Thrombomodulin-independent Activation of Protein C and Specificity of Hemostatically Active Snake Venom Serine Proteinases

CRYSTAL STRUCTURES OF NATIVE AND INHIBITED AGKISTRODON CONTORTRIX CONTORTRIX PROTEIN C ACTIVATOR

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Protein C activation initiated by the thrombin-thrombomodulin complex forms the major physiological anticoagulant pathway. Agkistrodon contortrix contortrix protein C activator, a glycosylated single-chain serine proteinase, activates protein C without relying on thrombomodulin. The crystal structures of native and inhibited Agkistrodon contortrix contortrix protein C activator determined at 1.65 and 1.54 Å resolutions, respectively, indicate the pivotal roles played by the positively charged belt and the strategic positioning of the three carbohydrate moieties surrounding the catalytic site in protein C recognition, binding, and activation. Structural changes in the benzamidine-inhibited enzyme suggest a probable function in allosteric regulation for the anion-binding site located in the C-terminal extension, which is fully conserved in snake venom serine proteinases, that preferentially binds Cl\(^{-}\) instead of SO\(_4\)\(^{2-}\).

Hemostasis, a complex system responsible for maintaining the fluidity of blood under physiological conditions, is primed to react rapidly to vascular injury, stemming blood loss by locally sealing the injured vessel wall (1). The highly regulated hemostatic system functions in equilibrium between two extremes: coagulation and fibrinolysis (2). On one hand by initiating the coagulation cascade, which comprises a network of highly, correlated and controlled reactions and is triggered either by the extrinsic (tissue factor) or the intrinsic pathways (Factor XII), activating thrombin to generate cross-linked fibrin polymers (1). On the other hand, in the fibrinolytic system, the protein C pathway comprises the major physiological anticoagulant mechanism and is activated on the surface of endothelial cells by the thrombin-thrombomodulin complex (3). Activated protein C subsequently binds protein S and inhibits coagulation by degrading FVIIa and FVa on the surface of negatively charged membranes by stimulating fibrinolytic activity (4, 5). Deficiencies or defects in the protein C (PC)\(^3\) anticoagulant pathway are associated with the increased risk of venous thromboembolism (6). The PC pathway also plays an important role in inflammatory processes and activated PC has been implicated in anti-apoptotic and neuroprotective activities (7).

Snake venom serine proteinases belong to the trypsin subfamily of enzymes, share significant sequence identity (50–70%), and display high specificity toward macromolecular substrates. These enzymes interfere in the control and regulation of the hemostatic system at different key points ranging from the coagulation cascade to the fibrinolytic feedback system (8) and are characterized as activators of the fibrinolytic system (plasminogen activators), procoagulant (thrombin-like enzymes), anti-coagulant (PC activators), and platelet-aggregating enzymes (9). Trimergusurus stejnegeri venom plasminogen activator converts plasminogen to plasmin by cleavage of the peptide bond Arg\(^{560}\)–Val\(^{562}\) with high substrate specificity and is resistant to inhibition (10, 11). Batroxobin, a thrombin-like enzyme from Bothrops atrox venom used for the treatment of thrombotic diseases (12), converts fibrinogen to fibrin by cleaving fibrinopeptide A (Aα 1–16) at the N-terminal portion of the Aα-chain forming a non-cross-linked “soft clot” that is rapidly removed from the circulatory system by the fibrinolytic mechanism resulting in a defibrinogenating effect. The PC activator from Agkistrodon contortrix contortrix venom, commercially referred to as Proteac\(^8\), specifically converts PC to activated PC by hydrolyzing the Arg\(^{560}\)–Leu\(^{561}\) bond, functioning independently of plasmatic factors in comparison to the physiological activation of PC by thrombin which is dependent on the participation of thrombomodulin (13). The PC activator, from A. contortrix contortrix venom, induced zymogen activation is clinically used in assays of functional PC determination, total protein S content, and other protein S functional assays (14) in plasma, since catalytically active PC can easily be detected by coagulation tests or by utilizing chromogenic substrates.

