REVIEW ARTICLE

Anticoagulant proteins from snake venoms: structure, function and mechanism

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Over the last several decades, research on snake venom toxins has provided not only new tools to decipher molecular details of various physiological processes, but also inspiration to design and develop a number of therapeutic agents. Blood circulation, particularly thrombosis and haemostasis, is one of the major targets of several snake venom proteins. Among them, anticoagulant proteins have contributed to our understanding of molecular mechanisms of blood coagulation and have provided potential new leads for the development of drugs to treat or to prevent unwanted clot formation. Some of these anticoagulants exhibit various enzymatic activities whereas others do not. They interfere in normal blood coagulation by different mechanisms. Although significant progress has been made in understanding the structure–function relationships and the mechanisms of some of these anticoagulants, there are still a number of questions to be answered as more new anticoagulants are being discovered. Such studies contribute to our fight against unwanted clot formation, which leads to death and debilitation in cardiac arrest and stroke in patients with cardiovascular and cerebrovascular diseases, arteriosclerosis and hypertension. This review describes the details of the structure, mechanism and structure–function relationships of anticoagulant proteins from snake venoms.

Key words: anticoagulant, C-type lectin, metalloproteinase, phospholipase A_2, snake venom, three-finger toxin.

INTRODUCTION

Snake venoms are complex mixtures of pharmacologically active proteins and polypeptides. They play an important role in incapacitating and immobilizing, as well as in digesting, prey [1,2]. Thus toxins have evolved to specifically target various critical points in the physiological systems of prey animals. Neuromuscular and circulatory systems are the two main physiological systems that are targeted by a great many toxins, as interruption(s) in these systems make the prey succumb to the venom in a short time. Over the years, a number of toxins that affect blood circulation have been isolated and characterized from various snake venoms [3–6]. Some of them affect platelet aggregation (for recent reviews, see [7–9]), whereas others affect blood coagulation. Studies of these factors have contributed immensely to the deciphering of various molecular mechanisms involved in the physiological processes. In addition, these studies have helped us in the development of various new therapeutic agents for the treatment of cardiovascular and haematological disorders [10,11]. Venom proteins affecting blood coagulation can functionally be classified as pro-coagulant or anticoagulant proteins on the basis of their ability to shorten or prolong the blood-clotting process. Pro-coagulant proteins are either serine proteinases or metalloproteinases. Their sizes vary between 24 kDa and 300 kDa. They induce blood coagulation either by specifically activating zymogen, one of the blood coagulation factors, or by directly converting soluble fibrinogen into an insoluble fibrin clot. Structural and functional details of these pro-coagulant proteins from snake venoms have been recently reviewed [12–15].

Snake venom toxins that prolong blood coagulation are proteins or glycoproteins with molecular masses ranging from 6 kDa to 350 kDa. These factors inhibit blood coagulation by different mechanisms. Some of these anticoagulant proteins exhibit enzymatic activities, such as PLA_2 (phospholipase A_2) and proteinase, whereas others do not exhibit any enzymatic activity. The mechanism of anticoagulant activity of only a few of these proteins is well understood. Further research is required to delineate the structure–function relationships and mechanism of a number of new anticoagulant proteins. Studies on such anticoagulants contribute to our understanding of 'vulnerable' sites in the coagulation cascade. Thus these studies help us to design novel strategies to develop anticoagulant therapeutic agents. Earlier reviews, dealing with snake venom proteins affecting thrombosis and haemostasis, have only marginally dealt with anticoagulant proteins [3–8,12]. This review attempts to provide an overview of the current understanding of the structure, function and mechanism of anticoagulant proteins (Table 1).

ANTICOAGULANT PROTEINS WITH ENZYMATIC ACTIVITY

Several proteins with enzymatic activity, such as PLA_2 and proteinases, inhibit blood coagulation. Some of them inhibit clot formation by the physical destruction of a factor that contributes directly to the coagulation. In these cases, the mechanisms appear to be simple and are directly dependent on the respective enzymatic activity. The study of such factors, in general, may not significantly contribute to our understanding of blood coagulation. However, at times, a careful examination of their mechanisms may be not only important, but also essential. For example, conventional wisdom suggests that PLA_2 enzymes exert their anticoagulant effects by the hydrolysis and physical destruction of

Abbreviations used: FVa etc., Factor Va, etc; Gla, γ-carboxyglutamic acid; PLA_2, phospholipase A_2; $HsPLA_2$, human secretory PLA_2; TF–FVIIa complex, tissue factor–Factor VIIa complex; TLE, thrombin-like enzyme.

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Table 1 Anticoagulant proteins from snake venom

