Snake Venom Activators of Factor X: An Overview

Guido Tans · Jan Rosing

Cardiovascular Research Institute Maastricht (CARIM), Maastricht University,
Maastricht, The Netherlands

Key Words

Snake venom · Blood coagulation factor X · Factor X activator · Metalloprotease · Serine protease

Abstract

Activators of blood coagulation factor X have been described in the venom of many snake species belonging to the genus Viperidae and Crotalidae as well as from a few Elapid species. Based on the structural and functional properties of purified activating principles, factor X activators are either metalloproteases or serine proteases. The best known activator is RVV-X from Russell's viper (*Daboia russelli*), a metalloprotease consisting of a heavy chain containing the catalytic domain and two light chains which share homology with C-type lectins and which are thought to exert a regulatory function in the Ca²⁺-dependent activation of factor X. This activator is also one of the best

examples of the use of exogenous activators in coagulation research and in addition it is used in many diagnostic research kits. In this paper, an overview is given of the structural and functional properties of snake venom factor X activators thus far described in the literature.

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Introduction

Blood coagulation factor X is one of the key components of the haemostatic system. Factor X is synthesized as a single-chain glycoprotein of 488 amino acids including a 40-amino-acid pre-pro leader peptide. Upon secretion, the leader sequence is cleaved off, but in addition a tripeptide (Arg-Lys-Arg) is also removed and, thus, the mature protein circulates in the blood as a two-chain protein (fig. 1). The light chain contains 11 γ -carboxyglutamic acid (Gla) residues which are the

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G. Tans, PhD, Department of Biochemistry
Cardiovascular Research Institute Maastricht, Maastricht University
PO Box 616, NL-6200 MD Maastricht (The Netherlands)
Tel. +31 43 3881677, Fax +31 43 3884159
E-Mail g.tans@bioch.unimaas.nl

Fig. 1. Schematic representation of the primary sequence and domain structure of blood coagulation factor X. Each circle represents an amino acid residue. The structure is shown without the pre-pro leader sequence and without the tripeptide (Arg¹⁴⁰-Lys¹⁴¹-Arg¹⁴²) which connects the heavy chain to the light chain. The catalytic triad (Ser195, His57 and Asp102) is indicated.

result of post-translational carboxylation of specific glutamic acid residues at the amino-terminal end (Gla domain), which are essential for the Ca^{2+} -dependent binding of factor X(a) to procoagulant (negatively charged) membranes. The Gla domain is followed by two EGF-like domains, the first of which contains a β -hydroxy aspartic acid residue at position 63 which is also introduced by post-translational modification. The heavy chain

of factor X contains the catalytic triad Ser195-His57-Asp102 (chymotrypsin numbering [1]), typical of serine proteases. Activation of factor X is brought about by cleavage of a single peptide bond at Arg194-Ile195 (fig. 1) which removes the heavily glycosylated first 52 amino-terminal residues of the heavy chain and upon which the active site becomes fully exposed. Under physiological conditions factor X activation is a membrane-bound process in

Table 1. Snake species with factor-X-activating principles in the venom

Species	Reference
<i>Bothrops</i>	<i>atrox, brazili, columbiensis, insularis, marajoensis</i>
	<i>cotiara</i>
	<i>erythromelas</i>
	<i>alternatus, jararaca, jararacussu, moojeni</i>
	<i>n. paranaensis, n. pauloensis, n. urutu</i>
	<i>n. neuwiedi, n. diporus, n. matogrossensis</i>
	<i>n. pubescens, pirajai, pradoi, venezuelensis, megaera</i>
<i>Bungarus</i>	<i>fasciatus</i>
<i>Cerastes</i>	<i>cerastes, vipera</i>
<i>Calloselasma</i>	<i>rhodostoma</i>
<i>Daboia</i> ^a	<i>russelli russelli, russelli siamensis</i>
<i>Echis</i>	<i>carinatus, ocellatus</i>
<i>Naja</i>	<i>naja atra</i>
<i>Ophiophagus</i>	<i>hannah</i>
<i>Vipera</i>	<i>berus berus, aspis aspis, ammodytes ammodytes lebetina</i>

^a Only the initial original references are given.

which factor X is cleaved by the tissue factor-factor VIIa complex during the initiation phase of coagulation via the extrinsic pathway. This initial phase is efficiently attenuated by tissue factor pathway inhibitor (TFPI) but, depending upon the initial strength of the triggering event, the activation of factor X at the membrane can be sustained by the tenase complex (factor IXa together with factor VIIIa). However, even before the blood coagulation cascade hypothesis was formulated, it was already recognized that certain snake venoms were capable of very efficiently clotting blood [2]. Nowadays, it is recognized that such venoms can contain combinations of activating principles that can initiate and interfere with many steps of the blood coagulation cascade. Indeed, many of the activating principles have greatly facilitated research aimed at understanding and unravelling the mechanism of blood coagulation reactions. One of the targets to initiate or interfere with

blood coagulation appears to be at the level of factor X or factor Xa since many proteins have been identified in snake venoms that are capable of activating factor X or inhibiting factor Xa. Some of the inhibitors show a remarkable specificity and have been found in blood-sucking animals such as vampire bats [3], ticks [4] or leeches [5, 6].

