Proexosite-1-dependent Recognition and Activation of Prothrombin by Taipan Venom*

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An activator complex from the venom of Oxyuranus scutellatus scutellatus (taipan venom) is known to rapidly activate prothrombin to thrombin. To determine whether, similar to prothrombinase, taipan venom utilizes proexosite-1 on prothrombin for a productive complex assembly, the activation of proexosite-1 mutants of prothrombin-1 by the partially purified venom was studied. It was discovered that basic residues of this site (Arg35, Lys36, Arg67, Lys70, Arg73, Arg75, and Arg77) are also crucial for recognition and rapid activation of the substrate by taipan venom. This was evidenced by the observation that the $k_{\text{on}}$ and $k_{\text{cat}}$ values for the activation of the charge reversal mutants of prothrombin-1 (in particular K36E, R67E, and K70E) were markedly impaired. Competitive kinetic studies with the Tyr-sulfated hirudin peptide revealed that although the peptide inhibits the activation of the wild type zymogen by taipan venom with a $K_D$ of ~2 μM, it is ineffective in inhibiting the activation of mutant zymogens ($K_D$ > 4-30 μM). Interestingly, an ~50-kDa activator, isolated from the taipan venom complex, catalyzed the activation of prothrombin in a factor Va-dependent manner and exhibited identical activation kinetics toward the substrate in the presence of the hirudin peptide. These results suggest that, similar to prothrombinase, proexosite-1 is a cofactor-dependent recognition site for taipan venom.

Prothrombin is a vitamin K-dependent serine protease zymogen that can be proteolytically converted to thrombin by factor Xa (FXa) that has been assembled into the prothrombinase complex (cofactor Va, negatively charged phospholipid vesicles and Ca$^{2+}$) (1–5). Factor Xa must cleave two peptide bonds at the P1 Arg273 and P1 Arg322 sites to convert prothrombin to thrombin (1). Although FXa can by itself catalyze the cleavage of both peptide bonds on the substrate, its catalytic efficiency is dramatically impaired (13). Further support for a direct interaction between basic residues of proexosite-1 with a complementary site of factor Va was provided by the observation that the mutagenesis of a hirudin-like sequence on the C-terminal heavy chain of factor Va dramatically compromised the cofactor function of factor Va in accelerating the FXa activation of prothrombin by the prothrombinase complex (14).

To determine whether, similar to activation by the prothrombinase complex, the basic residues of proexosite-1 constitute a recognition site for interaction with the taipan venom complex, the kinetics of activation of the proexosite-1 charge reversal mutants of prothrombin-1 (prothrombin lacking both the Gla and Kringle-1 domains) by the partially purified venom was studied. The prothrombin-1 mutants in which Arg35, Lys36, Arg67, Lys70, Arg73, Arg75, and Arg77 (chymotrypsinogen num-

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The abbreviations used are: FXa, factor Xa; PC, phosphatidylcholine; PS, phosphatidylserine; GPR-pNA, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide; TBS, Tris-buffered saline.
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Bering (15) were substituted with a Glu in individual constructs were expressed in mammalian cells and purified to homogeneity as described (13). The taipan venom activator complex was partially purified by the QAE-Sephadex ion exchange chromatography as described (8, 10). The SDS-PAGE and a functional activity assays revealed that both the FXα-like activator and the higher molecular weight cofactor of taipan venom are copurified by these methods. The activation kinetic data suggested that, similar to activation by the prothrombinase complex, the $K_m$ and $k_{cat}$ values for the activation of the charge reversal mutants of prethrombin-1 (in particular K36E, R67E, and K70E) have been markedly impaired. Further kinetic studies in the presence of the proexosite-1 specific peptide Tyr$^63$-sulfated hirudin$^{54—65}$ suggested that the hirudin peptide inhibits the taipan venom activation of prethrombin-1 with a $K_m$ of ~2 μM. However, the competitive inhibitory effect of the hirudin peptide on the activation of the mutants was impaired at varying degrees, which correlated well with the extent of the impairments observed in the activation of mutant zymogens. In agreement with previous results (10), the catalytically active subunit of the taipan venom complex was determined to be a ~50-kDa molecule that could catalyze the rapid activation of prothrombin in a factor Va-dependent manner. Similar to the factor Va-dependent inhibition of FXα, the hirudin peptide inhibited the activation of prothrombin by the isolated venom activator in the presence of factor Va. These results suggest that the basic residues of proexosite-1 are specific recognition sites for a factor Va-like cofactor in the taipan venom complex. The results further suggest that an interaction between the basic proexosite-1 on prothrombin and an acidic region on factor Va accounts for the mechanism of the rate accelerating effect of the cofactor in the prothrombinase complex.