We present the first crystal structures of the PC activator from the venom of the copperhead snake A. contortrix contortrix (ACC-C), a single-chain glycosylated serine proteinase that is a fast-acting PC activator, both in the native and inhibited states at 1.65 and 1.54 Å resolutions, respectively. These results provide information at the molecular level concerning the alternative PC activation pathway that is independent of thrombomodulin, the central physiological barrier against thrombosis and could serve as a basis for the structure-based design of clinically useful molecules and in the treatment of thromboembolism.

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The atomic coordinates and structure factors (codes 2AIP and 2AIQ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: PC, protein C; TSV-PA, Trimergusurus stejnegeri venom plasminogen activator; ACC-C, protein C activator from Agkistrodon contortrix contortrix venom; cmk, chloromethyl ketone; TME45, thrombomodulin epidermal growth factor-like domains 4 and 5; NAP5, nematode anticoagulant protein 5 from Ancylostoma caninum; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; r.m.s., root mean square.
**TABLE ONE**

Data collection and refinement statistics

Statistical values for the highest resolution shells are indicated in parentheses. LNLS, Laboratório Nacional de Luz Síncrotron.

| Crystal preparation          | Native state                          | Inhibited state                        |
|-----------------------------|---------------------------------------|----------------------------------------|
| Cryoprotectant solution     | Mother liquor + 20% glycerol          | Mother liquor + 20% glycerol + 50 mM benzamidine |
| Soaking time                | 30 s                                  | 45 s                                   |
| Data collection             |                                       |                                        |
| Wavelength (Å)              | 1.438                                 | 1.438                                  |
| Temperature (K)             | 100                                   | 100                                    |
| Detector                    | MARCCD                                | MARCCD                                 |
| Synchrontron radiation source | CPr beamline/LNLS-Brazil             | CPr beamline/LNLS-Brazil               |
| Space group                 | C 2                                   | C 2                                    |
| Unit cell parameters (Å, °) | a = 79.87, b = 63.30, and c = 48.24; β = 99.80 | a = 80.52, b = 63.46, and c = 48.22; β = 99.85 |
| Number of molecules in the asymmetric unit | 1                                        | 1                                      |
| Solvent content (%)         | 42.4                                  | 41.7                                   |
| V_M (Å³/Da)                 | 2.15                                  | 2.12                                   |
| Number of reflections       | 487,208                               | 409,068                                |
| Number of unique reflections | 28,664 (2835)                         | 35,447 (5817)                         |
| (I/σ[I])                    | 20.0 (2.9)                            | 18.3 (3.5)                            |
| Multiplicity                | 7.8 (7.5)                             | 6.4 (5.8)                             |
| Completeness (%)            | 99.8 (99.6)                           | 99.9 (99.8)                           |
| R_merge (%)                 | 9.0 (64.5)                            | 8.4 (49.2)                             |
| Ramachandran plot analysis  |                                       |                                        |
| Most favored regions (%)    | 83.9                                  | 82.8                                   |
| Allowed regions (%)         | 15.1                                  | 16.1                                   |
| Generously allowed regions (%) | 1.0                                  | 1.0                                    |

* R_merge = Σ[I(h)] – ⟨|I(h)|⟩/Σ[I(h)], where I_obs is the observed intensity of the i/r measurement of reflection h, and ⟨|I(h)|⟩ is the mean intensity of reflection h calculated after scaling.

