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## REVIEW ARTICLE

**Functional implications of the unique disulfide bond in venom factor V from the Australian common brown snake *Pseudonaja textilis***Daniël Verhoef<sup>1</sup>, Xiaoxun Yang<sup>1</sup>, Sudharsan Parthasarathy<sup>2,3</sup>, Pieter H. Reitsma<sup>1</sup>, Rodney M. Camire<sup>2,3</sup>, and Mettine H. A. Bos<sup>1</sup><sup>1</sup>Department of Thrombosis and Hemostasis, Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands, <sup>2</sup>Department of Pediatrics, Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA, USA, and <sup>3</sup>School of Medicine, The University of Pennsylvania, Philadelphia, PA, USA**Abstract**

The venom of the Australian brown snake *Pseudonaja textilis* contains a prothrombinase-like initiator of blood coagulation, which has evolved into a potent weapon through several gain-of-function adaptations. Here we examined the functional implications of a disulfide bond exclusively found in the factor (F)Va-like cofactor component, ptFV. We found that this remarkable structural feature is not required for the procoagulant properties of ptFV. The nearly identical liver-derived plasma ptFV that lacks this disulfide link displayed a similar procoagulant profile. Whether the unique disulfide bond imposes conformational constraints essential to other aspects of the venom FV life cycle remains to be determined.

**Keywords**

Coagulation, factor V, hemostasis, prothrombin activation, pseudonaja textilis, snake venom

**History**

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**Introduction**

Many snake venoms contain toxins that disrupt the intricate balance between the pro- and anticoagulant mechanisms in blood (i.e. hemostasis), thereby effectively contributing to their lethal potency. One of these potent toxins that impacts hemostasis is the procoagulant prothrombin activator pseutarin C from the venom of the Australian common brown snake *Pseudonaja textilis* (Bos & Camire, 2010). Consistent with the procoagulant nature of pseutarin C, *P. textilis* envenomation triggers venom-induced consumption coagulopathy, a rapid and systemic activation of blood coagulation resulting from uncontrolled turnover of prothrombin to thrombin that exhausts the coagulation system and can result in fatal bleedings (Masci et al., 1988; Sutherland & Tibballs, 2001). The prothrombin activating complex in *P. textilis* venom consists of a factor (F)Va- and FXa-like subunit and structurally and functionally resembles the prothrombinase complex which is essential for thrombin formation during coagulation (Rao & Kini, 2002; Speijer et al., 1986).

The enzymatic activity of mammalian prothrombinase (FVa–FXa) is tightly controlled and follows from a sequential series of steps involving zymogen to protease and procofactor

to cofactor transitions, and assembly of the macromolecular enzyme complex on a negatively charged phospholipid membrane surface (Camire & Bos, 2009; Krishnaswamy, 1990). In contrast, the venom-derived prothrombinase-like complex has escaped hemostatic control through several regulatory and structural modifications (Bos & Camire, 2010). Making use of recombinant venom-derived *P. textilis* FV (ptFV), we have previously demonstrated that ptFV has uncoupled the preservation of the procofactor state and exists as a constitutively active cofactor through loss of B-domain sequences that autoinhibit FV cofactor function (Bos et al., 2009, 2012). We further uncovered that ptFV has bypassed the membrane-dependence of mammalian prothrombinase, as the *P. textilis* venom FV–FXa complex assembles and functions in solution (Bos et al., 2009). Moreover, ptFV is functionally resistant to inhibition by the anticoagulant protease activated protein C (APC), despite APC-dependent proteolysis of its A2-domain (Bos et al., 2009). These gain-of-function adaptations likely enable the enzyme to move unrestrained through the vasculature of the host and initiate indiscriminate clotting. Interestingly, since ptFV shares 99% sequence identity with its hemostatic counterpart that is expressed in the liver by a separate gene (Minh Le et al., 2005), this variant may comprise one or more of the features considered unique to ptFV.

