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Procoagulant Proteins from Snake Venoms

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Abstract
Several procoagulant proteins from snake venoms have been isolated and characterized. They are either serine proteinases or metalloproteinases, which activate specific zymogens of coagulation factors and initiate the coagulation cascade. These procoagulant proteins are useful in treating various thrombotic and hemostatic conditions and contribute to our understanding of molecular details in the activation of specific coagulation factors. Recent studies have shown that the prothrombin activators with serine proteinase activity are structurally and functionally similar to mammalian coagulation factors. Their structural studies should provide us insight into prothrombinase complex formation. Here, we present an overview of snake venom procoagulant factors.

Introduction

Snake venoms are rich sources of pharmacologically active proteins and peptides. These toxins interfere in several vital physiologic functions; some act as agonists and behave similarly to a natural ligand or activator of a specific step, whereas others act as antagonists and interfere in the function of a natural ligand or activator. In general, the toxins exhibit high specificity and have evolved to attack the specific, and most likely, key step in the physiologic pathway. Thus, they affect various physiologic functions with high specificity and high potency. Therefore, snake ven-

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om toxins provide several highly specific 'tools', which are useful in deciphering molecular details in various physiological processes. Some of these toxins are also useful in developing prototypes of therapeutic agents. Further, the study of toxins helps in developing protective measures to counter mortality and morbidity associated with snake envenomation.

The circulation of blood is essential for life and the integrity of the process is crucial for the survival of the organism. The integrity of this closed system is strictly regulated by the interplay of platelet aggregation, blood coagulation and vasoconstriction. These processes are intertwined and synergistic, and occur almost simultaneously to prevent blood loss from the injured vessel. Several snake venoms and their toxins have evolved to interfere in the mechanism of thrombosis and hemostasis. Some of these toxins affect platelet aggregation [1–4], whereas others affect blood coagulation [5–7]. In this review, we will describe structural and functional properties of procoagulant toxins.

Procoagulant Proteins from Snake Venom

Procoagulant venoms contain components that hasten clot formation. All the procoagulants characterized to date are proteinases; they activate a zymogen of specific coagulation factors in the coagulation cascade. Some venom proteinases also activate the protein cofactor, factor V. For example, Russell's viper *(Daboia russelli)* venom (RVV-V) and thrombocytin *(Bothrops atrox)* activate factor V, the cofactor in the prothrombinase complex [reviewed in ref. 8 and 9].

Factor VII Activation

So far, snake venom proteins that specifically activate only factor VII are not known. However, oscutarin, a group C prothrombin activator (see below) from *Oxyuranus scutellatus* venom activates factor VII [10]. Gel electrophoresis analysis of the cleavage products indicated that activation occurred at a site similar to the natural cleavage site. The activation of factor VII, similar to that of prothrombin, was greatly potentiated by Ca^{2+} ions and phospholipids, and was not dependent upon the presence of its factor Va-like subunit [10]. The structural details of oscutarin and related proteins are given below.

Factor X Activation

Venoms from Viperidae, Crotalidae and Elapidae contain a variety of proteinases capable of activating factor X [for reviews, see ref. 11 and 12]. They are either metalloproteinases or serine proteinases. The factor X activator from RVV-X, first purified by Williams and Esnouf [13], is a metalloproteinase (93 K_d glycoprotein) with a heavy chain (catalytic subunit) and two distinct light chains [14]. The heavy chain (427 residues) consists of a metalloproteinase, a disintegrin and a cysteine-rich domain [15]. The light chains are homologous to C-type lectins, particularly to factor IX/X-binding protein from *Trimeresurus flavoviridis* venom [16]. They act as regulatory subunits and recognize the Ca^{2+}-bound conformation of the Gla domain of factor X [11]. This structure of RVV-X is very similar to that of the prothrombin activator, carinactivase-1, from *Echis carinatus leukogaster* (see below). RVV-X specifically cleaves the Arg_{52}-Ile_{53} bond in the heavy chain of factor X, and it requires Ca^{2+} ions at millimolar concentrations for optimal activity. RVV-X is unable to

activate factor X bound to negatively charged phospholipid bilayers; hence, it can be used to determine the binding of factor X to phospholipid vesicles [17]. Other metalloproteinase factor X activators from *B. atrox* and *Cerastes cerastes* venoms have structures similar to RVV-X [11]. Hence, they probably have similar catalytic mechanisms.

