In *Staphylococcus aureus*—caused endocarditis, the pathogen secretes staphylocoagulase (SC), thereby activating human prothrombin (ProT) and evading immune clearance. A previous structural comparison of the SC(1–325) fragment bound to thrombin and its inactive precursor prethrombin 2 has indicated that SC activates ProT by inserting its N-terminal dipeptide Ile1-Val2 into the ProT Ile16 pocket, forming a salt bridge with ProT’s Asp194, thereby stabilizing the active conformation.

We hypothesized that these N-terminal SC residues modulate ProT binding and activation. Here, we generated labeled SC(1–246) as a probe for competitively defining the affinities of N-terminal SC(1–246) variants preselected by modeling. Using ProT(R155Q,R271Q,R284Q) (ProTQOOQ), a variant refractory to prothrombinase- or thrombin-mediated cleavage, we observed variant affinities between ~1 and 650 nM and activation potencies ranging from 1.8-fold that of WT SC(1–246) to complete loss of function. Substrate binding to ProTQOOQ caused allosteric tightening of the affinity of most SC(1–246) variants, consistent withzymogen activation through occupation of the specificity pocket. Conservative changes at positions 1 and 2 were well-tolerated, with Val1-Val2, Ile1-Ala2, and Leu1-Val2 variants exhibiting ProTQOOQ affinity and activation potency comparable with WT SC(1–246). Weaker binding variants typically had reduced activation rates, although at near-saturating ProTQOOQ levels, several variants exhibited limiting rates similar to or higher than that of WT SC(1–246). The Ile16 pocket in ProTQOOQ appears to favor nonpolar, nonaromatic residues at SC positions 1 and 2. Our results suggest that SC variants other than WT Ile1-Val2-Thr3 might emerge with similar ProT-activating efficiency.

Blood clot formation by *Staphylococcus aureus* can be attributed to the combined effects of pathogen clumping and the generation of fibrin (Fbn). The latter is initiated by the secreted virulence factor, staphylocoagulase (SC). Based on the bacteria’s ability to promote clot formation in rabbit plasma, *S. aureus* is divided into coagulase-positive and -negative subgroups. Typing of bacterial isolates for SC is still performed today in clinical diagnosis. Coagulase-positive *S. aureus* is a potent human pathogen that causes conditions ranging from minor skin infections to life-threatening diseases, such as severe pneumonia, meningitis, and bone, joint, and heart infections. Each year ~500,000 patients in American hospitals contract staphyloccocal infections that lead to ~30,000 deaths (1, 2).

Turbulent blood flow can cause endothelial damage to heart valves, exposing subendothelium that leads to deposition of platelets and Fbn. The Fbn-platelet matrix deposited on damaged valves serves as a focus for adhering *S. aureus* bacteria circulating in the blood (3). The *S. aureus*-platelet interaction is facilitated by fibrinogen (Fbg), fibronectin, thrombospondin, and MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), such as protein A and Fbg-binding ClfA (clumping factor A) (4–11). In acute bacterial endocarditis, Fbn formation is mediated by the thrombin precursor prothrombin (ProT), conformationally activated by bacterial SC. This furthers aggregation of platelets and enlargement of platelet-Fbn-bacteria vegetations on the valves (3). These friable vegetations can break up and cause pulmonary embolism and stroke. The pathogens also utilize these vegetations to disseminate and avert clearance by the host immune system (12).
Acute bacterial endocarditis caused by *S. aureus* leads to 20–40% mortality despite antibiotic therapy (13).

Blood clotting is a highly regulated process, with a delicate balance between clotting and fibrinolysis. SC bypasses the clotting cascade by directly and nonproteolytically activating ProT, thereby shifting the balance to a procoagulant state. SC binds and conformationally activates ProT, forming a complex of catalytically active zymogen and activator, SC·ProT* (with the asterisk denoting a functional catalytic site). The activated complex cleaves Fbg to form Fbn clots involved in enlarging the vegetations. No physiological inhibitors of the SC·ProT* complex have been reported to date, and it is resistant to the plasma serpins, antithrombin-heparin and heparin cofactor II,7 α2-macroglobulin (14), and the leech inhibitor, hirudin (15).