Protein		Mechanism of action	Remarks
Enzymatic anticoagulant proteins			
1. Phospholipase A_2 enzymes	Strongly anticoagulant	Inhibits activation of FX to FXa by extrinsic tenase complex	By both enzymatic and non-enzymatic mechanisms; target protein not known
		Inhibits activation of prothrombin to thrombin by prothrombinase complex	By non-enzymatic mechanism; binds to FXa and interferes in the prothrombinase complex formation
	Weakly anticoagulant	Inhibits activation of FX to FXa by extrinsic tenase complex	By non-enzymatic mechanism through hydrolysis of phospholipids
2. Metalloproteinases (α-fibrinogenase)		Weaker soft clot formation due to physical destruction of fibrinogen	By cleaving Aα-chain of fibrinogen
3. Serine proteinases	Protein C activators	Inactivation of cofactors FVa and FVIIIa degradation	Directly activate protein C
	Thrombin-like enzymes	Deplete fibrinogen in the plasma	Releases either fibrinopeptide A or B; fibrin clots are removed leading to depletion of fibrinogen content
	Fibrinogenases	Physical destruction of fibrinogen	Mechanism not known
4. L-Amino acid oxidase		Inhibits FIX activity	Mechanism not known
Non-enzymatic anticoagulant proteins			
1. C-type lectin related proteins	FX and FIX binding proteins	Inhibit the formation of coagulation complexes	Bind to the Gla domain of FIX and FX, and interfere in their binding to phospholipids
	Bothrojaracin, bothroalternin	Inhibit the activity of thrombin	Bind to α-thrombin at both exosite-1 and exosite-2
2. Three-finger toxin	Cardiotoxins from *Naja nigricollis crawshawii* venom	Act on the extrinsic pathway of the clotting cascade	Mechanism not known
	Hemextin A and hemextin AB complex from *Hemachatus haemachatus* venom	Prevents clot initiation by inhibiting extrinsic tenase activity	Specifically binds to FVIIa

the membrane surface required for the formation of coagulation complexes. Interestingly, the anticoagulant activity of certain PLA_2 enzymes is due to their interaction with blood coagulation proteins and not phospholipid hydrolysis (for details, see below). Thus non-enzymatic mechanisms of these enzymatic proteins cannot be ignored.

PLA_2 enzymes

PLA_2 enzymes are esterolytic enzymes which hydrolyse glycerophospholipids at the $sn-2$ position of the glycerol backbone releasing lysophospholipids and fatty acids. Snake venoms are rich sources of PLA_2 enzymes. Several hundred snake venom PLA_2 enzymes have been purified and characterized. Amino acid sequences of over 280 PLA_2 enzymes have been determined [16,17]. (A database is available at http://sdmc.lit.org.sg/Templar/DB/snaketoxin_PLA2/index.html.) They are approx. 13 kDa proteins and contain 116–124 amino acid residues and six or seven disulphide bonds. They are rarely glycosylated. So far, three-dimensional structures of more than 30 PLA_2 enzymes have been determined (for a comprehensive list, see [18]). The structural data indicate that snake venom PLA_2 enzymes share strong structural similarity to mammalian pancreatic as well as secretory PLA_2 enzymes. They have a core of three α-helices, a distinctive backbone loop that binds catalytically important calcium ions, and a β-wing that consists of a single loop of antiparallel β-sheet. The C-terminal segment forms a semicircular 'banister', particularly in viperid and crotalid PLA_2 enzymes, around the Ca^{2+}-binding loop. In addition, they have a similar catalytic function in hydrolysing phospholipids at the $sn-2$ position. However, in contrast with mammalian PLA_2 enzymes, many snake venom PLA_2 enzymes are toxic and induce a wide spectrum of pharmacological effects [19–21]. These include neurotoxic, cardiotoxic, myotoxic, haemolytic, convulsive, anticoagulant, antiplatelet, oedema-inducing and tissue-damaging effects. Thus PLA_2 enzymes also form a family of snake venom toxins, which share a common structural fold but exhibit multiple functions. These factors make the structure–function relationships and the mechanisms of action intriguing, and pose exciting challenges to scientists.

Some snake venom PLA_2 enzymes inhibit blood coagulation [22–25]. Boffa and colleagues [22,23] studied the anticoagulant properties of a number of PLA_2 enzymes and classified them into strongly, weakly and non-anticoagulant enzymes. Strongly anticoagulant PLA_2 enzymes inhibit blood coagulation at concentrations below 2 μg/ml. Weakly anticoagulant PLA_2 enzymes show effects between 3 and 10 μg/ml. A number of venom PLA_2 enzymes do not prolong the clotting times significantly even at 15 μg/ml. Thus the anticoagulant activity of different PLA_2 enzymes varies significantly. Evans et al. [25] purified three anticoagulant proteins (CM-I, CM-II and CM-IV) from *Naja nigricollis* (black-necked spitting cobra) venom and showed their identity with PLA_2 enzymes. CM-IV shows at least 100-fold more potent anticoagulant activity than CM-I and CM-II [26]. On the basis of their anticoagulant properties, they were classified as strongly (CM-IV) and weakly (CM-I, CMII) anticoagulant PLA_2 enzymes respectively. Since phospholipids play a crucial role in the formation of several coagulation complexes, intuitively one might anticipate that the destruction of phospholipid surface would be the primary mechanism to account for anticoagulant effects of PLA_2 enzymes. However, strongly anticoagulant PLA_2 enzymes also affect blood coagulation by mechanisms that are independent of phospholipid hydrolysis (see below).

To explain the functional specificity and mechanism of induction of various pharmacological effects, the target model was proposed [21,27,28]. Accordingly, the susceptibility of a tissue to a particular PLA_2 enzyme is due to the presence of specific 'target sites' on the surface of target cells or tissues. These target sites are recognized by specific 'pharmacological sites' on the PLA_2 molecule that are complementary to 'target sites' in terms of charges, hydrophobicity and van der Waals contact surfaces

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[21,27,28]. Proteins (or glycoproteins) could act as specific target sites for PLA_2 enzymes. The affinity between PLA_2 and its target protein is in the low nanomolar range, whereas the binding between PLA_2 and phospholipids is in the high micromolar range. Such a four to six orders of magnitude difference in affinity between the protein–protein interaction and the protein–phospholipid interaction explains why the interaction of PLA_2 and its target protein governs the pharmacological specificity [27,28].