A few serine proteinases that activate factor X have also been isolated from the venoms of *Ophiophagus hannah*, *Bungarus fasciatus*, *C. cerastes* and *C. vipera* [11]. We have also isolated a serine proteinase factor X activator from the venom of the Malayan krait, *Bungarus candidus* [Joseph and Kini, unpubl. results]. However, currently no structural details are available on any of these proteins.

Prothrombin Activators

A large number of snake species contain prothrombin activators in their venoms [for an inventory, see ref. 18 and for reviews, see ref. 19–21]. Based on their structural properties, functional characteristics and cofactor requirements, they have been categorized into four groups [19, 22–23]. For a recent review on prothrombin activators, [see ref. 24].

Group A Prothrombin Activators

These metalloproteinases efficiently activate prothrombin without the requirement of any cofactors, such as Ca^{2+} ions, phospholipids or factor Va [19]. They are widely distributed in many kinds of viper venoms. The best-known example is ecarin, from the venom of the saw-scale viper *E. carinatus* [25]. The sequence of this protein was deduced from the sequences of cDNA clones [26]. The mature protein is a metalloproteinase with 426 amino acids and shares 64% identity with the heavy chain of RVV-X. It consists of three domains: a metalloproteinase, disintegrin and a cysteine-rich domain. It is a highly efficient enzyme with a low K_m for prothrombin and a high K_{cat} [21]. It cleaves the Arg_{320}-Ile_{321} bond in prothrombin and produces meizothrombin, which is converted to α-thrombin by autolysis. Ecarin also activates descarboxyprothrombin that accumulates in plasma during warfarin therapy. Other prothrombin activators in this class [18, 19], for example those isolated from the *Bothrops* species, also have similar properties [21].

Group B Prothrombin Activators

These are Ca^{2+}-dependent prothrombin activators, such as carinactivase-1 [23] and multiactivase [27] from *E. carinatus leukogaster* and *E. multisquamatus* venom, respectively. They consist of two subunits held noncovalently: a 62 K_d metalloproteinase and a 25 K_d C-type lectin-like disulfide-liked dimer. In contrast to the group A activators, carinactivase-1 requires millimolar concentrations of Ca^{2+} for activity; it has virtually no activity in the absence of Ca^{2+}. Also, unlike ecarin, it does not activate prothrombin derivatives in which Ca^{2+}-binding has been perturbed: prethrombin-1 and descarboxyprothrombin. This property is used to develop a chromogenic assay for normal prothrombin in the plasma of warfarin-treated individuals [28]. The metalloproteinase catalytic subunit taken in isolation does not require Ca^{2+} for activity and its reconstitution with the C-type lectin-related subunit restores Ca^{2+} dependence. They recognize the Ca^{2+}-bound conformation of the Gla domain in prothrombin via the 25 K_d regulatory subunit.

Group C Activators

These are serine proteinases found apparently exclusively in the venoms of Australian elapids [18, 19], and they require only Ca^{2+} ions and negatively charged phospholipids, but not factor Va, for maximal activity. They

have been purified and characterized from *O. scutellatus* [29–31] and *Pseudonaja textilis* venoms [32] [Rao and Kini, unpubl. obs.]. The native activators have a molecular mass of approximately 300 K_d and consist of a factor Xa-like catalytic subunit (60 K_d) and a factor Va-like cofactor subunit (approximately 200 K_d). The two chains of the factor Xa-like catalytic subunit are linked by disulfide bonds, whereas the two chains of the factor Va-like cofactor are held together by noncovalent interactions. The catalytic subunit, similar to factor Xa, has weak catalytic activity in isolation, which is greatly stimulated by the presence of the factor Va-like subunit. Bovine factor Va can also substitute for the cofactor subunit of the enzyme [31, 32] [Rao and Kini, unpubl. obs.]. These activators cleave at both Arg_{271}-Thr_{272} and Arg_{320}-Ile_{321} bonds of prothrombin [31], converting it to mature thrombin, in contrast to group A and B activators, which only convert prothrombin to meizothrombin. Recent structural studies indicate that the group C prothrombin activators structurally and functionally resemble the mammalian factor Xa-Va complex.