SC is a bifunctional protein with a molecular mass of ∼75,000 Da. Its N-terminal region binds ProT (16), whereas the C-terminal region contains seven 27-amino acid-repeat sequences that bind Fbg (Fig. 1) (17, 18). We previously described that the SC fragment consisting of N-terminal residues 1–325, SC(1–325), binds ProT extremely tightly (∼17–72 ps) and noncovalently in a 1:1 stoichiometry to form the active SC(1–325)·ProT* complex (19). A comparison of the crystal structures of SC(1–325) with thrombin and the inactive zymogen prothrombin 2 (Pre2, prothrombin without the fragments 1 and 2) showed that the first six residues of the SC fragment were fully defined by electron density in the complex with Pre2 but not with thrombin. SC activates the zymogen by inserting its N-terminal Ile1-Val2 (I1V2 amino acid single-letter code) residues into the Ile16 pocket of Pre2, forming a salt bridge with Asp194 and inducing a functional active site in the zymogen (19). Formation of the SC(1–325)·ProT* and SC(1–325)·Pre2* complexes partially blocks exosite 1, the Fbg-binding site, but expresses a new Fbg substrate recognition site that facilitates Fbg binding and cleavage (Fig. 2). SC(1–325) consists of two three-helix-bundle domains (D1 (residues 1–146) and D2 (residues 147–325)) with a boomerang-like structure. The D1 domain interacts with the 148-loop of thrombin or Pre2 and the south rim of the catalytic site, and the D2 domain binds (pro)exosite 1 on Pre2 and thrombin. We report here that SC(1–325), with a partially truncated D2 domain, is capable of ProT activation and binds ProT with a *K*~D~ of ∼1 nM, which made it a suitable probe for determining the affinities of mutant SC constructs by competitive equilibrium binding. The I1V2 residues are critically involved in SC-mediated ProT activation, and a comparison of the SC N-terminal residues from 12 different

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7 I. M. Verhamme, P. Panizzi, and P. E. Bock, unpublished observations.
ProT\textsuperscript{QQQ} with affinities similar to that of WT SC(1–246) and with ProT\textsuperscript{QQQ} activation potencies that were similar or up to 1.8-fold greater. Activation potencies of mutants with both weak equilibrium binding and activation-based affinities were typically reduced. Select mutants carrying nonpolar residues at position 1 bound moderately to ProT\textsuperscript{QQQ} when measured by equilibrium binding but exhibited tight (approximately nano-molar) binding when measured by ProT\textsuperscript{QQQ} activation, indicating a difference in affinity when binding to a disordered Ile\textsuperscript{16} pocket (equilibrium binding) and an Ile\textsuperscript{16} pocket conformationally stabilized by substrate occupation of the specificity site. These mutants had limiting activation rates similar to or exceeding that of WT SC(1–246). Overall, the Ile\textsuperscript{16} pocket of ProT\textsuperscript{QQQ} favors nonpolar, nonaromatic residues at positions 1 and 2, however with specific restrictions governed by steric complementarity between the N terminus of SC and the Ile\textsuperscript{16} pocket of ProT. Our results, including efficient activation of ProT by weaker binding mutants at saturating concentrations, suggest that SC variants might emerge with similar or higher efficiency to activate ProT.

**Results**

**Characterization of SC(1–246)-BODIPY and equilibrium binding of labeled and unlabeled SC(1–246) to ProT\textsuperscript{QQQ}**

SC(1–246)-BODIPY had a labeling ratio of 0.87 BODIPY-FL thiol-sensitive probe to SC(1–246) with a S7C substitution for covalent probe binding (Fig. 3A). Incubation of ProT\textsuperscript{QQQ} with SC(1–246)-BODIPY showed binding in an approximately 1:1 ratio as observed by native PAGE (Fig. 3B). Competitive equilibrium binding studies were performed to determine the affinity and stoichiometry of four separate ProT\textsuperscript{QQQ} preparations for unlabeled SC(1–246) and SC(1–246)-BODIPY. Unlabeled SC(1–246) bound very tightly to the ProT\textsuperscript{QQQ} preparations, with $K_D$ of 0.6–1.0 nM, and a 1:1 stoichiometry (Fig. 4 and Table 1). SC(1–246)-BODIPY bound ProT\textsuperscript{QQQ} with $K_D$ of 2.9–5.9 nM and a stoichiometry of 1:1. This weaker affinity is attributed to the BODIPY-FL label. Batch-to-batch variability of ProT\textsuperscript{QQQ} was modest, as reflected by the consistent affinity values for unlabeled and labeled SC(1–246). The maximum fluorescence intensity was 0.6 ± 0.1 for all of the ProT\textsuperscript{QQQ} preparations.
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Table 1