Here we explore the functional linkage between the distinct procoagulant properties of ptFV and a disulfide bond covalently connecting its A2–A3 domains. This structural element is not conserved throughout vertebrate

Address for correspondence: Mettine H. A. Bos, Department of Thrombosis and Hemostasis, Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Albinusdreef 2, C7-Q, 2333 ZA Leiden, The Netherlands. Tel: +31 71 526 8133. E-mail: M.H.A.Bos@lumc.nl

evolution, not even in liver ptFV, and is exclusive to ptFV (Bos et al., 2009). Since the A2–A3 domains comprise binding regions for FXa and APC in human FV (Lee et al., 2011; Segers et al., 2008), we examined the contribution of the disulfide bond to both the lipid-independent ptFVa–FXa activity and functional APC resistance of ptFV.

## Materials and methods

### Proteins

DAPA and human prothrombin, prethrombin-1 and APC were from Haematologic Technologies (Essex Junction, VT). Following synthesis (GenScript; Piscataway, NJ) of the base pairs encoding venom-derived *P. textilis* FXa (ptFXa) (Rao et al., 2004), recombinant ptFXa was prepared, purified and characterized as described (Camire, 2002). All functional assays were performed at 25 °C in 20 mM Hepes, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>, 0.1% polyethylene glycol 8000, pH 7.5.

### Preparation of ptFV variants

Upon synthesis of the base pairs encoding venom-derived (ptFV) or liver-derived (ptFV-liver) *P. textilis* FV (Minh Le et al., 2005; Rao et al., 2003), recombinant FV was prepared, purified and characterized (Bos et al., 2009). The variants ptFV-SS and ptFV-liver-CC (Figure 1A) were generated employing mutagenic complementary oligonucleotides as described (Bos et al., 2009). Protein purity was assessed by SDS-PAGE using pre-cast 4%–12% gradient gels and the MOPS buffer system (Invitrogen; Carlsbad, CA). For pre-treatment with thrombin, FV variants (1000 nM) were incubated at 37 °C for 15 min with 50 nM thrombin.

### Macromolecular substrate activation

Steady-state initial velocities of macromolecular substrate cleavage were determined discontinuously at 25 °C as described (Camire, 2002). For progress curves of prethrombin-1 activation, PCPS (50 μM; 75:25 w/w) (Higgins &

Mann, 1983), DAPA (10 μM), and prethrombin-1 (1.4 μM) were incubated with the various ptFV variants (1.0 nM), and the reaction was initiated with ptFXa (0.1 nM).

### APC inactivation of ptFV variants

Inactivation of the various proteins (500 nM; ptFV, ptFV-SS, ptFV-liver or ptFV-liver-CC) in the presence of PCPS (50 μM) was initiated by APC (750 nM). Aliquots of the reaction mixtures were withdrawn at the indicated time intervals and analyzed by SDS-PAGE and through assessment of the residual FV cofactor activity. Assay mixtures contained prothrombin (1.4 μM), PCPS (50 μM), DAPA (10 μM), FV (1.0 nM), ptFXa (0.1 nM), and prothrombin activation was determined as described under the section ‘‘Macromolecular substrate activation’’.

## Results

### SDS-PAGE analysis of ptFV variants

Recent crystallization of ptFV not only confirmed our earlier observation that the A2 and A3 domains are covalently linked via a Cys–Cys bond, but also disclosed the identity of the cysteines involved (Cys642–Cys1002) (Kumar et al., 2011; Lechtenberg et al., 2013). To assess the putative allosteric contribution of the A2–A3 link to the unique procoagulant features of ptFV, we eliminated this connection by substituting the cysteines involved for serines (ptFV-SS; Figure 1A). In addition, we generated liver-expressed FV required for normal *P. textilis* hemostasis (ptFV-liver) that, despite being almost identical to ptFV, does not comprise a homologous A3-domain cysteine. The latter was introduced in ptFV-liver-CC to facilitate disulfide linkage similar to ptFV. Absence of the disulfide bond in ptFV-SS and ptFV-liver was confirmed by non-reducing SDS-PAGE analysis as demonstrated by the characteristic FVa heterodimer subsequent to thrombin activation, whereas ptFV and ptFV-liver-CC predominantly migrated as a single-chain species similar to the untreated molecules (Figure 1B).