The target proteins such as membrane-bound receptors/acceptors are identified through studies using radiolabelled PLA_2 enzymes and specific binding studies, as well as photoaffinity labelling techniques (for details, see [29]). Anticoagulant PLA_2 enzymes, on the other hand, target one or more soluble proteins or their complexes in the coagulation cascade. Furthermore, the enzymes may interact with the active, but not the zymogen, form of the coagulation factor. Therefore different strategies have been used to identify the soluble target protein in order to understand the mechanism of anticoagulant effects of PLA_2 enzymes.

Mechanism of anticoagulant effects

A simple 'dissection approach' was used to identify the specific stage of the coagulation cascade that is inhibited by anticoagulant PLA_2 enzymes (for details, see [30,31]). In this approach, the effects of an anticoagulant on three commonly used clotting time assays, namely prothrombin time, Stypven (Russell viper venom) time and thrombin time, were studied to identify the stage in the extrinsic coagulation cascade. The anticoagulant will prolong clotting times when the cascade is initiated 'upstream' of the inhibited step, whereas it will not affect the clotting times when the cascade is initiated 'downstream' of the inhibited step. Since the above clotting assays specifically initiate the coagulation cascade at three different stages, it is easier to pinpoint the specific step(s) that is (are) inhibited by the anticoagulant (for details, see [18,30,31]). Using this strategy as well as the inhibition studies of specific reconstituted complexes, it was shown that the extrinsic tenase [TF–FVIIa (tissue factor–Factor VIIa)] complex is inhibited by all three anticoagulant PLA_2 enzymes from *N. nigricollis* venom (i.e. CM-I, CM-II and CM-IV), whereas the prothrombinase complex is inhibited only by CM-IV. Thus the strongly anticoagulant enzyme CM-IV inhibits both the extrinsic tenase and prothrombinase complexes, creating two 'bottlenecks' in the coagulation cascade, whereas the weakly anticoagulant enzymes CM-I and CM-II only inhibit the extrinsic tenase complex, and create only a single bottleneck [30].

The prothrombinase complex is inhibited by the strongly anticoagulant PLA_2 enzyme CM-IV via a non-enzymatic mechanism [32,33]. The inhibition of this complex does not increase with an increase in incubation time with CM-IV. In contrast, weakly anticoagulant CM-I and CM-II fail to inhibit the prothrombinase complex even after 30 min incubation [32,33]. Despite the complete hydrolysis of phospholipids by CM-I and CM-II, thrombin formation is not significantly reduced. Thus the inhibition of the prothrombinase complex is independent of phospholipid hydrolysis. Alkylation of the active-site residue His^{48} in CM-IV results in the loss of enzymatic activity, but it retains more than 60 % of the inhibition of the prothrombinase complex. Furthermore, CM-IV inhibits thrombin formation more strongly in the absence of phospholipids than in their presence (for details, see [33]). Studies of inhibition kinetics indicate that CM-IV is a non-competitive inhibitor [33]. Thus CM-IV neither competes with prothrombin to bind to the active site of the prothrombinase complex nor binds to prothrombin. However, CM-IV competes with FVa (Factor Va) and interferes with complex formation [32,34]. The addition of increasing amounts of FVa to the mixture reverses the inhibition, suggesting that CM-IV may compete with FVa for binding to FXa (Factor Xa) [34]. Direct binding studies using isothermal calorimetry showed that CM-IV forms a 1:1 complex with FXa (K_d 500 nM) and blocks the formation of the prothrombinase complex [34]. Thus the strongly anticoagulant PLA_2 enzyme CM-IV inhibits the key step in blood coagulation by a novel non-enzymatic mechanism, and FXa is the target protein for the anticoagulant activity of this enzyme [32,34].

All three PLA_2 enzymes from *N. nigricollis* venom inhibit the extrinsic tenase (TF–FVIIa) complex [35]. Interestingly, the strongly anticoagulant enzyme CM-IV inhibits the tenase by both enzymatic and non-enzymatic mechanisms. On the other hand, weakly anticoagulant CM-I and CM-II inhibit the complex mostly by their enzymatic activity (for details, see [35]). Further studies are needed to clarify the non-enzymatic mechanism of inhibition by CM-IV.

Structure–function relationships

PLA_2 enzymes share similar protein folds and three-dimensional structures, but exhibit diverse biological properties. Thus understanding their structure–function relationships and identifying their functional sites is a subtle, complicated and challenging task. Using a combination of theoretical and experimental approaches, we and others have successfully identified some of the functional sites in PLA_2 enzymes (for references and details, see [21,27,28]).

The anticoagulant region was identified by a systematic and direct comparison of the amino acid sequences of strongly, weakly and non-anticoagulant enzymes [26]. To minimize phylogenetic and structural parameters, we initially restricted the sequence comparison to seven PLA_2 enzymes isolated from venoms of the genus *Naja* with different anticoagulant potencies, and then extended this to others. The region between residues 54 and 77 is positively charged in strongly anticoagulant PLA_2 enzymes, but negatively charged in weakly and non-anticoagulant enzymes. At both ends of the region is a pair of lysine residues that are replaced by neutral or negatively charged amino acid residues [26]. This region is located on the surface and is accessible for interaction (Figure 1A). The modification of lysine residues by carbamoylation [36], ethoxyformylation or guanidination [37] affects the anticoagulant properties of the basic PLA_2 of *N. nigricollis* venom. Neutralization of the positive charges of lysines by carbamoylation resulted in almost complete loss of anticoagulant activity, whereas the enzymatic activity was not affected significantly [36]. In contrast, guanidination of lysine residues, which leads to a retention of the positive charges, reduced the anticoagulant potency of the enzyme by only 50 % [37]. This supports the essential nature of the positive charges in the proposed anticoagulant site.