EXPERIMENTAL PROCEDURES

Concentration and Expression of Mutant Proteins—The expression of wild type prothrombin-1 (prothrombin lacking both γ-carboxyglutamic acid and Kringle-1 domains) by the pNUT-PL2 expression/purification vector system in baby hamster kidney cells has been described previously (13, 16). Prethrombin-1 mutants in the chymotrypsinogen numbering system (15), R33E, K36E, R67E, K70E, R73E, R75E, and R77E, were prepared by PCR mutagenesis methods and expressed by the same vector system as described (13). Both the zymogenic and enzymatic properties of mutant proteins have been extensively characterized in previous studies (13, 16).

Human plasma proteins, antithrombin, factor Va, and FXα were purchased from Hematologic Technologies Inc. (Essen Junction, VT). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (17). The chromogenic substrates S2238, S2765, and S2222 were purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). The chromogenic substrate N$_2$-tosyl-Gly-Pro-Arg-nitroanilide (GPR-pNA), Tyr$^63$-sulfated hirudin$^{54—65}$ (Hir$^{54—65}$(SO$_3$)), and O. scutellatus scutellatus (taipan venom) were purchased from Sigma. Unfractionated heparin (heparin sodium injection, 10,000 units/ml) from beef lung and the active anti-thrombin-binding pentasaccharide fragment of heparin (fondaparinux sodium) were purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). The prothrombin activating complex was partially purified from the crude venom by an ion exchange chromatography using QAE-Sephadex A-50 (Amerham Biosciences) as described previously (8, 10).

Thus, 25 mg of crude venom was dissolved in 2 ml of 0.1 M NaCl and 50 mM NaOAc, pH 5.8, and applied to a QAE-Sephadex ion exchange column (10 × 1 cm) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of 0.1—0.6 M NaCl using a fast protein liquid chromatography system.

SDS-PAGE—Purified fractions from two QAE-Sephadex A-50 peaks were analyzed on 10% SDS-PAGE under nonreducing conditions. To identify the component of the activator complex, the gel was briefly rinsed with 0.1 M NaCl and 20 mM Tris-HCl, pH 7.5 (TBS), and then overlaid with 1 ml S2222 in 1% low melting agarose. Following 15—30 min of incubation at room temperature, the active subunit of the taipan venom was identified by its ability to cleave the chromogenic substrate S2222 and thus generate a yellow dye in the gel. The gel was then developed with Coomassie Blue. To isolate the active subunit, 100 μg of the partially purified taipan venom was applied on a 10% preparative polyacrylamide gel, and following electrophoresis, the protein band possessing amidolytic activity was sliced out of the gel and transferred to a dialysis bag containing 1 ml of 20 mM Tris-HCl, pH 7.5, and electroeluted at 4 °C. The concentration of the venom activator was determined by stoichiometric titration of the enzyme with a known concentration of antithrombin in complex with an unfractionated high molecular weight heparin as described (18).

