, His, and Lys) over acidic (18 Asp and Glu) residues. Thus, by this criterion, our ACC-C preparation would appear to be distinct from Protac.

Another significant difference lies in the apparent sensitivity to serine protease inhibitors. Protac has been reported to be insensitive to DFP, benzamidine, TLCK, and TPCK (Stocker et al., 1987). In contrast, the amidolytic and anticoagulant activities of ACC-C were readily neutralized following incubation with dansyl-Glu-Gly-Arg-chloromethyl ketone, Phe-Pro-Arg-chloromethyl ketone, PMSF, aPMSF, and antithrombin III in the presence and absence of heparin. Furthermore, ACC-C covalently bound [^{3}H]DFP (Kisiel et al., 1987b). The preparation of protein C activator (PCA) described by Orthner et al. (1988) was also sensitive to serine protease inhibitors, such as NPGB, PMSF, and PPACK. Thus, Protac does not appear to be identical with ACC-C and/or PCA (Orthner et al., 1988). Further work is needed to determine whether or not the Southern Copperhead venom conceivably contains two or more distinctly different protein C activators.

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Registry No. Serine proteinase, 37259-58-8; serine proteinase (*Agkistrodon contortrix contortrix* venom reduced), 117774-52-4; factor XIV, 60202-16-6.

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