Parameters for SC(1–246)-BODIPY and unlabeled WT SC(1–246) binding to four separate ProTQQQ preparations

Reference titrations of SC(1–246)-BODIPY with four separate ProTQQQ batches were obtained at two fixed probe concentrations. The competitive binding data for the ProTQQQ preparations were obtained by titrations of fixed concentrations of SC(1–246)-BODIPY probe, and SC(1–246) as competitor, with ProTQQQ. Data were fit simultaneously by the cubic equation to obtain the dissociation constant for ProTQQQ and SC(1–246)-BODIPY (Kd, probe) and the competitor SC(1–246) (Kc, competitor); the stoichiometric factor for SC(1–246)-BODIPY (n) and SC(1–246) (m); and the maximum fluorescence intensity (ΔFmax/Fo). Experimental error represents ± 1 S.D. Competitive equilibrium binding studies and data analysis were performed as described under "Experimental procedures." SF, stoichiometric factor.

| SC(1–246) | SF (n or m) | Kd, or Kc | ΔFmax/Fo |
|-----------|-------------|-----------|----------|
| SC(1–246)-BODIPY (n) | 0.90 ± 0.03 | 2.9 ± 0.7 | 0.60 ± 0.01 |
| SC(1–246) (m) | 1.10 ± 0.05 | 0.7 ± 0.2 | |
| SC(1–246)-BODIPY (n) | 0.90 ± 0.02 | 5.9 ± 0.8 | 0.59 ± 0.01 |
| SC(1–246)-BODIPY (n) | 1.10 ± 0.06 | 1.0 ± 0.3 | |
| SC(1–246)-BODIPY (n) | 1.00 ± 0.04 | 4.1 ± 1.1 | 0.57 ± 0.01 |
| SC(1–246) (m) | 1.10 ± 0.05 | 0.7 ± 0.2 | |
| SC(1–246)-BODIPY (n) | 1.01 ± 0.06 | 3.0 ± 1.4 | 0.58 ± 0.01 |
| SC(1–246) (m) | 1.20 ± 0.08 | 0.6 ± 0.4 | |

Binding prediction and competitive equilibrium binding of SC(1–246) N-terminal mutants to ProTQQQ

Computational modeling used Rosetta’s ΔΔG functionality to calculate the predicted change in binding energy for each mutation (21), sampling all 20 amino acids at positions 1–3. The fixed backbone design strategy allowed discrimination between steric clashing and nonclashing sequences, and based on the resulting energies in Rosetta energy units (REU), a representative double and triple N-terminal mutant panel was selected for experimental binding studies, with expected Kd values between 1 and ~1000 nM. Equilibrium binding showed that the double mutants V1V2, I1A2, and L1V2 bound to ProTQQQ very tightly, with affinities of ~1 nM, similar to that of WT SC(1–246) (Fig. 5, A–C). Their excellent ProTQQQ activation potency also indicated efficient salt bridge formation with ProTQQQ Asp194 and formation of the ProTQQQ active site. The stoichiometric factor was ~1, indicating that 1 mol of ProTQQQ binds to 1 mol of SC(1–246) mutant. The Kd values from equilibrium binding were measured in the absence of a conformationally stabilizing tripeptide thrombin substrate, and they reflect global binding to ProTQQQ with a disordered Ile16 pocket and specificity subsite. The V1V2, I1A2, and L1V2 combinations of nonpolar residues had comparable equilibrium binding and activation Kd values, suggesting a favorable steric complementarity to the Ile16 pocket. Mutants with weaker equilibrium Kd values may be governed mainly by the D1 and truncated D2 domain interactions with ProTQQQ, although double mutants with Ile, Val, Leu, and Thr in combination with polar, nonpolar aromatic, bulky, or small residues at position 2 exhibited considerable tightening of the binding to ProTQQQ when an Ile16 pocket-stabilizing substrate was present (Fig. 5 and Tables 2 and 3). The A1S2 and A1T2 mutants exhibited weak equilibrium binding affinity; however, the presence of a chromogenic substrate during ProTQQQ activation caused these mutants to bind with ~30–100-fold tighter Kd, respectively, and exhibit WT-like activation potency. This suggests some conformational flexibility of the ordered Ile16 pocket. Mutants with charged residues (Lys, Arg, Asp, Glu, His) in positions 1 and/or 2; Gly or Trp in position 1; or Pro in position 2 typically bound weakly and were poor activators, with G1G2 the weakest binder (Fig. 6 and Tables 2 and 3). Although disrupted binding for Pro was expected, even some pairs with small, hydrophobic residues in positions 1 and 2 may not optimally fit, illustrating the steric specificity of the Ile16-binding pocket.

