Structural Basis for Reduced Staphylocoagulase-mediated Bovine Prothrombin Activation*

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Staphylocoagulase (SC) is a protein secreted by the human pathogen, Staphylococcus aureus, that activates human prothrombin (ProT) by inducing a conformational change. SC-bound ProT efficiently clot fibrinogen, thus bypassing the physiological blood coagulation pathway. The crystal structure of a fully active SC fragment, SC-(1–325), bound to human prothrombin 2 showed that the SC-(1–325) N terminus inserts into the Ile19 pocket of prothrombin 2, thereby inducing expression of a functional catalytic site in the cognate zymogen without peptide bond cleavage. As shown here, SC-(1–325) binds to bovine and human ProT with similar affinity but activates the bovine zymogen only very poorly. By contrast to the ∼2-fold difference in chromogenic substrate kinetic constants between human thrombin and the SC-(1–325)-human (pro)thrombin complexes, SC-(1–325)-bovine ProT shows a 3,500-fold lower $k_{cat}/K_m$ compared with free bovine thrombin, because of a 47-fold increase in $K_m$ and a 67-fold decrease in $k_{cat}$. The SC-(1–325)-bovine ProT complex is ∼5,800-fold less active compared with its human counterpart. Comparison of human and bovine fibrinogen as substrates of human and bovine thrombin and the SC-(1–325)-(pro)thrombin complexes indicates that the species specificity of SC-(1–325) cofactor activity is determined primarily by differences in conformational activation of bound ProT. These results suggest that the catalytic site in the SC-(1–325)-bovine ProT complex is incompletely formed. The current crystal structure of SC-(1–325)-bovine thrombin reveals that SC would dock similarly to the bovine proenzyme, whereas the bovine (pro)thrombin-characteristic residues Arg144 and Arg145 would likely interfere with insertion of the SC N terminus, thus explaining the greatly reduced activation of bovine ProT.

Pathogenic bacteria exploit and subvert several host processes and signaling pathways (1). For example, some secreted or cell wall-bound bacterial proteins can efficiently activate trypsin-like serine proteinase zymogens circulating in the blood plasma of the host. From a mechanistic point of view, these bacterial activators can be grouped into proteolytic and nonproteolytic ones (2). The former class comprises proteinases capable of cleaving host zymogens at their physiologic Arg15-(Ile/Val)16 activation sites (using the chymotrypsinogen numbering for the catalytic domain residues of serine proteinases). This cleavage liberates a new N terminus with a typical (Ile/Val)16/Val/Ile17 sequence, which inserts into the preformed "Ile" pocket of the zymogen and engages in a strong salt bridge with the Asp194 carboxylate. The corresponding rotation of the Asp194 side chain induces formation of a functional active site (for a recent review on zymogen activation mechanisms see Ref. 3). The plasminogen (Pg)4 activator from Yersinia pestis (i.e. Pla proteinase), and related membrane-bound ompTinms belong to this class of bacterial activators (4, 5).

The second group of activators is formed by several nonenzymatic proteins, which upon binding induce functional active sites in their cognate serine proteinase zymogens. Proteolysis of the Arg15-(Ile/Val)16 bond is not needed for activation but can occur as an epiphenomenon of the activation mechanism (6, 7). Notably, these bacterial activators can modify the specificity of the bound host proteinase toward macromolecular substrates by providing novel docking sites for substrate recognition (8). The mechanism of nonproteolytic, cofactor-induced activation has been intensively investigated for streptokinase (SK), a Pg activator secreted by β-hemolytic streptococci (9, 10), as well as for staphylocoagulase (SC), a prothrombin (ProT) activator from Staphylococcus aureus (11–13). Another Pg activator from S. aureus, staphylokinase, is structurally related to SK (8, 14) but requires active plasmin (Pm) to form an activator complex of free Pm molecules (15, 16).