Prothrombin and Prethrombin-1 Activation—The initial rate of prothrombin and prothrombin-1 activation by taipan venom was studied by incubating different concentrations of the substrate (0.2–30 μM) with QAE-Sephadex purified activator complex (140 μM) in TBS containing 0.1 mg/ml bovine serum albumin, 0.1% polyethylene glycol 8000, and 5 mM CaCl$_2$ (TBS/Ca$^{2+}$). Following 3—30 min of incubation at room temperature, small aliquots of the activation reactions were transferred to wells of a 96-well assay plate containing 20 mM EDTA, and the rate of thrombin generation was determined from the cleavage of S2238 (200 μM) at 405 nm by a V$_{max}$ Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). The concentration of the generated thrombin was determined from standard curves prepared from the cleavage rate of S2238 (200 μM) by known concentrations of wild type and mutant thrombins as described (13). The apparent $K_m$ and $k_{cat}$ values for prethrombin-1 activation were calculated from the Michaelis-Menten equation. In all reactions, it was ensured that less than 10% of prethrombin-1 was activated at all concentrations of the substrates.

The factor Va-dependent activation of prothrombin by the isolated ~50 kDa venom activator was utilized to evaluate the affinity of the venom activator for the human cofactor. Thus, the activation of prothrombin (0.5 μM) by the isolated activator (6.5 μM) was monitored on PC/PS vesicles (35 μM) in TBS/CA$^{2+}$ as a function of increasing concentration of human factor Va (0.05—10 μM). Following 3—5 min of incubation at room temperature, EDTA was added to a final concentration of 20 mM, and the rate of thrombin generation was determined by an amidolytic assay using S2238 as described above. The kinetics of prothrombin activation by the isolated activator in complex with a saturating concentration of factor Va (30 nM) was also studied as a function of increasing concentrations of prothrombin (7.8—1,000 nM) by the same procedures.

Prothrombin-1 Activation in the Presence of Hirudin$^{54—65}$(SO$_3$) —The inhibitory effect of the hirudin peptide on the kinetics of prothrombin-1 activation by the taipan venom complex was studied as described (12, 13). Briefly, the rate of activation of each prethrombin-1 derivative (1 μM) by the QAE-Sephadex purified venom (140 pM) was monitored in the presence of increasing concentrations of the hirudin peptide (0—30 μM) in TBS/CA$^{2+}$. The concentration of thrombin generated in each reaction was calculated from standard curves as described above except that GPR-pNA was used as the chromogenic substrate. It is known that the cleavage rate of this substrate is not affected by the occupancy of the hirudin peptide. The hirudin peptide inhibition of prothrombin-1 activation by the ~50-kDa isolated active subunit was also monitored in the presence of a saturating concentration of factor Va (30 nM) on PC/PS phospholipid vesicles (35 μM) in TBS/CA$^{2+}$. To simplify comparisons of the hirudin peptide dependence of the activation reactions, the data for all activation reactions were normalized to maximal thrombin generation in the absence of the peptide. The dissociation constants ($K_D$) for the interaction of the hirudin peptide with prethrombin-1 derivatives were calculated from Equations 1 and 2 as described (12).

$$V_{obs} = (V_{max} - V) \frac{[Pre-1-Hir]}{[Pre-1]} + V$$ (Eq. 1)

$$K_D + [Pre-1-Hir] + \frac{[Pre-1]-[Hir]}{2} - \frac{4[Pre-1][Hir]}{2}$$ (Eq. 2)

$V_{obs}$ is the observed initial rate of prothrombin-1 (Pre-1) activation; $V_{max}$ is the limiting rate at a saturating hirudin peptide concentration; Hir is hirudin peptide; $V$ is the initial rate of activation in the absence of the hirudin peptide; $K_D$ is the dissociation constant for the hirudin peptide binding to prethrombin-1; and $[Pre-1-Hir]$ represents the prothrombin-1-hirudin peptide complex concentration.