Group D Prothrombin Activators

These are also serine proteinases found exclusively in the venoms of Australian elapids and their activities are strongly stimulated by Ca^{2+} ions, factor Va and negatively charged phospholipid vesicles [33–35]. They are serine proteinases with molecular mass ranging from 45,000 to 47,000 (based on mass spectrometry and primary structure [36], or from 52,000 to 58,000, SDS-PAGE, [37–41]). They have two chains held together by disulfide bonds. The serine proteinase active site is located in the heavy chain [36, 37].

They exhibit potent procoagulant effects, comparable to mammalian factor Xa, through activation of prothrombin [36, 37]. Similar to factor Xa, they cleave prothrombin at two sites, Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} [36, 37]. Their poor activator activity is stimulated by more than a million-fold by negatively charged phospholipids, factor Va and Ca^{2+} ions [36, 37]. They also hydrolyze factor Xa-specific chromogenic substrates [36, 37]. Recently, we determined the complete amino acid sequences of trocarin [34] and hopsarin D [Rao et al., unpubl. data]. They share high identity (53–60%) and homology (62–70%) and hence similar domain architecture with factor Xa [36]. Their light chains are homologous to the light chains of vitamin K-dependent coagulation factors, especially that of factor Xa (53–59% identity, 60–67% homology with factor Xa) [36]. Their light chains consist of an N-terminal Gla domain (residues 1–39), followed by two epidermal growth factor (EGF)-like domains, EGF-I (residues 50–81) and EGF-II (residues 89–124). Thus, group D venom prothrombin activators are true structural and functional homologues of blood coagulation factors.

Thrombin-Like Enzymes

These fibrinogen-clotting enzymes are widely distributed within several pit viper genera *(Agkistrodon, Bothrops, Lachesis* and *Trimeresurus)*, as well as some true vipers *(Bitis* and *Cerastes)* and the colubrid, *Dispholidus typus* (for an inventory, see [42] and for recent review, see [43, 44]). The thrombin-like enzymes (TLEs) are single-chain serine proteinases (for example [45]), except for the enzyme from *C. cerastes*, which is reported to consist of two identical disulfide-linked chains [46]. Some of them are glycoproteins. They share a high degree of sequence identity among themselves (approximately 67%). However, they show less than 40% homology with human thrombin. The presence of Asp 189 in all the TLEs explains their cleavage of

arginyl bonds in fibrinogen, as this is the specificity pocket residue common to all serine proteinases that hydrolyze peptide bonds after basic residues [44]. They preferentially release either fibrinopeptide A or B, rarely both with equal efficiency, unlike thrombin [43, 47]. Secondary cleavages are also often seen. They also show a fair degree of species specificity in the efficiency of fibrinogen conversion. Classical serine proteinase inhibitors inhibit TLEs, but most of them are not inhibited by thrombin inhibitors like antithrombin III and hirudin [5, 43, 47]. They act on blood plasma usually forming friable and translucent clots, presumably due to a lack of cross-linking of fibrin by factor XIIIa. TLEs often act on coagulation factor XIII as well, but appear to degrade rather than activate it [5]. Unlike thrombin, TLEs do not activate other coagulation factors [47]. Thus, although TLEs 'resemble' thrombin to an extent, they are structurally and functionally dissimilar to the coagulation factor [5, 43, 24]. These unique properties enable their clinical use as defibrinogenating agents; for example, ancrod (Arvin®; *Calloselasma rhodostoma*) and batroxobin (Defibrase®; *B. atrox moojeni*; [48]), [reviewed in ref. 43 and 49]. Since the fibrin formed is not cross-linked, it is readily degraded by the fibrinolytic system. TLEs are clinically well tolerated with no or only minimal side effects [43].

Importance of Procoagulant Venom Proteins

Procoagulant venom proteins activate the coagulation cascade at specific steps. This property has enabled their use in diagnosis of coagulation factor deficiencies by the determination of coagulation times of blood plasma treated with these proteins. As mentioned above, TLEs are also used for clinical defibrinogenation of blood plasma. The unique sequence specificity of cleavage of group D prothrombin activators can be exploited in cleavage of recombinant fusion proteins in molecular biology. Potentially, these proteins can also be used to selectively detect fully carboxylated prothrombin and lupus anticoagulant [49, 50] and possibly factor Va deficiency in human plasma. Finally, characterization of structure-function relationships in group C and D prothrombin activator will enable us to better understand interactions within the prothrombinase complex and the mechanisms of prothrombin activation.

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