The N-terminal sequences of SK and SC (8,11-Ala-Gly and SC11–Val-Thr, respectively; superscripts identify cofactor residues) mimic those of mature trypsin-like catalytic domains. These conformational zymogen activators are thought to be necessary to spread and maintain infections by their respective Gram-positive pathogens. Species-specific zymogen activation by SK and SC is well documented and poorly understood covaet to their cofactor function. SK is highly specific for conformational activation of human Pg among other species, but the catalytic SK-Pg/Pm complexes proteolytically activate Pg from a broad range of species (17). The resistance of murine Pm to activation by SK has impeded the development of versatile animal models of human streptococcal infection. As elegantly shown recently in a transgenic mouse

4 The abbreviations used are: Pg, plasminogen; SC, staphylocoagulase; SC-(1–325), staphylocoagulase fragment, residues 1–325; D1, crystallographically defined domain 1 of SC residues 1–142; D2, crystallographically defined domain 2 of SC residues 150–281; Flbg, fibrinogen; [Glu]FPR-Pro, Nα-[jaceothyethyl]acetyl-o-Phe-Pro-Arg-chloromethyl ketone-inactivated ProT labeled with 5-(and 6)-iodoacetamido-2,7'-difluorofluorescein; pNA, p-nitroaniline; ProT, prothrombin; Pm, plasmin; SK, streptokinase; Pre 2, prothrombin 2, the product of cleavage of ProT at Arg271-Thr272.

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model expressing only human Pg, tissue invasion by the human pathogen *Streptococcus pyogenes* is critically dependent on human Pg, SK, and the bacterial Pg/Pm-binding M-like protein (i.e. PAM) to generate Pm that degrades fibrin and the extracellular matrix (18). Given the conservation in sequence and fold exhibited by members of the chymotrypsinogen family of serine proteinases across species, the high specificity of SC for activation of human ProT is similarly quite remarkable. Here we present for the first time peptide substrate and Fbg clotting studies that establish limits on the ability of SC-(1–325) to activate bovine ProT compared with human ProT and the effect of SC-(1–325) binding on bovine thrombin activity. The results suggest that compared with SC-(1–325):human ProT, the catalytic site in SC-(1–325):bovine ProT is incompletely formed. Surprisingly, equilibrium binding results indicate that SC-(1–325) binds bovine ProT with a 1 to 1 stoichiometry and only ~12-fold weaker than to the humanzymogen. We also present the crystal structure of SC-(1–325) bound to bovine α-thrombin, along with a more detailed description and comparison of the atomic interactions in the SC-(1–325):human (pre)thrombin and SC-(1–325):bovine thrombin complexes to define structural differences responsible for the species specificity of ProT activation by SC.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Characterization**—SC Ile1-Gln325 (SC-(1–325)) from *S. aureus* Newman, Tager strain 104, or strain BB were expressed and purified according to published procedures (12) or purified by diethyl aminomethyl chromatography, respectively. SC-(1–325) was shown previously to activate human ProT and to form an SC-(1–325):ProT complex with similar Fbg clotting activity to α-thrombin (12, 19). Human ProT was labeled specifically at the catalytic site with 5-(and 6)-(iodoacetamido)-2’7’-difluorofluorescein (Oregon Green 488 iodoacetamide) ([OG]FPR-ProT) as described previously (20). Bovine ProT and α-thrombin (Hematologic Technologies Inc.) were dialyzed into 50 mM Hepes, 110 mM NaCl, 5 mM CaCl₂, 1 mg/ml polyethylene glycol 8000, pH 7.4, quick-frozen, and stored at −80 °C. Protein concentrations were determined by absorbance at 280 nm with the following absorption coefficients (mg/ml)^−1 cm^−1) and molecular weights: human ProT, 1.47, 71,600; human thrombin, 1.74, 36,600; human Fbg, 1.54, 340,000; bovine Fbg, 1.5, 330,000; SC-(1–325), 1.00, 38,000; bovine ProT, 1.44, 72,100; and bovine thrombin, 1.95, 36,700 (20–22).

**Chromogenic Substrate Kinetics and Fbg Clotting Activity**—Hydrolysis of the thrombin substrate, H-D-Phe-Pip-Arg-pNA by bovine α-thrombin and the SC-(1–325):bovine (pro)thrombin complexes was followed by monitoring the absorbance increase at 405 nm in 50 mM Hepes, 110 mM NaCl, 5 mM CaCl₂, 1 mg/ml polyethylene glycol 8000, pH 7.4 buffer containing 1 mg/ml bovine serum albumin and 0.1 mg/ml soybean trypsin inhibitor, at 25 °C. Stoichiometric factors (n) were determined by fitting of the quadratic binding equation including competitive product inhibition to full reaction progress curves determined at H-D-Phe-Pip-Arg-pNA concentrations of 1.5, 31, and 62 μM with 1 nM bovine thrombin in presence or absence of saturating SC-(1–325). For SC-(1–325):bovine ProT cleavage of H-D-Phe-Pip-Arg-pNA, kinetic parameters were determined by fitting the Michaelis-Menten equation to initial rates collected over a substrate concentration range of 0–4 mM. In all of the assays, SC-(1–325) and bovine (pro)thrombin were preincubated for at least 20 min, and the reactions were initiated by the addition of sub-