RESULTS

Expression and Purification of Recombinant Proteins—Wild type and mutant prothrombin-1 derivatives were expressed in...
The prothrombin activating complex was partially purified from the crude venom by a QAE-Sephadex ion exchange chromatography as described (8, 10). The second peak, which contained four or five major protein bands with apparent molecular masses of ~50–250 kDa (Fig. 1B, lane 1) activated prothrombin rapidly and also exhibited amidolytic activity toward FXa-specific chromogenic substrates. The venom activator hydrolyzed S2765 with $K_m$ and $k_{cat}$ values of 0.5 mM and 20 s$^{-1}$, respectively. Previously, the gel filtration of the crude venom on the Sephadex G-200 followed by a QAE-Sephadex ion exchange chromatography has also fractionated the prothrombin activating complex into four or five similar major protein bands (8, 10). However, the gel filtration step with the commercial crude venom, prior to the ion exchange chromatography, did not yield any further purification advantage in this study (data not shown).

To identify the active subunit of the prothrombin activating complex, SDS-polyacrylamide gel electrophoresis of the QAE-Sephadex isolated proteins was incubated with 1 mM S2222 dissolved in TBS containing 1% agarose. The active subunit was identified by its electrophoretic mobility and its ability to release $p$-nitroaniline and was thus identified by the appearance of a yellow band in the gel at the point corresponding to the catalytically active enzyme. As shown in Fig. 1C, the active subunit of the taipan venom activator complex migrated at an apparent molecular mass of ~50 kDa. This band was sliced out of the preparative gel and transferred to a small dialysis bag (12–14 kDa molecular mass cut-off) containing 1 ml of 20 mM Tris-HCl. The active protein was eluted at 4 °C and concentrated by Centricron (Millipore Corp., Bradford, MA), and its activity toward S2222 was determined by the absorbance at 405 nm, as determined by the amount of $p$-nitroaniline released. Each lane contains equal amount of protein (~10 μg) as determined by a Bio-Rad protein assay.

**Reaction with Antithrombin and Active Site Titration of Prothrombin Activator**—Noting that the ~50-kDa activator from taipan venom exhibited FXa-like properties, it was rationalized that the activator may also react with antithrombin. However, the incubation of the isolated ~50-kDa activator or the taipan venom activation complex partially purified by the ion exchange chromatography with 1 μM serpin for 1 h at room temperature did not result in a significant decline in the amidolytic or the proteolytic activities of enzymes. Nevertheless, it was discovered that both the isolated and the activator complex react with the serpin in the presence of a full-length heparin with second order association rate constants of 4.8 ± 0.5 × 10$^3$ M$^{-1}$ s$^{-1}$, thus making it possible to reliably determine the concentration of the active site of the venom activators by their stoichiometric titration with known concentrations of antithrombin. It should be noted that SDS-PAGE analysis of the inhibition reactions indicated that, similar to FXa, the venom activator forms a stable high molecular weight complex with the serpin with no evidence for the enzyme recognizing the serpin as a substrate. Further study revealed that the rate
Following to a final concentration of 20 mM, and the rate of thrombin generation was impaired at varying degrees. Thus, in contrast to an apparent oosite-1 mutants by taipan venom was impaired at varying degrees. The venom activator activation complex are presented in Fig. 3. The venom activator activation of prethrombin-1 derivatives by the taipan venom Complex and R73E mutants, they were also impaired. The kinetic parameters for K36E, R35E, R73E, R75E, and R77E mutants, they were also impaired.

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The initial rates of concentration dependence of the acceleration and the observational activation of antithrombin but through a template accelerating effect of heparin is not mediated through a conformational activation of antithrombin but through a template mechanism, as evidenced by heparin exhibiting an optimal concentration dependence for the acceleration and the observation that the pentasaccharide fragment of the high affinity heparin did not enhance the inhibition reaction. The optimal concentration of heparin was determined to be 0.5–1 µM in the reaction.