Strongly anticoagulant PLA_2 enzymes exhibit high penetrating ability, whereas non-anticoagulant ones show weak penetrating ability [23,24]. Furthermore, strongly anticoagulant PLA_2 enzymes bind to phospholipid vesicles with a significant increase in their intrinsic fluorescence, whereas poor anticoagulants show little or no effect [26,38]. The changes in the fluorescence were correlated to the microenvironment of Trp^{68} in the anticoagulant region (for details, see [26]). On the basis of a comparison of three-dimensional structures of class II PLA_2 enzymes, three independent groups of researchers supported the predicted anticoagulant site [39–41]. This region shows conformational similarity, and the presence of a positively charged residue free for intermolecular interactions at the corner of the molecule corresponding to the stretch of residues at positions 55 to 67 seems to be a common feature of most of the anticoagulant PLA_2 enzymes.

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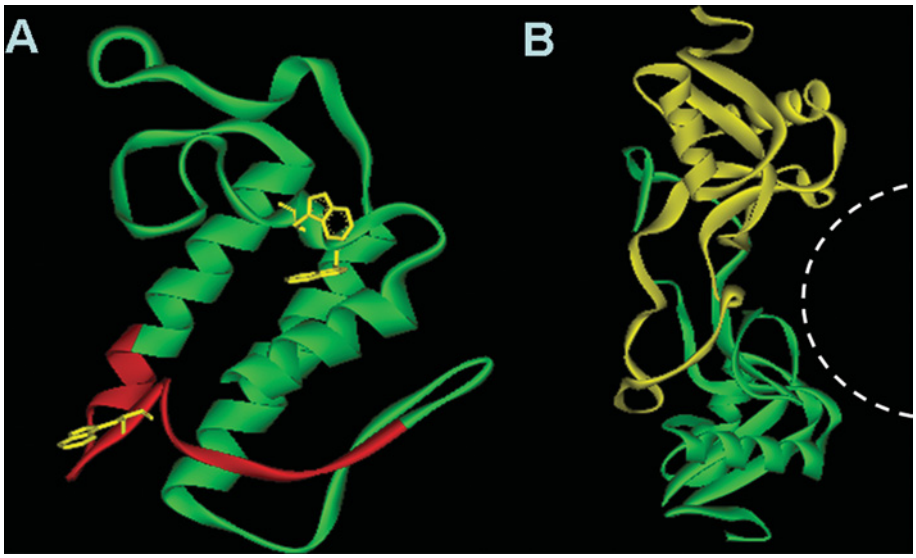

Figure 1 Structure–function relationships of anticoagulant proteins

(**A**) The predicted anticoagulant region of anticoagulant PLA_2 enzymes [26]. The ribbon model of *N. naja* PLA_2 was generated from the PDB file (#1POA) [171]. The predicted anticoagulant region (highlighted in red) is fully exposed on the surface and easily accessible for interaction. Tryptophan residues are shown in yellow. (**B**) The binding site of FIX/FX-binding protein. The ribbon model was generated from the PDB file (#1IXX) [99]. The concave central region formed through domain swapping between the two subunits is the binding site for the Gla domain [108,109], and is indicated by the white broken line).

As mentioned above, the strongly anticoagulant PLA_2 enzyme interferes with the activities of prothrombinase and extrinsic tenase complexes [33,35]. Therefore the predicted anticoagulant region of CM-IV was compared with the amino acid sequences of bovine FX, FV, prothrombin and TF. No homology was detected between the anticoagulant region of CM-IV and that of either prothrombin or FX. However, the anticoagulant region showed partial homology with a region of the light chain of FVa and with a section of TF [28,32]. This site in the light chain of FVa is closer to the site that binds to activated protein C [42]. The fact that binding of FXa to the light chain of FVa protects FVa from subsequent cleavage of the heavy chain by activated protein C [43] could be explained by the potential steric hindrance that arises when they compete for binding to two separate sites that are close to each other. The second half of the predicted anticoagulant region shares similarity with TF [28,32,44]. Thus it appears that there are two separate sites in the predicted anticoagulant region, each of the sites sharing similarity with FVa and TF respectively. We hypothesized that these sites are responsible for targeting to the prothrombinase and extrinsic tenase complexes respectively [44].

The predicted anticoagulant region is strongly supported by site-directed mutagenesis studies [45] as well as using synthetic peptides [46]. Insertion of a positive charge into porcine PLA_2 (a D59R/S60G mutant) increased its ability to inhibit the prothrombinase complex, whereas a K56Q mutant of $HsPLA_2$ (human secretory PLA_2) lost 3-fold activity [45]. However, the affinity of $HsPLA_2$ for FXa upon mutation of the residues Lys^{53}, Arg^{54}, Lys^{57} and Arg^{58} to negatively charged residues did not change significantly [47]. In contrast, mutations in the clusters of basic residues located on the interface binding site led to the loss of anti-prothrombinase activity of $HsPLA_2$. Thus the authors proposed that several clusters of basic residues probably play an important role in the electrostatic interaction of $HsPLA_2$ with FXa [47]. Recent studies using site-directed mutagenesis have also shown that the basic residues in the C-terminal tail and the β-wing of ammodytoxin A are responsible for its binding to FXa and the inhibition of the prothrombinase complex [48].