**Staphylocoagulase Species Specificity**

**FIGURE 1. Effect of SC-(1–325) binding on activation of bovine ProT and the activity of bovine α-thrombin.** Initial velocities ([OA]_abs/min) for hydrolysis of 100 μM H-D-Phe-Pip-Arg-pNA by 50 nM bovine ProT (BProT, ○) and 1 nM bovine α-thrombin (BT, □) plotted a function of the ratio of total concentrations of SC-(1–325) to BProT or BT ([SC-(1–325)]/[BProT or BT]). The assays were performed, and the results were analyzed as described under “Experimental Procedures.”

**RESULTS**

**Activation of Bovine ProT by SC-(1–325) and Its Effect on Bovine α-Thrombin Activity**—There have been conflicting reports regarding the species specificity of SCs from different isolates. SC from strain...
TABLE 1
Effect of SC-(1–325) binding to bovine ProT and α-thrombin on the hydrolysis of H-D-Phe-Pip-Arg-pNA
Michaelis-Menten kinetic parameters determined for hydrolysis of H-D-Phe-Pip-Arg-pNA by the indicated enzyme species are listed. The parameters were obtained by nonlinear least squares fitting of the Michaelis-Menten equation to the initial rates for the SC-(1–325):bovine ProT complex or fitting of the integrated equation to progress curves for bovine α-thrombin in the presence and absence of saturating concentrations of SC-(1–325). The experiments were performed, and the data were analyzed as described under “Experimental Procedures.”

| Enzyme                        | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) | \( K_i \) |
|-------------------------------|---------------|-----------|-----------------|---------|
| α-Thrombin                    | \( 94 ± 2 \)  | \( 3 ± 1 \) | \( 34,700 \)  | \( 32 ± 2 \) |
| SC-(1–325):ProT               | \( 1.4 ± 0.1 \) | \( 140 ± 50 \) | \( 10 \)    |         |
| SC-(1–325):α-thrombin         | \( 68 ± 1 \)  | \( 9 ± 1 \)  | \( 7,400 \)  | \( 39 ± 4 \) |

TABLE 2
Effect of SC-(1–325) binding on Fbg clotting activity of human and bovine (pro)thrombin
Fbg clotting activities were determined relative to human α-thrombin for the indicated enzyme species. The experiments were performed, and the data were analyzed as described under “Experimental Procedures.” ND represents not detectable.

| Species | Enzyme | Bovine Fbg | Human Fbg |
|---------|--------|------------|-----------|
| Human   | α-Thrombin | 25         | 100       |
|         | SC-(1–325):ProT | 25         | 70        |
|         | SC-(1–325):α-thrombin | 30         | 111       |
| Bovine  | α-Thrombin | 31         | 67        |
|         | SC-(1–325):ProT | ND         | 0.07      |
|         | SC-(1–325):α-thrombin | 30         | 96        |

The results demonstrated that SC-(1–325) activates bovine ProT but that the activity of the complex \( (k_{cat}/K_m) \) toward H-D-Phe-Pip-Arg-pNA compared with the SC-(1–325):bovine thrombin complex is 740-fold lower, whereas the SC-(1–325):complex with human ProT is ~2-fold more active than SC-(1–325):human thrombin measured with the same substrate (20).

Fibrinogen Clotting Activities of Human and Bovine Thrombin and SC-(1–325):Pro(thrombin) Complexes—Fbg clotting assays were performed to assess the role of the low catalytic activity of the SC-(1–325):bovine ProT complex in bovine and human Fbg substrate recognition (Table 2). Compared with human thrombin, the SC-(1–325) complexes with human (pro)thrombin had indistinguishable activity toward human Fbg and also exhibited a similar, 3–4-fold lower activity with bovine Fbg. Bovine thrombin and SC-(1–325):bovine thrombin complex had essentially the same activities of 67% and 96%, respectively, as human thrombin toward human Fbg and lower activities of ~30% toward bovine Fbg. By contrast, the SC-(1–325):bovine ProT complex exhibited only 0.07% of the clotting activity of human thrombin toward human Fbg. Moreover, the clotting activity of the bovine complex with bovine Fbg was undetectable at concentrations up to 46 μM. These results indicated a small 3–4-fold dependence of the clotting activity on the species of Fbg for human and bovine thrombin and the corresponding SC-(1–325):thrombin complexes. In these results, the 5-fold lower activity of the bovine ProT complex in comparison with the human thrombin complex is a result of the lower affinity of the bovine ProT complex with human Fbg (20).