**MATERIALS AND METHODS**

ACC-C, provided by Pentapharm (Basel, Switzerland), was dissolved to a concentration of 10 mg/ml in a 20 mM Hepes (pH 7.5) buffer that also contained 10 mM sodium chloride and 1 mM dithiothreitol. Crystals were obtained at 18 °C by equilibration of the protein solution (1 μl) against a reservoir solution containing 2.0 M ammonium sulfate and 100 mM sodium acetate (pH 4.6) utilizing the hanging-drop vapor diffusion method. The benzamidine-inhibited ACC-C complex was obtained by soaking crystals for 45 s in a cryo-protectant solution (20% (v/v) glycerol that additionally contained 50 mM benzamidine. Dose-dependent x-ray diffraction data were collected from cryo-protected crystals at 100 K at a synchrotron radiation source (CPr Beamline-Laboratório Nacional de Luz Síncrotron, Campinas, Brazil) where the wavelength was fixed at 1.438 Å, diffraction intensities were measured utilizing a Mar CCD165 detector (Mar Inc.), and the diffraction intensities were reduced and scaled using the DENZO/SCALEPACK suite of programs (15). Both the native and benzamidine-inhibited crystals are isomorphous, belong to the monoclinic space group C2, and contain one protein molecule in the asymmetric unit. The crystal structure of ACC-C was solved by molecular replacement (AMoRe) (16) initially using the atomic coordinates of T. stejnegeri venom plasminogen activator (Protein Data Bank entry: 1BQY) (11) as a search model. Positional and restrained isotropic B-factor refinements were performed using REF-MAC5 (17), all model building was carried out utilizing TURBO-FRODO (18), and PROCHECK (19) was utilized to evaluate the stereochemistry of the final models. The data collection, processing, and refinement statistics are presented in TABLE ONE.

**RESULTS**

The crystal structures of both native and benzamidine-inhibited ACC-C have been refined at 1.65 and 1.54 Å resolutions to crystallographic residuals of 16.7% (R_free = 19.6%) and 16.7% (R_free = 19.1%), respectively. An analysis of the stereochemistry of the final models indicates that the main-chain dihedral angles for all residues are located in the permitted regions of the Ramachandran diagram and that the root mean square (r.m.s.) deviations from ideal values are distributed within the expected ranges for well refined structures (TABLE ONE).

In analogy to the structures of trypsin-like serine proteinases, the structure of ACC-C consists of two domains (S and S’), each containing a six-stranded β-barrel and two short α-helices (residues: 165–173 and 235–244, sequence numbering is based on chymotrypsinogen) (20) (Fig. 1, A and B). The catalytic triad (His^57, Asp^102, and Ser^195) is located at the junction of both barrels and is surrounded by the conserved 70-, 148-, and 218-loops, and the non-conserved 37-, 60-, 99-, and 174-loops (Fig. 1, A and B).

ACC-C contains 16% carbohydrate, including glucosamine, neura-
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minic acid, and neutral hexose (13, 21), the consensus signal sequence for the attachment of carbohydrate moieties to asparagines (Asn-X-Thr/Ser, where X represents any amino acid) was identified at positions 38, 96A, and 148. In both the structures, clear electron density was observed for the \( N^\text{acetyl-D-glucosamine} \) monosaccharide, which is \( N \)-linked to Asn\(^{38} \) and Asn\(^{148} \); however, the moiety linked to Asn\(^{38} \) was inserted based on the presence of diffuse electron density (Fig. 1B). The three carbohydrate moieties are strategically positioned at the tips of the 37-, 99-, and 148-loops, which form the entrance to the active-site pocket (Fig. 1B) and modulate the selectivity toward macromolecular substrates (discussed below). Two snake venom serine proteinase isoforms, AaV-SP-I and AaV-SP-II, from \( Agkistrodon acutus \), also posses an \( N \)-linked carbohydrate group (Asn\(^{35} \)) that is considered to interfere with the binding of macromolecular inhibitors (22). In contrast, \( T. stejnegeri \) venom plasminogen activator (TSV-PA) has a unique glycosylation site at Asn\(^{278} \) located on the opposite face (23) and apparently does not play a role in the binding macromolecular substrates at the interface.