Metalloproteinases

Snake venom metalloproteinases are endoproteolytic enzymes. Their catalytic activity is dependent on Zn^{2+} ions. On the basis of size and domain structure characteristics, they are classified into P-I, P-II, P-III and P-IV classes [49,50]. P-I proteinases contain only a metalloproteinase domain, P-II proteinases contain metalloproteinase and disintegrin domains, P-III proteinases contain metalloproteinase, disintegrin-like and cysteine-rich domains, and P-IV proteinases contain the P-III domain structure plus lectin-like domains connected by disulphide bonds. To date, the sequences of over 40 metalloproteinases from snake venoms have been determined [50]. Six crystal structures of snake venom metalloproteinases are available, but all of them are from the P-I class. They are structurally similar to elastases and matrix metalloproteinases. They have a central core of a five-stranded β-sheet mixed with α-helices. There is a characteristic methionine-turn structure between the αD and αE helices. The structure is organized as an upper and lower domain with the substrate-binding cleft running between them. In addition to their role in the digestion of prey, they exhibit several biological effects, including haemorrhagic, pro-coagulant, anticoagulant and antiplatelet effects [50].

Some of the snake venom metalloproteinases inhibit blood coagulation. Most metalloproteinases are fibrinogenases and they release peptides from the C-terminal of fibrinogen. They are classified into α- and β-fibrinogenases on the basis of their specificity for the Aα or Bβ chain of fibrinogen [51]. α-Fibrinogenases inhibit blood coagulation, because truncated fibrinogen does not form as strong a fibrin clot as the native fibrinogen. Thus the subtle physical destruction leads to the anticoagulant action of metalloproteinases. The structure–function relationships of

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these metalloproteinases with respect to their anticoagulant effects have not been studied yet.

Serine proteinases

Snake venom serine proteinases, in addition to their contribution to the digestion of prey, affect various physiological functions. They affect platelet aggregation, blood coagulation, fibrinolysis, the complement system, blood pressure and the nervous system [6–9,12–15,52]. Among the serine proteinases, only protein C activators exhibit direct anticoagulant effects. Physiologically, the zymogen of protein C circulating in the blood is activated by thrombin. This activated protein C degrades FV/FVa and FVIII/FVIIIa, and releases a tissue-type plasminogen activator. It also stimulates fibrinolysis through its interaction with plasminogen activator inhibitor [53–55]. Venoms from snake species belonging to the genus *Agkistrodon* [copperhead snakes: *A. contortrix contortrix* (southern copperhead), *A. contortrix mokasen* (northern copperhead), *A. contortrix pictigaster* (Trans-Pecos copperhead), *A. piscivorus* (cottonmouth), *A. piscivorus leucostoma* (western cottonmouth), *A. halys halys* (Siberian moccasin), *A. blomhoffi ussuriensis* (Ussurian mamushi) and *A. bilineatus* (cantil)] contain protein C activators. These are glycoproteins with a molecular mass of approx. 36–40 kDa. They activate protein C at low salt concentrations in the absence of Ca^{2+} ions. High salt concentrations and the presence of Ca^{2+} ions inhibit their ability to activate protein C [56–58]. So far, the amino acid sequence of only the protein C activator from *A.c. contortrix* venom has been determined [59]. They prolong clotting times [60,61] and thrombus formation in the arteriovenous shunt [62] *in vivo*. So far, no significant data are available on the structure–function relationships of this class of proteinases.

Another group of serine proteinases, namely TLEs (thrombin-like enzymes), deplete the fibrinogen and makes the plasma unclottable. They are widely distributed within several pit viper genera (*Agkistrodon*, *Bothrops*, *Crotalus*, *Lachesis* and *Trimeresurus*), as well as some true vipers (*Bitis* and *Cerastes*) and the colubrid, *Dispholidus typus* (for an inventory and reviews, see [63–65]). They are single-chain proteins or glycoproteins (for example, see [66]) with a molecular mass of 26–33 kDa. They share a high degree of sequence similarity among themselves ($\approx$67 %). However, they show less than 40 % similarity to human thrombin. They preferentially release either fibrinopeptide A or B, although rarely both with equal efficiency, unlike thrombin [64,67]. Classical low-molecular-mass serine proteinase inhibitors inhibit them, but most are not inhibited by thrombin inhibitors like antithrombin III and hirudin [4,64,67]. They act on blood plasma and induce friable and translucent clots, presumably due to lack of cross-linking of fibrin by FXIIIa. They often also act on the coagulation factor FXIII, but appear to degrade rather than activate it [4]. Unlike thrombin, they do not activate other coagulation factors [67]. Thus, although TLEs 'resemble' thrombin to an extent, they are structurally and functionally dissimilar to the coagulation factor [4,14,15,64]. Furthermore, flavoxobin, a TLE from *Trimeresurus flavoviridis* (Habu snake) venom, activates complement C3 protein and acts as a heterologous C3 convertase [68]. These unique properties enable their clinical use as defibrinogenating agents; for example, ancrod [Arvin®; from *Calloselasma rhodostoma* (the Malayan pit viper)] and batroxobin [Defibrase®; from *Bothrops moojeni* (the Brazilian lancehead snake)] (reviewed in [69,70]). Since the fibrin formed is not cross-linked, it is readily degraded by the fibrinolytic system.