The effect of SC-(1–325) on the chromogenic substrate activity of bovine thrombin was examined further by full progress curve analysis to determine Michaelis-Menten kinetic parameters for α-thrombin and the SC-(1–325):α-thrombin complex. In the presence of SC-(1–325), the specificity constant \( (k_{cat}/K_m) \) for bovine α-thrombin decreased 5-fold, because of a 1.4-fold decrease in \( k_{cat} \) and a 3-fold increase in \( K_m \) (Table 1). Kinetic constants determined for the SC-(1–325):bovine ProT complex by initial rates indicated a 3,500-fold lower \( k_{cat} \) and a 5-fold lower \( K_m \) relative to human ProT. The results indicate significantly weaker substrate binding, \( k_{cat} \) for hydrolysis of H-D-Phe-Pip-Arg-pNA by the SC-(1–325):bovine ProT complex was 1.4 ± 0.1 s⁻¹ (Table 1), reduced 67-fold compared with α-thrombin. The reason for this puzzling observation is clarified below.

Chromogenic substrate kinetic studies were performed to determine the effects of SC-(1–325) on activation of bovine ProT and on the activity of bovine thrombin. SC-(1–325) activated bovine ProT with a stoichiometric factor of 0.85 ± 0.07 mol SC-(1–325)/mol ProT and with a maximal rate of 0.051 ± 0.003 ΔA₄₀₅ nm/min at 50 nM SC-(1–325):bovine ProT complex and 100 μM H-D-Phe-Pip-Arg-pNA (Fig. 1). SC-(1–325) binding to bovine α-thrombin resulted in a 1.9-fold hyperbolic decrease in the rate of substrate hydrolysis by the complex, characterized by a stoichiometry of 1.1 ± 0.6 mol SC-(1–325)/mol thrombin and an apparent dissociation constant \( (K_{D}) \) of 0.3 ± 0.2 nM (Fig. 1). The results demonstrated that SC-(1–325) bound both bovine ProT and α-thrombin and that the SC-(1–325):bovine ProT complex was ~60-fold less active as compared with the SC-(1–325):human ProT complex under the conditions of the assays, indicating that SC-(1–325) is a much more effective activator of the humanzymogen.

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$k_{cat}/K_m$ of the SC-(1–325)-bovine thrombin complex for chromogenic substrate compared with bovine thrombin (Table 1) was not reflected in the clotting activity (Table 2). Remarkably, however, the 3,470-fold lower $k_{cat}/K_m$ for chromogenic substrate hydrolysis by the SC-(1–325)-bovine ProT complex was correlated with a 1,400-fold lower clotting activity toward human Fbg and an undetectably lower activity toward bovine Fbg. These results indicated that the greatly reduced Fbg clotting activity of the SC-(1–325)-bovine ProT complex results from its low catalytic activity and suggested a minor role for species-specific recognition of substrate Fbg.

### Competitive Binding of SC-(1–325) to Native Bovine ProT and [OG]FPR-ProT

Competitive binding experiments were carried out to determine the affinity of SC-(1–325) for bovine ProT by the use of human [OG]FPR-ProT as a competitive binding probe. Simultaneous fits of direct and competitive titrations determined that SC-(1–325) bound human [OG]FPR-ProT with a stoichiometry of 1.0 ± 0.1 mol SC-(1–325)/mol labeled ProT and a dissociation constant ($K_d$) of 16 ± 9 nM, consistent with values determined in the companion paper (20). Analysis of competitive binding of [OG]FPR-ProT and native bovine ProT demonstrated that SC-(1–325) bound native bovine ProT with a $K_d$ of 0.2 ± 0.1 nM (Fig. 2). These results indicated that SC-(1–325) binds very tightly to bovine ProT and suggested that high affinity binding may not be the sole determinant of effective zymogen activation by SC-(1–325).