Snake Venom Activators of Factor X

Since the publication of McFarlane, who was the first to identify a factor X activating principle in the venom of Russell's viper [7], many other snake venoms were found to contain a factor X activator. Table 1 gives a comprehensive list of the snake species reported. Factor X activators appear widespread in Viperid and Crotalid venoms and, more recently, a few Elapid venoms were also found to contain factor-X-activating principles. Colu-

Table 2. Structural and functional properties of purified factor-X-activating principles

Species	Enzyme class	M _r	Subunits	Cofactor(s)	Inhibitors
<i>D. russelli</i>	metalloprotease	92,880	heavy chain two light chains	Ca ²⁺	chelating agents/PL
<i>C. cerastes</i>	metalloprotease	75,000	heavy chain ^a two light chains	Ca ²⁺	chelating agents/PL
	serine protease	40,000	two subunits		DFP
	serine protease	14,000	single chain		
<i>C. vipera</i>	serine protease	12,500	single chain		PMSF
<i>B. atrox</i>	metalloprotease	75,000	heavy chain ^a two light chains	Ca ²⁺	chelating agents
<i>V. berus berus</i>	metalloprotease	38,000		Ca ²⁺	chelating agents
<i>V. aspis aspis</i>	metalloprotease	75,000	multiple chains	Ca ²⁺	
<i>V. lebetina</i>		102,000 ^b	heavy chain two light chains		
<i>O. hannah</i>	serine protease	64,500		Ca ²⁺	PMSF, TPCK, DFP
<i>B. fasciatus</i>	serine protease	70,000		Ca ²⁺	PMSF, DFP

^a A heavy chain and two light chains were reported which makes it likely that these activators consist of three subunits.

^b Calculated from the M_r reported for the heavy chain (67,000) and the two light chains (16,200 and 18,800).

brid venoms do not appear to contain factor X activators because thus far none have been reported and efforts to identify them in venom from Rhabdophis and Dispholidus genera failed [8]. Nahas et al. [9] screened the venom of 26 Bothrops species and found that only six venoms (*B. atrox asper*, *B. cotiara*, *B. fonsecai*, *B. ittapetiningae*, *B. castelnaudi*) were not able to activate factor X [9]. However, in a later screening Furtado et al. [10] did find a factor X activator in the venom of *B. cotiara* (especially in young snakes), but not in that of *B. jararacussu* (table 1). Thus, apart from some minor differences reported, the presence of factor X activators is rather widespread in snake venoms and apparently offers some advantage to the snake in captivating and/or killing its prey. From these many venoms, only a limited number of activating principles have been purified to homogeneity and

characterized well. Based on what is known about their functional and physical properties, snake venom factor X activators are either metalloproteases or serine proteases (table 2). With only a few exceptions, efficient activation of factor X critically depends upon the presence of CaCl₂. Dose-response curves for CaCl₂ typically show a sigmoidal dependency with Hill coefficients ranging from 2.4 to 7.9 [11–13]. Since the mechanism of factor X activation by the activator present in Russell's viper venom (RVV-X) is quite well understood and can be regarded as an example for the other venom activators belonging to the group of metalloproteases, we will describe this activator in more detail.

The Activator from Russell's Viper Venom

By far the strongest and most important factor X activator is the one found in the venom of Russell's viper (*Daboia russelli*, formerly known as *Vipera russelli*). Venom preparations of the major two subspecies, i.e. *Daboia russelli russelli* and *Daboia russelli siamensis*, show potencies that are approximately the same and that are at least ten- to a hundred-fold higher than in any other venom [8]. The purified factor X activator (RVV-X) contains some 13% carbohydrate and runs as a single band with 79,000 apparent M_r on SDS-PAGE [14]. RVV-X activates factor X by cleaving the Arg-Ile bond at position 194 (cf. fig. 1) and is also able to activate factor IX and protein C by specific cleavage of Arg-Ile and Arg-Val bonds [14, 15]. The exact molecular weight, sugar composition as well as the composition of the subunit structure following reduction was clarified by Gowda et al. [16] who showed that RVV-X ($M_r = 92,880$) consists of a heavy chain of 57,600 and two light chains of 19,400 and 16,400 M_r , respectively, held together by disulfide bond(s). The molecule contains six N-linked oligosaccharides, four of which are located in the heavy chain and one in each light chain. Removal of easily accessible sugars does not appear to change the functional capacity of RVV-X, but removal of the core sugars with N-glycanase causes a virtually complete loss of factor-X-activating capacity, apparently as a result of major conformational changes in the molecule [17].