Two anticoagulant serine fibrinogenases from *Vipera lebetina* (blunt-nosed viper) venom have been characterized [71]. One is a basic (pI > 10) α-fibrinogenase, whereas the other is an acidic (pI < 3) β-fibrinogenase [72–74]. Both enzymes are structurally similar to other snake venom serine proteinases [71]. They have the catalytic triad, and, in both enzymes, Asp^{189}, which is located in the bottom of the primary specificity pocket, is replaced by Gly^{189}. (For more details on proteinases affecting thrombosis and haemostasis, their structure and properties, see [52,74–76].)

L-Amino acid oxidases

L-Amino acid oxidases catalyse the oxidative deamination of a number of L-amino acids and generate hydrogen peroxide (H_2O_2). It is widely known that these enzymes affect haemostasis by modulating platelet function [77–80]. Recently, Sakurai et al. [81] showed that L-amino acid oxidase purified from *Agkistrodon halys blomhoffii* exhibits anticoagulant activity. This enzyme affects only the intrinsic pathway, having little effect on the extrinsic pathway. Furthermore, they showed that it selectively inhibits FIX activity. H_2O_2 production does not appear to be involved in the inactivation. Interestingly, L-amino acid oxidase does not bind or interact directly with FIX, as shown by surface plasmon resonance [81]. Further studies are needed to clarify the mechanism of inactivation.

NON-ENZYMATIC ANTICOAGULANT PROTEINS

Several snake venom proteins with no 'detectable' (known or tested) enzymatic activity inhibit blood coagulation. A number of non-enzymatic anticoagulant proteins have been purified and characterized. These proteins inhibit the coagulation process through their direct interaction with a specific coagulation factor. The mechanisms appear to be simple, and these proteins interfere in either complex formation or inhibit the activity of one of the proteinases. The study of such factors significantly contributes to our understanding of blood coagulation. Furthermore, the structure–function relationships of these proteins and identification of the functional sites may be useful in the development of new anticoagulant agents.

C-type lectin-related proteins

C-type lectins are homodimers and possess the ability to agglutinate red blood cells through their interaction with carbohydrate moieties. C-type lectin-related proteins, on the other hand, are heterodimers or oligomeric complexes of heterodimers and do not possess lectin-like activity [82–84]. At times, they are also found in the snake venom as a complex with metalloproteinases. C-type lectin-related proteins form the integral part of pro-coagulant proteins, such as FX activator from *Daboia russelli* (Russell's viper; formerly *Vipera russelli*) venom and prothrombin activators from *Echis carinatus* (saw-scaled viper) and *Echis multisquamatus* (Central Asian sand viper) venoms [85–88]. In all these cases, C-type lectin-related subunits act as regulatory subunits and are involved in determining the substrate specificity in the presence of Ca^{2+} ions (for details, see [89]).

FX and FIX-binding proteins

Anticoagulant C-type lectin-related proteins were among the first non-enzymatic proteins to be isolated and characterized from snake venoms. They were first isolated and purified from *Deinagkistrodon acutus* (hundred-pace pit viper; formerly *Agkistrodon acutus*) and *Trimeresurus stejneri* (Stejneger's bamboo viper; formerly, *Trimeresurus gramineus*) venoms [90,91].

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They showed that these anticoagulant proteins inhibit prothrombin activation by non-enzymatic mechanisms [92,93]. However, these studies were not followed by detailed studies on their structure and mechanism of action. Atoda and Morita [94] purified an anticoagulant protein from *T. flavoviridis* venom using a FX-affinity column. This protein binds to FX/FXa as well as to FIX/FIXa. This anticoagulant was shown to be the first C-type lectin-related protein on the basis of its amino acid sequence and disulphide linkages [95,96]. Subsequently, they also purified and characterized a specific FIX-binding protein and FX-binding protein from *T. flavoviridis* and *D. acutus* venoms respectively [97,98]. These are heterodimeric proteins with α- and β-chains. Both chains share the common structural scaffold of C-type lectin [99,100]. The core structure is similar to the carbohydrate-recognition domain of mannose-binding protein, a C-type lectin [101,102]. A distinctive structural feature among C-type lectin-related proteins [99,100,103–106] is that the central loop of the individual subunit extends away from the core structure and forms a large open loop. This central loop forms the dimeric interface through domain swapping; a domain from the α subunit replaces essentially an identical domain in the β subunit. At the same time, this domain from the β subunit is swapped for the same domain in the α subunit. This is the first demonstrated example of three-dimensional swapping in the central region, whereas all other domain swapping occurs at the N- or C-terminus [107]. Furthermore, domain swapping is found mostly in the formation of homodimers or homo-oligomers, but not in the formation of heterodimers, as in the case of C-type lectin-related proteins [99,100,103–106]. This swapped dimeric interface, along with core structures of the α and β subunits, forms the concave ligand-binding site (see below).