### The Two Helical Domains of SC-(1–325) Form a Continuous Structure

We have previously presented crystal structures of SC-(1–325) bound to human -thrombin and to its immediate precursor, Pre 2.
We have now solved the structure of the SC-(1–325) complex with bovine thrombin to low but still satisfactory resolution. A summary of crystallographic parameters and refinement statistics for this crystal structure is given in Table 3. All of the stereochemical parameters were better than average in structures solved at this resolution, as assessed with PROCHECK (www.biochem.ucl.ac.uk/prot判/check.html). Here we present a detailed analysis of the three crystal structures, as a basis for understanding the species specificity of SC and the mechanism of Fbg processing by SC(pro)thrombin complexes.

Each boomerang-shaped SC-(1–325) molecule consists of two α-helical domains, which stick together at an angle of about 110°. The N-terminal domain D1 (residues SCIle1 to SCGln142) essentially comprises three α-helices (1D1 to 3D1), which range in length from 25 to 41 residues (Fig. 3). The three α-helices are slightly wound around each other to form a left-handed α-helical coiled-coil. The C-terminal SC domain D2 comprises residuesSCThr150 to SCGly281 and also contains a three-helix bundle formed by helices 1D2 to 3D2. The two major helix bundles are topologically similar (Fig. 4A) but are only distantly related at the sequence level, pointing to an early gene duplication event. The centers of the helix bundles are occupied by a number of medium-sized hydrophobic side chains, which are mainly leucine residues in D1 but more varied in D2. These side chains pack together in a “knobs-in-holes” manner, creating a quite hydrophobic core free of buried water molecules or polar groups. In addition, D2 is C-terminally extended by three shorter α-helices. Together, 75% of all defined residues of SC-(1–325) are found in segments with helical conformation in agreement with results obtained by circular dichroism (not shown).

D1 and D2 contact each other along an interface centered on the side chain of the strictly conserved linker residueSCLeu146, burying a surface area of about 1,150 Å² from bulk solvent (Fig. 4B). The SCLeu146 side chain is enwrapped by the aliphatic parts of polar/charged residues donated by the C-terminal ends of helices α1D1 and α3D1 (e.g. the strictly conserved SC Tyr55) and the N-terminal parts of α1D2 and α5D2. Several of these side chains engage in a network of hydrogen bonds and salt bridges, most notably the superficial interdomain salt bridge, SC Glu144 ... SC Arg209. Together, this interface is well packed and rigid, so...
that the two SC domains would presumably maintain their relative orientation in solution also in the free cofactor.

The C Terminus of SC-(1–325) Is Intrinsically Disordered—The C-terminal SC-(1–325) segments from SCGlu282 to SCLeu325 are not defined by appropriate electron density in any of the six equivalent SC-(1–325) moieties of the human (pre)thrombin and bovine thrombin complexes, suggesting enhanced flexibility. Three-state secondary structure prediction methods (cubic.bioc.columbia.edu/predictprotein/), which correctly identify all major a-helices within the defined SC-(1–325) fragment, indicate with high probability that this molecular region is almost entirely solvent-exposed and devoid of regular secondary structure. An inherent disorder of this C-terminal peptide is also in agreement with VL-XT predictor analyses (www.pondr.com/), according to which most of the SC region following SCGln300 is intrinsically disordered. Around the defined C termini of both SC-(1–325) molecules, the crystals contain large solvent-filled cavities, which would seem to accommodate this disordered SCGlu282 to SCLeu325 segment without steric hindrance.

The D1-Thrombin Interface Differs in the Bovine and Human Thrombin Complexes—Upon SC-(1–325) thrombin complex formation, ~3,400 Å² of accessible surface is removed from contact with the bulk solvent (Fig. 3). Domain 1 contacts the cognate thrombin molecule primarily at the 148 loop that borders the “south” rim of the thrombin active site (with respect to the standard orientation) (27). Of note, this loop moves considerably compared with its position in human FPR-thrombin (27), Gly149 being displaced to a maximum of about 11.5 Å (Fig. 5). SC-(1–325) helices α₁D₁ and α₂D₁ as well as the α₂D₁-α₃D₁ connector engage in particularly extensive contacts with the indole moiety of Trp144. In addition to these hydrophobic interactions, a few polar/charged contacts are observed at this SC-(1–325)/thrombin interface. For example, the thrombin-internal Gln146...Arg211 salt bridge becomes embedded in a hydrophobic pocket formed by the strictly or well conserved residues SCLeu74, SCLys81, and SCPhe105, with the Arg211 guanidyl group frontally opposing the SCAsp106 carboxylate.