Prethrombin-1 Activation by the Taipan Venom Activation Complex—The initial rates of concentration dependence of the activation of prothrombin-1 derivatives by the taipan venom activation complex are presented in Fig. 3. The venom activator rapidly activated prethrombin-1 to thrombin in the absence of an exogenous cofactor. However, the activation of all proexosite-1 mutants by taipan venom was impaired at varying degrees. Thus, in contrast to an apparent $K_m$ and $k_{cat}$ values of 2.2 ± 0.1 µM and 122.1 ± 2.1 nM/min/nM, respectively, for the activation of wild prethrombin-1, the $K_m$ values were impaired ~4–7-fold for R35E, R73E, R75E, and R77E mutants (Table I). Although the $k_{cat}$ values were minimally affected for the R35E and R73E mutants, they were also impaired ~3–5-fold for the R75E and R77E mutants. The kinetic parameters for K36E, R67E, and R70E could not be determined because the rate of thrombin generation with these mutants was dramatically impaired and remained linear for up to 10–30 µM substrate (the highest concentration available) (Fig. 3). These results suggest that the basic residues of proexosite-1 on prothrombin are critical recognition sites for interaction with the venom activation complex.

Inhibition of Activation by Hir54–65(SO3)2—The Tyr53 sulfated hirudin peptide has been previously used to probe the interaction of different ligands with (pro)exosite-1 of (pro)thrombin (12, 19, 20). Thus, to determine whether the loss of specific interactions of proexosite-1 residues with the taipan venom activation complex accounts for the defective catalytic reactions with different prethrombin-1 derivatives, the initial rates of activation of mutant substrates were measured in the presence of increasing concentrations of the hirudin peptide.

The results presented in Fig. 4A and Table I indicated that the hirudin peptide inhibits the initial rate of wild type prethrombin-1 activation with a $K_I$ of 2.4 µM. However, the ability of the peptide to inhibit activation of prethrombin-1 mutants was impaired at varying degrees. The degree of impairment in $K_I$ values correlated well with the degree of impairments observed in the activation rates of mutant substrates (Table I). Thus, the hirudin peptide exhibited no inhibitory effect toward the activation of either R67E or R70E mutants, which were also ineffective substrates for the venom activator complex (Fig. 4A).

Because the taipan venom activator complex did not require factor Va, but the isolated ~50-kDa venom subunit did require factor Va for the activation of the substrate, the competitive effect of the hirudin peptide on the activation of wild type prethrombin-1 by the ~50-kDa venom activator-factor Va complex was also studied. Interestingly, the hirudin peptide effectively inhibited the cofactor effect of factor Va in prethrombin-1 activation by the isolated venom activator (Fig. 4B). The inhibitory effect of the hirudin peptide was specific for the presence of factor Va in the reaction because it did not influence the rate of substrate activation in the absence of the cofactor (data not shown). These results suggest that the hirudin peptide blocks the cofactor functions of both factor Va and an unidentified cofactor in the taipan venom activator complex by binding to the proexosite-1 of the substrate.

DISCUSSION

The results presented above suggest that the basic residues of proexosite-1 on prothrombin are recognition sites for interaction with taipan venom. In a recent study, we showed that the same basic residues are also factor Va-dependent recognition sites for the prothrombinase complex (13). Thus, both the
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Table I

| Kinetic constants for the activation of prethrombin-1 derivatives by the taipan venom activator complex and dissociation constants (K_D) for the binding of Hir^44--65(SO_3^-) to prethrombin-1 derivatives |
|---|---|---|
| | K_{miapp} | h_{cat} | K_D |
| | µM | nM/min/nM | µM |
| Wild type | 2.1 ± 0.1 | 122.1 ± 2.1 | 2.4 ± 0.5 |
| R35E | 8.1 ± 1.5 | 115.9 ± 10.1 | 3.8 ± 0.7 |
| R67E | ND | 11.1 ± 3.6 | 10 |
| K70E | ND | ND | > 30 |
| R73E | 14.9 | ND | > 30 |
| R75E | 9.3 ± 2.5 | 147.9 ± 15.6 | > 30 |
| R77E | 15.4 ± 2.2 | 22.0 ± 3.7 | 7.3 ± 2.9 |