The electron density (2 \( \sigma \) level) for the benzamidine molecule bound to the carboxylate oxygen atoms of Asp\(^{189} \) is clearly defined (Fig. 2A) and indicates a mode of interaction similar to that observed in the structures of benzamidine-inhibited trypsin-like enzymes (24). A sulfate ion is coordinated by His\(^{57} \), Arg\(^{60} \), Glu\(^{193} \), Ser\(^{195} \), and two water molecules both in the native state and benzamidine-inhibited states (Fig. 2B), which suggests that the catalytic triad is unaffected by benzamidine binding and could account for the residual amidolytic activity observed in benzamidine-inhibited ACC-C toward small substrates (25).

The binding of benzamidine at the S1 subsite results in significant, structural modifications in the C-terminal extension (244–245e), 99-loop (Cys\(^{91} \), Leu\(^{92} \), and Asn\(^{95} \)), 174-loop, and in the 37-loop (r.m.s. deviations up 2.97 Å) (Figs. 1B and 3). The anion-binding pocket formed between the highly conserved C-terminal extension and the 99-loop, stabilized by a disulfide bridge (Cys\(^{91} \)–Cys\(^{245e} \)) and a salt bridge (between Pro\(^{245g} \) and Lys\(^{101} \)), binds either \( SO_4^{2-} \) or \( Cl^- \) ions depending on the presence or absence of a benzamidine molecule at the active site (Figs. 1B and 3). In native ACC-C, Arg\(^{82\#} \) (# indicates amino acids from a symmetry equivalent molecule) also participates in the coordination of the sulfate ion by forming a hydrophilic pocket (between Arg\(^{82\#} \), Arg\(^{83\#} \), and Phe\(^{64\#} \), Fig. 3) and binds a water molecule. In benzamidine-inhibited ACC-C, Arg\(^{82\#} \) also coordinates the \( Cl^- \) ion but adopts a different conformation thereby modifying the Arg\(^{82\#} \), Arg\(^{83\#} \), Phe\(^{64\#} \) loop. The phenyl ring of Phe\(^{64\#} \) functioning as a lid moves inwards creating a local hydrophobic environment that prevents the binding of the fore mentioned water molecule. These structural changes suggest the possible existence of an allosteric mechanism functioning between the active-site pocket and the C-terminal extension that preferentially binds \( Cl^- \) instead of \( SO_4^{2-} \) in response to substrate or inhibitor binding at the S1 subsite.

Superpositioning of the structure of ACC-C with TSV-PA results in 1799 topologically equivalent Cx positions with r.m.s. deviations of 0.54 Å, indicating a high degree of structural similarity in agreement with the observed sequence identity of 75% (Fig. 1A). The S domain is highly conserved and the notable structural differences are in the surface loops surrounding the active-site pocket, mainly, the 37-, 60-, and 99-loops which form the S1 domain (Fig. 4). The 37-loop located at southeast corner of the S’ domain, is stabilized by a disulfide bridge (Cys\(^{42} \)–Cys\(^{58} \)), presents a single deletion at position 37 and a N-linked Asn at position 38, which results in the formation of a truncated loop when compared with TSV-PA (r.m.s. deviation up 5.5 Å). The 60-loop is positively charged in ACC-C due to Arg\(^{60} \) and Arg\(^{64} \), whereas in TSV-PA it is negatively charged (Fig. 4). The positioning of Arg\(^{60} \) and Asp\(^{189} \) creates a polar environment between the S and S’ domains (Fig. 5). Thus, twist-erzonic inhibitors, such as 4-(4-amidinephenyl)butanoic acid, that contain a guanidine head group and a carboxylic tail could bind simultaneously to Asp\(^{189} \) and Arg\(^{60} \) blocking the S1 subsite and restricting access to the catalytic triad.