Equilibrium binding Kd values of triple mutants varied from ~4 to ~55 nM (Table 4). The mutant H1L2V3 bound ProTQQQ with a Kd of 4 ± 3 nM (Fig. 7A) and activated ProTQQQ at an appreciable rate, suggesting that the ProTQQQ Ile16 pocket accommodates these nonpolar residues with reasonable fit, conducive to forming a salt bridge with ProTQQQ Asp194. The mutants R1H2W3, F1L2Q3, E1S2W3, D1D2Y3, G1G2G3, and E1L2K3 had Kd values of 36–55 nM (Fig. 7, B–G) but activated ProTQQQ poorly. Interestingly, substituting Gly3 for WT Thr3 rescued equilibrium binding ~10-fold, compared with G1G2, but with no improvement in activation potency.
Table 2

Characteristics of N-terminal residues in relation to mutant SC(1–246) affinity and ProTQO activation potency

| N-terminal mutants | Residue 1 characteristics | Residue 2 characteristics | Affinity range (K_D) | Activation potency (V_{lim}) | Free energy (∆G) |
|--------------------|--------------------------|--------------------------|---------------------|-----------------------------|------------------|
| II1V2 WT           | Nonpolar                  | Nonpolar                 | 0.7 ± 0.2           | 1.0                         | −12.48           |
| V1V2, I1A2, and L1V2| Similar nonpolar          | Similar nonpolar         | −0.9±1.3            | −1.0±1.8                     | −12.33 to −12.11  |
| I1T2, I1W2, I1L2, and V1G2 | Similar nonpolar          | Polar, nonpolar, indole | −5.100              | −0.9±1.6                     | −11.9 to −9.55    |
| T1V2, T1P2, and T1A2| Polar                     | Nonpolar, pyridoline     | −11.100             | −0.4±1.70                    | −10.88 to −9.55   |
| L1T2, I1Q2, L1K2, and L1P2 | Similar nonpolar          | Nonpolar, pyridoline, charged | −19.220              | −0.3±1.6                     | −10.15 to −9.08   |
| WI1A2 and W1E2     | Indole                    | Nonpolar, charged        | −117−191            | ND                          | −9.45 to −9.17    |
| M1L2, M1W2, M1K2, and M1E2 | Bulky nonpolar          | Nonpolar, indole, charged | −127−514              | −0.06±1.1                    | −9.40 to −8.58    |
| GIH2, G1A2, G1P2, G1D2, G1G2, A1W2, A1K2, A1S2, and A1T2 | Small nonpolar          | Small nonpolar, polar, charged | −153−503              | −0.02±1.0                     | −9.29 to −8.62    |
| Q1K2, T1D2, S1K2, Q1L2, and N1D2 | Polar                   | Charged, nonpolar        | −169−493              | −0.02−0.24                    | −8.94 to −8.58    |
| R1R2, K1A2, E1T2, R1Q2, and E1S2 | Charged, nonpolar        | Polar                    | −379−650              | −0.04−0.11                    | −8.75 to −8.44    |