Notably, human and bovine thrombin diverge considerably in the 148 loop sequence, with mostly nonconservative substitutions Leu144→Arg, Lys149→Arg, Ala149A→Thr, Asn149B→Ser, Gly149D→Ala, Glu149E→Lys, and Gly150→Val. These variations in turn provoke changes at the SC-thrombin interface. For example, the 149–150 segment adopts different conformations in the two thrombin complexes, but in neither case seem to make particularly specific interactions with the SC moiety (Fig. 5).

The exchanges of Leu144 and in particular of Lys149 by Arg residues in the bovine species have more dramatic consequences. In SC-human (pre)thrombin, the distal ammonium group of Lys149, framed by SCLeu67, SCGln71, and the Glu146-Thr147 main chain segment, is fixed through favorable hydrogen bonds with SCGln71 Oε1, SCAsp70 Oδ1, and Glu146 O. By contrast, steric clashes of the bulkier Arg145 side chain of bovine (pre)thrombin would prohibit it to be similarly buried at the interface with SC. Indeed, Arg145 extends away from the thrombin moiety in the SC-bovine thrombin complex (shown in yellow in Fig. 6).

To insert into the Ile16 pocket (marked by the Asp194 carboxylate group at its bottom; Fig. 6) and to induce functional active and substrate binding sites, the N-terminal SC segment would have to change its direction at SCLys5 to circumvent the Arg145 side chain. Because of collisions of the SC N terminus with the Arg144 and Arg145 side chains, its activator capability toward the bovine zymogen would be impaired or at least considerably weakened, as observed.

**Comparison of Exosite I Interactions in the SC-(1–325)-Bovine and Human Thrombin Complexes**—The positively charged anion-binding exosite I is extremely similar in both human and bovine (pre)thrombin. It is a slightly notched surface depression “east” of the thrombin active site centered on the 70–80 loop and bordered by the 37 loop (Fig. 3) (28, 29). Its central Arg73, Arg75, Arg79, and Arg77A side chains are surrounded by other positively charged residues such as Arg35, Lys36, Lys81, Lys109, and Lys110, only interspersed by a few hydrophobic side chains such as the phenolic group of Tyr76. The vast majority of residues that form exosite I are strictly conserved in human and bovine thrombin. The edge residue Asn78 (in the human enzyme) represents an exception to this pattern of conservation, because it is replaced by a lysine in bovine thrombin. This side chain, however, is not involved in direct contacts with SC. Consequently, SC interactions with exosite I are virtually identical in the human and bovine (pre)thrombin complexes, in...
agreement with the similar SC affinities for both thrombin species (see above).

## DISCUSSION

Although SC-(1–325) activates bovine ProT, the SC-(1–325)-bovine ProT complex is about 5,800-fold less active than its human counterpart, consistent with early reports that *S. aureus* isolates activated human ProT better than bovine ProT (25). Our studies demonstrated a low intrinsic activity of the SC-(1–325)-bovine ProT complex toward a tripeptide-pNA substrate, which may explain early reports of no activation of bovine ProT by SC (30).

The role of substrate Fbg and (pro)thrombin species specificity in the clotting activities of the SC-(1–325)-(pro)thrombin complexes was evaluated by comparing the activities of human and bovine thrombin and the corresponding SC-(1–325)-(pro)thrombin complexes toward both human and bovine Fbg. The results demonstrate a relatively small, 3–4-fold preference of both human and bovine thrombin as well as SC-(1–325)-(pro)thrombin complexes for bovine Fbg. Although cofactor complexes with human thrombin and its precursor have similar activity, the bovine SC-(1–325)-ProT complex shows a drastically reduced activity toward both human and bovine Fbg. The results support the conclusion that the low catalytic activity of SC-(1–325)-bovine ProT is mainly responsible for its low Fbg clotting activity. The species specificity of SC-(1–325) cofactor activity in ProT activation and Fbg clotting is evidently primarily due to species-specific differences in conformational activation of ProT in the SC-(1–325)-ProT complexes.