Dansyl-Glu-Gly-Arg-chloromethyl ketone (dansyl-EGR-cmk) and D-Phe-Pro-Arg-cmk rapidly inhibit both the amidolytic and anticoagulant activities of ACC-C (13). The structure of ACC-C was superimposed on the structure of the human single-chain tissue plasminogen
complexed with dansyl-EGR-cmk (Protein Data Bank entry: 1BDA) (26), and the relative position of dansyl-Glu-Gly-Arg-cmk was subjected to energy minimization. This indicates that the interaction between Arg4 from dansyl-Glu-Gly-Arg-cmk and Asp189 at the S1 subsite is maintained by hydrogen bonds formed between the carbonyl oxygen of Gly3, Lys192Ne, and the carboxylate group of Glu2 and Asn218N

The dansyl naphthalene ring forms hydrophobic interactions with the side chains of Trp99, Leu172, and Val215. These interactions could account for the efficient inactivation of ACC-C by Arg-chloromethyl ketone derivatives.

Binding of natural macromolecular inhibitors (bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor) would be prevented by the steric clash caused by the indole ring of Trp99 in ACC-C and by the phenyl ring of Phe193 in TSV-PA (Fig. 5). Interactions of these inhibitors would also be restricted by the carbohydrate moieties present on the interfacial surface of ACC-C (Fig. 1B) and AaV-SP-1/II from Agkistrodon acutus venom (22).

Another significant feature of ACC-C, apart from the three carbohydrate moieties distributed strategically around the active-site pocket, is the significant positive charge present on the interfacial surface (Fig. 6A) due to the presence of several arginine residues exposed to the bulk solvent that could bind polyanionic compounds. The amidolytic activity of ACC-C is almost completely (98%) inhibited by heparin (13) probably as a result of binding at this site. This site is negatively charged in TSV-PA (Fig. 6B) and may account for the role of electrostatic interactions in the selectivity of snake venom serine proteinases.

Under physiological conditions, the primary binding site of the 37- and 70-loops of PC zymogen in the thrombin-thrombomodulin complex is formed by a number of polar and charged side chains, which form an extended solvent-exposed region on thrombomodulin epidermal growth factor-like domains 4 and 5 (TME45) (27). The negatively charged activation peptide (sequence QVDPRLIDGK) of PC was modeled based on the crystal structure of Gla-domainless activated PC (Protein Data Bank entry: 1AUT) (28) and the interactions formed by nematode anticoagulant protein 5 from Ancylostoma caninium (NAP5) with factor Xa. In this model, electrostatic interactions formed between the positively charged surface of ACC-C created by the eight arginine residues (at positions 56, 60, 65, 82, 83, 107, 110, and 113) and the acidic residues of the activation peptide and the interaction of Arg60 (discussed above) that forms the P3

Additionally, the three carbohydrate moieties that form prongs on the interfacial surface of ACC-C are probably important in the recognition and orientation of PC zymogen during activation. In physiological PC activation, the carbohydrate moieties are not considered to be important and are not required either for thrombin binding or for cofactor activity as indicated by the activity of thrombomodulin expressed in Escherichia coli (29, 30).

**DISCUSSION**

The vitamin K-dependent enzyme PC (31) is physiologically activated by cleavage of the Arg69-Leu72 bond by the thrombin/thrombomodu-
lin complex and functions as an anticoagulant enzyme by deactivating factors Va and VIIIa (32). Functional assays of PC activation are complicated either by incomplete activation by thrombin or by the interference of thrombin in chromogenic assays. The ACC-C commercialized by Pentapharm as Protac® does not rely on other plasmatic factors and is clinically used for PC assays by the direct chromogenic method, by the indirect chromogenic method based on activated partial thromboplastin time, or by a functional clotting assay (13, 14). Since the activation product of ACC-C is not influenced by the inhibitory effect of PC inhibitors, time-consuming adsorption steps to separate PC are not required (14).