Table 3

ProTQO binding and activation by SC(1–246) N-terminal double mutants

Reference titrations of SC(1–246)-BODIPY with ProTQO were obtained at two fixed probe concentrations. Competitive binding data were obtained by titrations of fixed concentrations of SC(1–246)-BODIPY probe, and mutant SC(1–246) as competitor, with ProTQO. Data were fit simultaneously by the cubic equation to obtain the dissociation constant for ProTQO and SC(1–246)-BODIPY (K_D(probe)) and mutant SC(1–246) (K_D(competitor)); the stoichiometric factor for SC(1–246)-BODIPY (n) and mutant SC(1–246) (n); and the maximum fluorescence intensity (ΔF_{max}/F_0). Experimental error ranges ± 2 S.D. Competitive equilibrium binding studies and data analysis were performed as described under “Experimental procedures.” ND, not determined; SF, stoichiometric factor.

The Gibbs free energy ∆G for binding of the mutants varied from −12.45 to −8.44 kcal/mol (Tables 2–4 and Fig. 8), with the lowest value for WT SC(1–246), calculated from the averaged K_D for binding to the four ProTQO batches. V1V2, I1A2, L1V2, I1T2, I1W2, and I1I2V3 had ∆G values similar to the WT protein, consistent with K_D values in the nanomolar range. A good correlation was observed between the predicted Rosetta energies and the ∆G values calculated from equilibrium binding (Fig. 9), except for a few outliers (T1P2, W1A2, M1W2, A1W2, W1E2, L1P2, and G1P2) that gave inconsistent Rosetta energies but also exhibited reduced activation potency (Fig. S1). These outliers occur in the presence of ProT or of Trp or display an off-scale energetic prediction because Rosetta is unable to fit them into the structure of the complex. This is not unexpected behavior; the introduction of a Pro or Trp residue may require such major conformational rearrangement, due to
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Figure 6. Competitive binding titrations of SC(1–246) N-terminal double mutants that bind weakly to ProTQQQ. Titrations of the probe, SC(1–246)-BODIPY (29 nm ○) and 502 nm ■) with ProTQQQ from Fig. 4 served as reference curves for competitive titrations of SC(1–246) mutants. The probe concentration for all of the competitive titrations (■) in A–F was 50 nm. Concentrations of competing SC(1–246) mutants were as follows: G1G2 8.12 μM (A), R1R2 9.06 μM (B), K1A2 9.50 μM (C), E1T2 9.84 μM (D), R1Q2 10.30 μM (E), and E1S2 11.38 μM (F). Titrations of probe and competitor were simultaneously analyzed by the cubic binding equation to obtain Kc, stoichiometry, and maximum fluorescence intensity (∆Fmax/∆F0) of the SC(1–246) mutants (Tables 2 and 3).

Discussion

In physiological blood coagulation, ProT is proteolytically cleaved in a multistep process to form the central clotting pro tease, thrombin (24). Proteolytic activation of ProT and Pre2 is initiated by cleavage of the peptide bond between Arg15 and Ile16 (chymotrypsinogen numbering). The newly formed I1V2 N terminus inserts into the Ile16 pocket of the zymogen, triggering the folding of zymogen activation domain residues 142–152, 186–194, and 216–226, and the α-ammonium of group of Ile1 forms a salt bridge with the carboxylate of Asp194. This generates the substrate recognition subsites and the oxyanion hole (25, 26). In contrast, SC, a virulence factor secreted by S. aureus, is a potent nonproteolytic ProT activator. Our structure-function studies on SC constructs in complex with ProT, Pre2, and thrombin demonstrate that the SC I1V2 residues are critical for ProT and Pre2 activation. As shown in the crystal structure of the SC(1–325)/Pre2 complex, these residues insert into the Ile16 pocket of Pre2, with SC Ile1 forming the salt bridge with Asp194, and conformational changes resulting in the gen-

steric clash or backbone geometry restriction, that the score penalty increases beyond the ability of the sampling protocol to incorporate it. Additional sampling might be necessary to create accurate models for these large or conformationally restricted amino acids. The only unexpected discrepancy was I1W2 with an REU score of 481 but exhibiting WT-like binding and ProTQQQ activation properties. The behavior of I1W2 is difficult to rationalize; the high Rosetta score reflects our expectation that inserting the steric bulk of a Trp residue at position 2 would be unfavorable. This prediction is consistent with the predictions and measurements of other mutants containing Trp at position 2. We can only conjecture that the I1W2 combination permits binding and activation via an unknown mechanism.