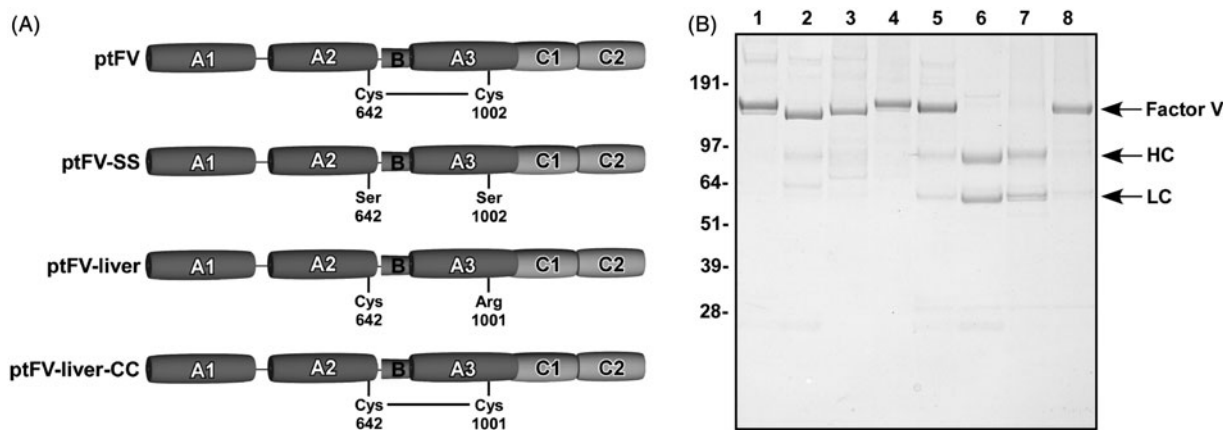


Figure 1. Schematic representation and SDS-PAGE analysis of rFV variants. *Panel A*, The common A1–A2–B–A3–C1–C2 domain structure of the recombinant *P. textilis* FV variants used in this study is shown schematically. Venom-derived *P. textilis* FV (ptFV) harbors a disulfide bond mediated by Cys642 and Cys1002 that covalently connects the A2 and A3 domains. In ptFV-SS, these Cys are substituted for Ser (Cys642Ser and Cys1002Ser). Liver-derived *P. textilis* FV (ptFV-liver) comprises an Arg at the position homologous to venom FV residue 1002, and in ptFV-liver-CC this region is modified to resemble venom FV (Arg1001Cys and Ala1002Glu). *Panel B*, Purified proteins (3 μg/lane) were subjected to SDS-PAGE under nonreducing conditions and visualized by staining with Coomassie Brilliant Blue R-250. *Lane 1*, ptFV; *lane 2*, ptFV-SS; *lane 3*, ptFV-liver; *lane 4*, ptFV plus thrombin; *lane 5*, ptFV-SS plus thrombin; *lane 6*, ptFV-liver plus thrombin. The single-chain FV species (‘‘Factor V’’), the heavy (‘‘HC’’) and light (‘‘LC’’) chains of the heterodimeric cofactor FVa, and the apparent molecular weights of the standards are indicated.

## Role of the disulfide bond in prothrombin-1 conversion

The ptFV variants were functionally assessed in the presence and absence of anionic phospholipids to examine whether the structural disulfide bond is linked to the remarkable ability of the venom-derived *P. textilis* FV–FXa complex to function in solution. To rule out the contribution of substrate-membrane binding to the rates of substrate conversion, we assayed activation of the prothrombin derivative prothrombin-1. Since prothrombin-1 lacks the membrane binding Gla and kringle-1 domains (Mann, 1976), lipid binding is not required for its efficient conversion to thrombin. All FV variants stimulated prothrombin-1 activation in an identical manner, irrespective of the availability of anionic membranes (Table 1). The apparent lack of loss- or gain-of-functions following either elimination or introduction of the Cys–Cys link suggests that the latter is not a functional requirement for the lipid-independent cofactor function of ptFV. Moreover, these data reveal that similar to venom FV, ptFV-liver exists as a constitutively active cofactor that is able to function in the absence of a lipid surface.