Analysis of the crystal structures of native and inhibited ACC-C indicates that binding of benzamidine does not involve the residues of the catalytic triad, which could account for the residual amidolytic activity observed when benzamidine and benzamidine derivatives are used as inhibitors (25). However, benzamidine binding induces significant structural changes: (i) the 37-loop adopts a different conformation, (ii) the anion-binding site located at the extended C terminus formed by Cys91, Leu92, Asn93, Cys245e, Thr245d, and Arg82# (from a symmetry equivalent molecule) preferentially binds Cl\(^-\) instead of SO\(_4\)\(^{2-}\), and (iii) when the N\(_92\) and N\(_93\) atoms of Arg82# coordinate the SO\(_4\)\(^{2-}\) ion, the phenyl ring of Phe84# shields the hydrophobic micro-environment formed between Arg82# and Phe84#. However, when the N\(_91\) and N\(_92\) atoms of Arg82# coordinate the Cl\(^-\) ion, the phenyl ring of Phe84# flips out and the resulting altered geometry at this site creates a hydrophilic microenvironment, which permits the binding of a water molecule.

**FIGURE 4.** Multiple sequence alignment of ACC-C (A. contortrix contortrix protein C activator, GenBank™ accession code P33588), bothrombin (thrombin-like enzyme from Bothrops jararaca, GenBank™ accession code P81661), batroxobin (thrombin-like enzyme from B. atrox, GenBank™ accession code CAA31240), TSV-PA (T. stejnegeri venom plasminogen activator, GenBank™ accession code Q91516), and GHV-PA (Gloydius halys venom plasminogen activator, GenBank™ accession code AAD01624). The numbering scheme refers to chymotrypsinogen. Residues colored in light gray are identities and in dark gray are highly conserved, and the loops discussed in the text are boxed.

**FIGURE 5.** Stereo view of the active-site pocket for the superimposed structures of ACC-C (white, carbon) and TSV-PA (yellow, carbon).
These structural changes indicate the possible existence of an allosteric mechanism.

The polar character of the ends of the active site observed in the crystal structure of ACC-C indicates that zwitterzonic inhibitors such as 4-(4-amidinephenyl)butanoic acid would bind optimally with the guanidine and carboxylic sections interacting with Asp189 and Arg40, respectively, thus contributing to substrate specificity. The potent inhibitory effects on both amidolytic and anticoagulant activities induced by D-Phe-Pro-Arg-cmk and derivatives such as dansyl-Glu-Gly-Arg-cmk and dansyl-Gly-Gly-Arg-cmk are mainly due to the hydrogen bonds formed to Lys192 and Asn218 and hydrophobic interactions with Trp99, Leu172, and Val215, which simultaneously occupy the S1 subsite and modulate access to the catalytic site.

The two significant differences between ACC-C and TSV-PA are: (i) the positively charged surface surrounding the catalytic site and (ii) the three carbohydrate moieties that protrude on the surface of ACC-C, which probably play a pivotal role in macromolecular substrate recognition or specificity. Both ACC-C and TSV-PA share a long circulating half-life and demonstrate high resistance to protein-type macromolecular inhibitors, making them interesting targets for drug design. Macromolecular substrates such as bovine pancreatic trypsin and soybean trypsin inhibitors are unable to bind to TSV-PA due the positioning of the phenyl ring of Phe193 and in AaV-SP-I/II due to the presence of extended N-linked carbohydrate groups in the 37-loop. In ACC-C, both these strategies are utilized simultaneously, whereas position 193 is occupied by a glycine, the indole of Trp99 and the N-linked oligosaccharides are positioned to prevent interaction with these macromolecular inhibitors.

Under physiological conditions, thrombin depends on thrombomodulin to switch between functioning as a coagulant or anticoagulant enzyme and the TME45 domains form the primary binding site of the PC zymogen, permitting the hydrolysis of the scissile bond between Arg169 and Leu170. In the thrombomodulin-independent activation of PC by ACC-C the positive charge present on the interfacial surface and carbohydrate moieties are important features that could play crucial roles in PC recognition/binding/activation.

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