The snake venom anticoagulant C-type lectin-related proteins inhibit the activity of the coagulation factors FIX and FX [96–98]. They bind to these coagulation factors with nanomolar and subnanomolar affinities. The Gla (γ-carboxyglutamic acid) domain peptides of FX (comprising residues 1–44 and 1–41) bind to FX-binding protein in the presence of Ca^{2+} with apparent dissociation constants of 1.0 and 100 nM respectively [108]. Thus most of the interaction occurs through the Gla domain. Interestingly, although FIX/FX-binding protein interacts with both FIX and FX, it has a low affinity for FX Gla domain peptides but binds to the Gla peptide of FIX-(1–46) [109]. The three-dimensional structures of the complexes [108,109] show that the Gla domains bind to the concave ligand-binding site between the two subunits (Figure 1B). The FX Gla domain has eight bound Ca^{2+} ions [108]. One of the Ca^{2+} ions participates in the binding interface between the Gla domain and the FX-binding protein. There are nine salt-bridges between the negatively charged Gla domain and the positively charged FX-binding protein, and 21 water molecules form an extensive network of hydrogen-bonds between the α-chain and the Gla domain [108]. Phe^4, Leu^5 and Val^8 in the N-terminal loop of the Gla domain interact with Arg^{112}, Met^{113} and Ile^{114} of the β-chain. Thus salt-bridges along with hydrophobic interactions and hydrogen bonds stabilize the complex between the Gla domain and the FX-binding protein (for details, see [108]). This binding interferes in the Ca^{2+}-dependent binding of FIX and FX to phospholipid membranes, and hence exhibits potent anticoagulant effects.

Bothrojaracin

A second group of snake venom anticoagulants belonging to C-type lectin-related proteins interact specifically with thrombin/prothrombin. They include bothrojaracin [110,111] and bothroalternin [112]. Bothrojaracin was purified from *Bothrops jararaca* venom as a component that inhibited thrombin-induced platelet aggregation [110], with IC_{50} values of 1–20 nM; it also inhibited binding of α-thrombin to fibrinogen with a K_i of 15 nM [110]. It, however, did not inhibit platelet aggregation induced by other agonists, such as collagen, platelet activation factor, arachidonic acid, ADP and cerestocytin, a TLE from *Cerastes vipera* (Sahara sand viper) venom. Bothrojaracin inhibited the binding of ^{125}I-labelled α-thrombin to platelets [110]. To test whether this inhibition is due to either the direct interaction with thrombin receptor(s) on the platelets or thrombin itself, Zingali et al. [110] performed an elegant experiment. They incubated 10 nM bothrojaracin (sufficient to completely inhibit thrombin-induced aggregation) with platelets and removed the supernatant. The platelets that were resuspended in a bothrojaracin-free medium aggregate when stimulated by thrombin. These results indicated that bothrojaracin interacted with thrombin, and not thrombin receptor(s) on platelets. Furthermore, they showed that ^{125}I-labelled bothrojaracin did not bind to platelets [110]. Bothrojaracin–thrombin complexes were identified on non-denaturing gel electrophoresis. Bothrojaracin inhibited thrombin effects on macromolecular substrates, but not on the small chromogenic substrate S-2238 [110]. It also neutralized the inhibition of amidolytic activity of thrombin by hirudin. From these results, Zingali et al. [110] concluded that it is an exosite inhibitor. The high-affinity binding of α-thrombin to immobilized bothrojaracin (K_d 0.6 nM) is inhibited by a C-terminal peptide (comprising residues 54–65) of hirudin, indicating that bothrojaracin binds to the exosite 1. On the other hand, γ-thrombin, in which exosite 1 is disrupted, binds only with a K_d of 0.3 μM [111]. As γ-thrombin still binds to bothrojaracin, Arocas et al. [111] examined its interaction at exosite 2 (the heparin-binding site). Bothrojaracin does not modify the rate of inhibition of α-thrombin by antithrombin in the absence of heparin. In contrast, it significantly lowers the rate of inhibition by antithrombin in the presence of heparin. Further, bothrojaracin does not bind to a heparin column, indicating that it interacts with thrombin at exosite 2 [111]. Data from solid-phase binding studies showed that γ-thrombin clearly supported the binding of bothrojaracin to exosite 2. Thus the high affinity of interaction is due to its ability to bind to both exosites 1 and 2 [111]. Structurally, it is a heterodimeric protein held together by an interchain disulphide bond. The A and B chains are similar to C-type lectin-related proteins [113]. More than one isoform of bothrojaracin has been identified in individual *Bothrops jararaca* venom [114–116]. It has been found in six *Bothrops* species [*B. atrox* (Barba Amarilla snake), *B. cotiara* (cotiara), *B. jararacussu* (jararacussu), *B. moojeni* and *B. neuwiedi* (jararaca pintada)] and in small amounts in *Lachesis muta* (bushmaster snake) venom, but not in *Crotalus durissus terrificus* (South American rattlesnake) venom [117]. In addition to its ability to inhibit thrombin directly, bothrojaracin exhibits its anticoagulant effects by inhibiting the feedback activation of coagulation factor FV [118] and activation of prothrombin to thrombin [119,120]. The inhibition of FV activation by thrombin is due to its ability to bind to exosite 1, as exosite 2 does not appear to play a direct role in FV recognition by thrombin [118]. In prothrombin, bothrojaracin binds to partially exposed anion-binding exosite (pro-exosite 1) [121]. By studying the effects of dithiothreitol and urea on subunit dissociation, unfolding and inactivation of bothrojaracin, Monteiro et al. [122] proposed a denaturation model for C-type lectin-related proteins. Overall, bothrojaracin is an excellent inhibitor of thrombin through its interaction with both the exosites, but not with the active site. Furthermore, it exhibits allosteric effects on the thrombin active site and provides a tool to study allosteric changes in thrombin [123].