Table 1. Prothrombin-1 activation.

| Cofactor species | Initial velocity <sup>a</sup>          |  |
|------------------|--|--|
|                  | +PCPS<br>nM II $\alpha$ /min/nM Enzyme | –PCPS<br>nM II $\alpha$ /min/nM Enzyme |
| ptFV             | 182 $\pm$ 19                           | 197 $\pm$ 23                           |
| ptFV–SS          | 163 $\pm$ 1                            | 220 $\pm$ 9                            |
| ptFV–liver       | 184 $\pm$ 35                           | 181 $\pm$ 48                           |
| ptFV–liver–CC    | 166 $\pm$ 8                            | 183 $\pm$ 5                            |

<sup>a</sup>The initial velocity of thrombin generation (nM II $\alpha$ /min/nM Enzyme) was determined as described in the section “Materials and methods”. The mean values  $\pm$  S.D. of at least two independent determinations are presented.

## Role of the disulfide bond in functional APC resistance

We next determined whether removal of the unique disulfide bond in ptFV would affect its functional resistance to APC. Using high APC concentrations, all ptFV variants were proteolyzed at the previously identified APC cleavage sites Lys507 and Arg742, which are equivalent to the human Arg506 and Arg709 sites (Figure 2A and C) (Bos et al., 2009). As anticipated, whereas the ptFV A2<sub>C</sub> and B–A3–C1–C2 fragments are held together by the A2–A3 connection, these proteolysis products are not covalently linked in the absence of the disulfide bond (Figure 2A). Interestingly, the \*A2<sub>C</sub> fragment is lacking in APC-treated ptFV–liver, suggesting that the associated cleavage site is not available in this variant. To our surprise, full cofactor activity was retained following APC cleavage for all ptFV variants, including liver-derived ptFV (Figure 2B), even though hemostatic FV is generally assumed to be subject to APC-dependent downregulation *in vivo*. Further evaluation of the endogenous regulatory mechanisms is currently hampered by the lack of information on *P. textilis* APC. Overall, these findings indicate that not the covalent A2–A3 link, but non-covalent interactions likely stabilize the A2<sub>C</sub> fragment such that APC-treated *P. textilis* FV remains fully functional.

## Discussion

A hallmark of the macromolecular enzyme complexes involved in blood coagulation is their functional assembly on anionic membranes, which localizes the enzyme to a physiological cell surface and dramatically reduces the reaction dimensions and complexity of the assembly process (e.g. 1000-fold enhanced affinity for mammalian FVa–FXa) (Krishnaswamy et al., 1988; Mann et al., 1990). Venom ptFV–FXa has escaped this regulatory requirement, which is