Prothrombin activation by SC(1–246) N-terminal double and triple mutants

Initial rates ν0 of p-nitroanilide formation upon chromogenic substrate cleavage by the ProTQQQ-SC complexes were linear for WT SC(1–246) and tight-binding mutants, and the titrations showed saturation around 20 nM SC(1–246) variant. A few mutants caused hysteresis-like lag phases in substrate hydrolysis by their complexes with ProTQQQ, and post-lag, linear ν0 rates were used for analysis of these mutants. The limiting velocity (Vlim) of WT SC(1–246) was 20 ± 5 mAbs/min, in good agreement for all four ProTQQQ batches, and used as the 100% value, or 1.00, for normalizing assays of slow-activating mutants to 1 nM ProTQQQ. Kp and Vlim values derived from the ProTQQQ activation analysis are given in Table 3. The V1V2, I1A2, and L1V2 mutants with affinities similar to WT SC(1–246) activated ProTQQQ with similar or higher potency than WT SC(1–246). I1A2 and L1V2 showed ∼81 and ∼41% increase in ProTQQQ activity, suggesting that Ala2 and Leu1 nonpolar residues bound tightly and fit optimally in the Ile16 pocket of ProTQQQ for Ile1 and Leu1 bonding with Asp194, resulting in increased ProTQQQ activity. Apparent Kp values for these tight-binding mutants, derived from the activation kinetics, were in good agreement with those measured by equilibrium binding (Fig. 10). Most mutants with a weaker equilibrium binding Kp for ProTQQQ also activated ProTQQQ weakly, with relative Vlim ≪ 1, although some, like M1L2, A1T2, A1S2, M1K2, T1A2, L1Q2, I1L2, L1T2, L1K2, V1G2, and T1V2 activated ProTQQQ similarly or up to 1.7-fold better than WT SC(1–246) at mutant concentrations approaching ProTQQQ saturation with regard to Kp calculated from the activation profiles (Tables 2–4). The affinities of these mutants, defined by ProTQQQ activation, were typically higher than their counterparts defined by equilibrium binding, due to allosteric modulation of the binding by the presence of the chromogenic substrate occupying the specificity pocket of thezymogen. This was previously also reported for vWbp binding to FPR derivatives of prothrombin, prethrombin 1 and 2 (22), and the binding of oligopeptides to trypsinogen with the specificity site occupied by a covalent ligand or a tight-binding inhibitor (23). Affinities defined for triple mutants that activated ProTQQQ poorly were not well-defined due to large experimental error. Overall, nonpolar hydrophobic residues were well-tolerated in position 1, whereas polar, aromatic, or charged residues generally diminished the ProTQQQ activation potency. Due to its conformational rigidity and unusual configuration, Pro in position 2 is thought to hamper efficient salt bridge formation of any residue at position 1, with a greatly diminished activation potency as a result.
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Table 4
ProT^{QQQ} binding and activation by SC(1–246) N-terminal triple mutants

Reference titrations of SC(1–246)-BODIPY with ProT^{QQQ} were obtained at two fixed probe concentrations. The competitive binding data were obtained by titrations of fixed concentrations of SC(1–246)-BODIPY probe, and triple mutant SC(1–246) as competitor, with ProT^{QQQ}. Data were fit simultaneously by the cubic equation to obtain the dissociation constant for ProT^{QQQ} and SC(1–246)-BODIPY (K_{D}, probe) and mutant SC(1–246) (K_{D}, competitor); the stoichiometric factor for SC(1–246)-BODIPY (n) and mutant SC(1–246) (m); and the maximum fluorescence intensity (ΔF_{max}/F_{o}). Experimental error represents ± 2 S.D. Competitive equilibrium binding studies and data analysis were performed as described under “Experimental procedures.”