In view of these results, the similar affinity of SC-(1–325) for bovine and human ProT was anticipated. Indeed, analysis of the competitive binding results indicated an approximate 12-fold higher affinity of SC-(1–325) for human [OG]/FPR-ProT compared with bovine ProT, whereas their affinities for the zymogen are 60- and 6-fold lower than SC-(1–325), respectively (12). These anticipated findings imply that presentation of a free N terminus that can reach into the Ile16 pocket is more critical for activation than both the exact nature of the N-terminal residue and the conformation of the preceding segment. The substantially higher affinity for activation of ProT by the truncated variant is explained by the close physicochemical similarity of its N-terminal SCVal2 to the natural SCile1 residue, as compared with the nonbranched SCMet0 of Met-SC-(1–325) and its ability to extend into the activation pocket.

Our crystal structures of SC-(1–325) bound to human and bovine thrombin, together with that of the SC-(1–325)-human Pre 2 complex, allow for a dissection of molecular events during ProT binding and activation. First, we notice that in these three complexes the interactions of SC-(1–325) domains D2 and D1 with exosite I and the 148 loop, respectively, are almost identical. Exosite I is in a precursor, low affinity state in ProT (proexosite I) (31), whereas the more solvent-exposed, longer 148 loop is commonly disordered in thrombin crystal structures. Therefore, considering the excellent electrostatic complementarity of the two surfaces, it appears reasonable to assume that docking of the more C-terminal D2 to proexosite I represents the initial binding event.

This strong electrostatic interaction stabilizes proexosite I in its active, thrombin-like conformation. Further, because D1 and D2 are rigidly connected, the N-terminal domain D1 would be appropriately positioned to dock the tip of the 148 loop into a shallow hydrophobic groove. The entropic penalty of stabilizing this long flexible loop is compensated for by a multitude of van der Waals and electrostatic interactions, most notably with the indole moiety of the thrombin-specific Trp148.

Finally, positioning of D1 locates the N-terminal activation hexapeptide SCile1-SCVal2 at the correct distance to insert into the Ile16 pocket of the zymogen, triggering the Asp194 side chain rotation linked to functional active site generation in a manner similar to the endogenous Ile16 N terminus formed upon proteolytic activation. The latter step is essential for cofactor-induced activation, because deletion of the SCile1-SCVal2 dipeptide reduces SC activator activity ~98% (12).

We reported previously that both Met-SC-(1–325) and the truncated form, SC-(2–325) generate complexes of only slightly different maximal activity with human ProT, whereas their affinities for the zymogen are 60- and 6-fold lower than SC-(1–325), respectively (12). These anticipated findings imply that presentation of a free N terminus that can reach into the Ile16 pocket is more critical for activation than both the exact nature of the N-terminal residue and the conformation of the preceding segment. The substantially higher affinity for activation of ProT by the truncated variant is explained by the close physicochemical similarity of its N-terminal SCVal2 to the natural SCile1 residue, as compared with the nonbranched SCMet0 of Met-SC-(1–325) and its ability to extend into the activation pocket.

Contrasting with these observations, SK-mediated Pg activation more strictly requires the N-terminal SCile1 residue (32). The crystal structure of SK bound to the Pm catalytic domain reveals that the first residue defined by electron density, SKSer12, is located ~21 Å away from the endogenous Ile16 residue (10). Considering that the similarity of catalytic domains of thrombin and Pm implies conserved interactions of the N-terminal cofactor residue with the Ile16 pocket, we predict that the N-terminal SK segment approaches Pg in a more extended conformation compared with the SC-(1–325)/Pre 2 complex and that the impaired activator activity of the SKile1-deleted variant results from the inability of SKAla2 to reach the zymogen Ile16 pocket. A previously unappreciated effect of fixing the (pro)thrombin 148 loop conformation by cofactor binding is the deflection of residues that might interfere with insertion of the activation peptide into the Ile16 pocket. Unexpectedly, bovine thrombin, although engaging in similar overall interactions with SC-(1–325), differs from its human counterpart in the location of several side chains of the 148 loop, most notably the pair of consecutive basic residues, Arg144 and Arg145. These bulky side chains would seem to interfere directly with an incoming SCile1-SCVal2 segment, both through direct steric hindrance and by generating a positively charged region that repels the N terminus of SC. This finding adds a new example to the reported phenomenon of coevolution of...
host and bacterial proteins, as recently reported for the SK-Pg system (33).

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