FIG. 4. Loss of the competitive effect of Hir^44--65(SO_3^-) on the taipan venom activation of prethrombin-1 derivatives. A, the inhibitory effect of the hirudin peptide was monitored by incubating each prethrombin-1 derivative (1 µM) with the second peak of the QAE-Sephadex purified taipan venom (140 µM) in the presence of increasing concentrations of the hirudin peptide shown on the x axis. The initial rate of thrombin generation was measured by an amidolytic activity assay using GPR-pNA, and the data were normalized to a percentage of activity at each concentration of the inhibitor (100% in the absence of the inhibitor) as described under “Experimental Procedures.” The symbols are: wild type prethrombin-1 (•), R35E (△), K36E (□), R67E (■), K70E (◇), R73E (○), R75E (◇), and R77E (△). The solid lines are best fit of data according to Equations 1 and 2. The K_{miapp} values, determined from the slope of these curves, are presented in Table I. B, the same data (A except that the competitive effect of the hirudin peptide on the wild type prethrombin-1 activation by the QAE-Sephadex purified venom complex (•) is compared with the same effect on the substrate activation by the ~50-kDa activator (6.5 µM) in complex with human factor Va (30 nM) (○).

The apparent K_{miapp} and h_{cat} values were determined from the concentration dependence of the activation of prethrombin-1 derivatives by taipan venom in TBS/CaCl_2 as described under “Experimental Procedures.” The K_{miapp} values for binding of Hir^44--65(SO_3^-) to prethrombin-1 derivatives were determined from nonlinear regression analysis of inhibition kinetic data (shown in Fig. 4) according to Equations 1 and 2 as described in the text. ND, not determinable at substrate concentrations of up to 30 µM for R36E and R67E and 11 µM for R76E. All of the values are the average of three measurements ± S.E.

Table II

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| | | |
most contribution for the substrate interaction with the venom activator complex. Thus, the same basic residues of proexosite-1 interact with the cofactors of both the physiological and nonphysiological activators to promote the activation of prothrombin by a similar mechanism. Previous biochemical and mutagenesis data have indicated that the complementary substrate interactive site on factor Va may reside in a hirudin-like sequence within the C-terminal heavy chain of the cofactor involving residues 659–698 (22–24). In support of this hypothesis, the recent mutagenesis of the 695DYDY698 motif of the factor Va heavy chain dramatically impaired the cofactor function of factor Va in the prothrombinase complex (14). Neither the sequence of the activator nor the cofactor of taipan venom has been determined to understand whether the same substrate interactive site is also conserved in the venom cofactor. However, a factor Va-like cofactor from another group C prothrombin activator from the eastern brown snake Pseudonaja textilis was recently cloned that has a significant sequence similarity to mammalian factor V and has a homologous domain structure composed of A1-A2-B-A3-C1-C2 domains (11). Although the Tyr residues of human factor Va are not conserved in the cofactor of P. textilis prothrombin activator, nevertheless, the C-terminal heavy chain of the cofactor contains several acidic residues that may also interact with the proexosite-1 on prothrombin (11). Further studies will be required to determine the validity of this hypothesis.

A full-length heparin accelerated the inhibition of the activator by the serpin by a template mechanism, suggesting that similar to FXa, the venom activator contains a basic heparin-binding exosite. However, the observation that the venom activator did not react with antithrombin at a detectable rate in either the absence or presence of the pentasaccharide fragment of the high affinity heparin is indicative of important differences in the structure of the active site and/or surface loops surrounding the active site pocket of the enzyme. The elucidation of the amino acid sequence of the venom activator and its cofactor will provide additional insight into the mechanism by which factor Xa interacts with the physiological substrates and inhibitors in the absence and presence of cofactors.

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