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Three-finger toxins

This is a family of non-enzymatic polypeptides containing 60–74 amino acid residues [124,125]. This family of proteins is found commonly in the venoms of elapids (cobras, kraits and mambas) and hydrophids (sea snakes). Recently, they have been found in colubrid venoms [126–130], but not those of vipers and crotalids (rattlesnakes) [131]. They contain four or five disulphide bridges, of which four are conserved in all the members [125]. Consequently, all proteins of this family show a similar pattern of protein folding: three β-stranded loops extending from a central core containing the four conserved disulphide bridges [132,133]. Because of this appearance, this family of proteins is called the three-finger toxin family. Despite the overall similarity in structure, at times they differ from each other in their biological activities. Members of this family include α-neurotoxins [133–135], κ-bungarotoxins [136], muscarinic toxins [137], fasciculins [138], calciseptine [139,140], cardiotoxins (cytotoxins) [124,141], dendroaspins [142] and anticoagulant proteins [143–145]. They exhibit such varied activities through interaction with different target protein receptors/acceptors, ion channels or phospholipids (for details, see [146]). Interestingly, several other non-venom proteins and polypeptides also belong to this superfamily of proteins [147–151]. Structure–function relationships of a number of these polypeptides have been well elucidated, and their functional sites are located on distinct surfaces (for details, see [146]).

Anticoagulant three-finger toxins

The anticoagulant and antiplatelet effects of three-finger toxins were first identified in cardiotoxins isolated from *Naja nigricollis crawshawii* (spitting cobra) venom [143,144]. The mechanism of antiplatelet action [152] and structure–function relationships of these cardiotoxins [153,154] have been well elucidated. We have recently initiated studies to characterize a number of three-finger toxins with anticoagulant effects (see below).

Hemextin AB complex

Recently, a novel anticoagulant complex was characterized from *Hemachatus haemachatus* venom [145]. It has two three-finger toxins, hemextin A and hemextin B, as subunits. Individually, hemextin A prolongs blood coagulation, but hemextin B does not show any effect on blood clotting. However, hemextin B forms a 1:1 complex and synergistically enhances the anticoagulant effects of hemextin A. The dissection approach [30,31] was used to identify the coagulation step that is (are) inhibited by hemextin AB complex. Hemextin A and hemextin AB complex prolong the prothrombin time, but not the Stypven or the thrombin time, and hence we proposed that they inhibit the extrinsic tenase complex [145]. Hemextin A inhibits the reconstituted extrinsic tenase (TF–FVIIa) complex. As expected, hemextin B by itself does not inhibit the complex, but through complex formation enhances the inhibitory effects of hemextin A. Hemextin AB complex non-competitively inhibits the TF–FVIIa complex with a K_i value of 50 nM [145]. Of the 12 serine proteinases tested, hemextin A and hemextin AB complex specifically inhibit FVIIa and its complexes. In addition, they mildly inhibit plasma kallikrein activity. Thus hemextin AB complex is a highly specific natural inhibitor of the initiation of blood coagulation. It is also the first anticoagulant complex isolated from snake venom [145].

Proteinase inhibitors

Several snake venoms contain a number of isoforms of serine proteinase inhibitors [155–158]. They contain 57–60 amino acid residues and three disulphide bridges, and belong to the Kunitz pancreatic trypsin-inhibitor family [156,157]. Some of the closely related polypeptides from snake venoms block potassium and calcium channels [159–162]. Overall three-dimensional structures of proteinase inhibitors and their isoforms are similar [163–166]. As all the proteinases in blood coagulation and fibrinolysis are serine proteinases, these group polypeptides were thought to be potential anticoagulants [155]. Textilinins from *Pseudonaja textilis textilis* (Australian common brown snake) venom are being investigated for their plasmin inhibitive and antifibrinolytic activities [167,168]. However, no specific inhibitors of proteinases in blood coagulation and fibrinolysis have been identified yet.

Recently, a new family of snake venom proteins, waprins, was isolated [169]. Members of this family contain 50–52 amino acid residues with four disulphide bridges. They show significant similarity to elafin (elastase inhibitor), and other whey acidic proteins. Nawaprin, the first member of this family, shows a structural fold similar to that of elafin [169]. Although cysteine residues are conserved, waprins differ from each other in their intercysteine segments. It would be interesting to study their ability to inhibit various proteinases in blood coagulation.

FUTURE PROSPECTS

Aberrations in normal blood coagulation functions can result in thrombotic disorders or haemorrhage. In thrombosis, largely unknown conditions promote the apparently spontaneous formation of clots large enough to block circulation. Formation of such blocks in the arteries supplying vital organs, such as the heart or brain, can cause myocardial infarction or stroke respectively. Thus a life-saving mechanism of blood coagulation becomes a potentially life-threatening disease mechanism. Several conditions, such as atherosclerosis, contribute significantly to promote the spontaneous initiation of clotting. Anticoagulants are pivotal for the prevention and treatment of thromboembolic disorders, and approx. 0.7 % of the Western population receives oral anticoagulant treatment [170]. With the increasingly aging population throughout the world, more people will require antithrombotic therapies in the future. Thus various new anticoagulant and antiplatelet agents are being sought after. Proteins from snake venom affecting blood coagulation and platelet aggregation can provide us with new lead compounds to design novel therapeutic agents, providing new paradigms in the treatment of thromboembolic disorders. So far, tremendous progress has been made in understanding the structure–function relationships and mechanisms of a number of anticoagulant proteins from snake venoms. In recent years, several new anticoagulant proteins have been isolated from snake venoms. Further studies are needed to decipher the structure–function relationships and mechanisms of newly isolated anticoagulant proteins. Studies on these anticoagulant proteins have potential in identifying new drug leads.

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