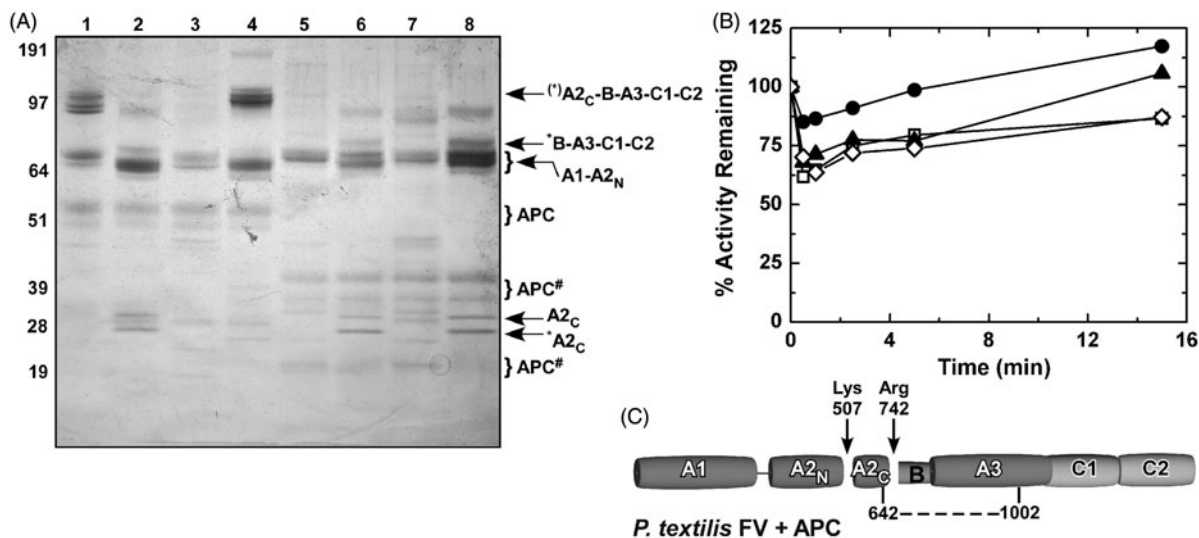


Figure 2. Inactivation of rFV variants by APC. Reaction mixtures containing 50  $\mu$ M PCPS and 500 nM ptFV (–●–), ptFV–SS (–□–), ptFV–liver (–▲–) or ptFV–liver–CC (–◇–) were incubated with 750 nM APC. At selected time intervals, samples were removed for cofactor activity (panel B) or SDS-PAGE under nonreducing (lanes 1–4) or reducing (lanes 5–8) conditions and visualized by silver staining (1  $\mu$ g/lane;  $t$  = 15 min.; panel A). Lanes 1, 5, ptFV plus APC; lanes 2, 6, ptFV–SS plus APC; lanes 3, 7, ptFV–liver plus APC; lanes 4, 8, ptFV–liver–CC plus APC. Protein bands are annotated according to the scheme in panel C: A2<sub>C</sub>–B–A3–C1–C2, 508–1430; B–A3–C1–C2, 743–1430; A1–A2<sub>N</sub>, 1–507; A2<sub>C</sub>, 508–742. Fragments denoted by \* comprise and additional cleavage site at their C-terminal ends. APC can be visualized under non-reducing and reducing conditions (#) as indicated. The functional measurements and gels are representatives of two similar experiments.



likely induced through a variety of changes in the primary structure. Our functional assessment of the A2–A3 domain disulfide link unique to ptFV revealed that it is not at the basis of the lipid-independent cofactor function. This concurs with observations made in the human system suggesting that introduction of a homologous Cys–Cys link does not promote soluble FVa–FXa activity (Verhoef et al., 2013). Functional analysis of other structural variations in ptFV or ptFXa may provide insight in this enigma (Lechtenberg et al., 2013).

Previously, we speculated that the ptFV disulfide bond stabilizes ptFV and prevents APC-mediated dissociation of the A2<sub>C</sub> fragment (Bos et al., 2009). In the current study, we showed that *P. textilis* FV variants either comprising or lacking this covalent link all proved functionally resistant to APC. This implies that a role for the disulfide bond in stabilization of the A2<sub>C</sub> fragment seems marginal at most. Whether the disulfide bond imposes conformational constraints essential to other aspects of the FV life cycle remains to be determined. Interestingly, sequence analysis revealed that a site homologous to the human APC cleavage site Arg306 is absent in both liver and venom *P. textilis* FV and is only preserved in mammals. In the mammalian system, cleavage at Arg506 results in partial (~80%) loss of cofactor activity while the A2<sub>C</sub> fragment is retained (Mann et al., 1997). Following Arg306 cleavage, FVa activity is completely lost due to A2-domain dissociation. Our observations on *P. textilis* FV cleaved at the position homologous to residue 506 demonstrated no significant loss of FV cofactor activity, thereby indicating that the A2<sub>C</sub> fragment is stabilized within the A2–A3 domain structure. Thus far we can only speculate if this structural and functional integrity results from non-covalent interactions only, or whether binding of venom ptFXa may contribute as well.

Using liver-derived ptFV that is nearly identical to its venom counterpart but lacks the Cys–Cys link, we uncovered that this variant shares the remarkable procoagulant features initially observed for venom ptFV. Liver ptFV appears to circulate in an active, cofactor state, can function in solution when assayed with venom ptFXa, and is functionally resistant to APC. The implications for normal coagulation in *P. textilis* are unclear at this point, but considering that FV can only function in the presence of the liver-derived protease FXa that is assumed to require membrane binding for its activity, the likelihood of unregulated initiation of coagulation is minimal. To compensate for the apparent functional inconsequence of APC, termination of clotting may be more dependent on coagulation inhibitors such as tissue factor pathway inhibitor- $\alpha$  which is modulated by FV in the human system (Duckers et al., 2008). Taken together, these findings invoke fundamental questions on the nature of the hemostatic regulatory processes in evolutionary distinct vertebrates.

### Declaration of Interest

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