The complete amino acid sequence of the heavy chain and the smallest of the two light chains have been determined [18]. The 427-residue heavy chain was found to contain three distinct domains: a metalloproteinase domain containing the conserved HExxH metalloprotease catalytic site sequence, a disinte-

grin (platelet aggregation inhibitor) domain and a cysteine-rich region, the function of which is as yet not completely clear. The disintegrin domain is presumably responsible for the strong antiplatelet-aggregating effect of RVV-X, although the specific RGD sequence is missing [18]. The light chain (123 residues) shares homology with C-type (Ca^{2+} -dependent) lectins and with the anticoagulant proteins factor IX/X binding protein of *Trimeresurus flavoviridis* [18, 19] and factor-X-binding protein of *Deinagkistrodon acutus* [19]. These latter findings are of great interest and can explain some of the mechanistic properties of RVV-X described earlier. The factor-IX/X- and X-binding proteins consist of two C-type lectin subunits held together by a single disulfide bond, probably as the result of domain swapping between the two subunits [20, 21]. These C-type lectin heterodimers specifically bind to the Gla domain of factor IX and factor X in a Ca^{2+} -dependent manner [19, 22] as a result of which the binding of these clotting factors to negatively charged membranes is likely blocked [23]. Mizuno et al. [23] recently published the crystal structure of the factor-X-binding protein of *D. acutus* in complex with the Gla domain (residues 1–44) of factor X and showed that the two C-type lectin domains form a concave region into which the Gla domain of factor X fits by interactions which involve a number of Gla residues and Ca^{2+} ions that form salt bridges with a cluster of positively charged residues in the factor-X-binding protein.

These data fit well with the model that Morita [24] proposed for RVV-X in which it is supposed that the two C-type lectin light chains of RVV-X serve as an exosite by which RVV-X recognizes and binds to the Gla domain of factor X (fig. 2). From the crystal structure of factor-X-binding protein from *D. acutus* with the Gla domain of factor X, a critical role for Ca^{2+} ions and Gla residues can be

normally carboxylated prothrombin Gla domain [29, 30]. Moreover, the isolated regulatory subunits of carinactivase recognize the Ca^{2+} -bound conformation of the prothrombin Gla domain [29] and binding of prothrombin to negatively charged lipids results in inhibition of prothrombin activation [29, 31].

Metalloprotease Activators

From the snake venom activators that were purified to homogeneity, a number were found to be both structurally and functionally similar to RVV-X (table 2). Although in the original publications concerning the activators purified from *B. atrox* and from *Cerastes cerastes* venom, it was proposed that the purified activators showed heterogeneity because two light chains were observed on SDS-PAGE [11, 32], it is very likely that these activators, like RVV-X, consist of a heavy chain containing the metalloprotease domain and two C-type lectin light chains held together by disulfide bond(s). Hoffmann and Bon [11] isolated two factor X activators from *B. atrox* venom, but apart from differences in the purification both activators show similar functional characteristics. In addition, it was noted that these activators cleave the heavy chain of factor X at two additional positions, an activity not seen with RVV-X. Very recently, an RVV-X like activator was purified from the venom of *Vipera lebetina* which, like RVV-X, was found capable of activating factor IX [33].

The activators from *Cerastes* venom deserve special attention. Franssen et al. [32] purified an activator which showed typically the same characteristics as RVV-X, but these authors failed to find other activators. In later publications, however, several other activators were identified which appear to be serine proteases (see below) [34, 35].

Vipera berus berus venom may contain several activators and one of these (VBFXAE) was purified to homogeneity and found to be a single-chain glycoprotein of 38,000 M_r [36]. This enzyme, which contains two Ca^{2+} ions, specifically activates factor X, although a weak hydrolysis of the insulin B chain was noted [36]. The latter activity was inhibited by EDTA, which indicates that this preparation contains a metalloprotease.

Komori et al. [37] purified an activator of 75,000 M_r from *Vipera aspis aspis* venom which upon reduction showed bands only at 14,000 and 16,000 M_r . These authors, therefore, suggested that the activator likely comprises a multitude of these subunits held together by disulfide bonds and the exact subunit composition awaits clarification [37].

Serine Protease Activators

Until the early 90s, factor X activators had only been identified in Crotalid and Viperid venoms. However, since then specific factor X activators have also been found in Elapid venoms (table 1, 2). These venom activators are enzymes that are quite different from the one found in *D. russelli*. They appear to be serine proteases since they are inhibited by phenylmethylsulphonyl fluoride (PMSF), diisopropylfluorophosphate (DPF) and chloromethylketones [12, 13]. Moreover, these enzymes are single-chain proteins as judged by SDS-PAGE in the absence and presence of reducing agents [12, 13]. However, they share with RVV-X an absolute dependence on Ca^{2+} ions and the effect of Ca^{2+} on the activation of factor X is strongly cooperative with Hill coefficients of 6.83 and 7.9 for the activator of *Ophiophagus hannah* and *Bungarus fasciatus*, respectively [12, 13]. Similar activators have been found in venoms from other *Bungarus* species as well [Kini, R.M., pers. commun., July 12, 2001].

The activators that were reported in *C. cerastes* and *Cerastes vipera* venom are somewhat atypical. Thus far, this has been the only species for which both an RVV-like metalloproteinase and serine protease factor X activators have been reported. Moreover, a number of the serine proteases reported are rather small (14,000 and 12,500 M_r [35]) when com-

pared to the other factor X activators described. Therefore, final sequence analysis of the various activators present in venom from *Cerastes* species will be necessary to reveal whether these low M_r enzymes reflect partially degraded products or whether these truly represent another class of activators.

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