| SC(1–246) mutants | \( K_{D} \) from ProT^{QQQ} activation | \( V_{	ext{max}} \) | ΔF_{max}/F_{o} | ΔG |
|-------------------|---------------------------------------|-----------------|----------------|-----|
| 1I12V3            | 4 ± 3                                  | 3 ± 1           | 0.80 ± 0.03   | −11.51 |
| R1H2W3            | 35 ± 23                                | 124 ± 48        | 0.31 ± 0.03   | −10.16 |
| F1L2Q3            | 39 ± 25                                | > 500           | 0.06 ± 0.05   | −10.11 |
| E1L2K3            | 42 ± 27                                | 309 ± 94        | 0.26 ± 0.01   | −10.07 |
| E1S2W3            | 48 ± 32                                | 28 ± 42         | 0.03 ± 0.01   | −9.98 |
| G1G2G3            | 48 ± 33                                | 29 ± 20         | 0.11 ± 0.01   | −9.98 |
| D1D2Y3            | 55 ± 36                                | 545 ± 145       | 0.19 ± 0.03   | −9.90 |

Figure 7. Competitive binding titrations of SC(1–246) N-terminal triple mutants with ProT^{QQQ}. Titrations of the probe, SC(1–246)-BODIPY (29 nM (C) and 502 nM (D)) with ProT^{QQQ} from Fig. 4 served as reference curves for competitive titrations of SC(1–246) mutants. The probe concentration for all of the SC(1–246) mutants were as follows: 1I12V3 2.84 μM (A), R1H2W3 7.59 μM (B), F1L2Q3 5.49 μM (C), E1S2W3 5.74 μM (D), D1D2Y3 7.65 μM (E), E1L2K3 5.77 μM (F), and G1G2G3 6.44 μM (G). Titrations of probe and competitor were simultaneously analyzed by the cubic binding equation to obtain \( K_{D} \), stoichiometry, and maximum fluorescence intensity (ΔF_{max}/F_{o}) of the SC(1–246) mutants (Table 4).
intact D1 domain but lacks residues 247–282 of the (pro)-thrombin (pro)exosite I-binding D2 domain, which is thought to result in weakened affinity compared with a construct with an intact D2 domain.

The mutants in this study can be categorized in four groups with regard to ProTQQQ binding and activation, compared with WT SC(1–246): (a) similar affinity, and similar or increased activation potency, in constructs with conserved or homologous nonpolar N-terminal residues; (b) modestly to significantly weaker equilibrium binding affinity, but substrate-induced, tight $K_D$ and induced fit to the Ile$^{16}$ pocket, and similar or increased activation potency; (c) modestly weaker affinity but significantly reduced activation potency due to poor fit of the N-terminal residues and mainly governed by partial D2 binding; and (d) significantly weaker affinity and significantly reduced or abolished activation potency in constructs with polar or proline-containing N termini, perhaps by triggering unfavorable long-range binding interactions through electrostatic or steric conformational changes. The overall affinity of our constructs for ProTQQQ is proposed to result from the combined effects of binding of N-terminal SC(1–246) residues to the ProTQQQ Ile$^{16}$ pocket, contact of the D1 domain with the ProTQQQ 148-loop, and binding of the truncated D2 domain to ProT proexosite I, whereas the activation potency is critically defined by the capacity of SC(1–246) residue 1 forming a salt bridge with ProTQQQ Asp$^{194}$ and an adequate and, if necessary, substrate-inducible fit of residue 2 in the ordered Ile$^{16}$ pocket. Our previous studies indicated that isolated D1 bound to ProT with modest affinity, $K_D \sim 780$ nM when measured by ProT activation and $K_D \sim 3.5 \mu M$ when measured by fluorescence equilibrium binding in the absence of a substrate. Isolated D2 binding to ProT proexosite I did not cause ProT activation due to the absence of the critical N-terminal residues; however, its affinity by equilibrium binding was $\sim 30 \text{ nM}$ (19, 30).

Active-site ligands with high affinity for the proteinase are known to induce a proteinase-like conformation in the zymogen. Occupation of the ProTQQQ specificity site by a thrombin substrate caused a $\sim 5$-fold allosteric tightening of the D1 binding, which is facilitated by favorable steric complementarity of the first two N-terminal residues of SC and the Ile$^{16}$-binding pocket (Fig. 11). A similar allosteric modulation is caused by binding of the S. aureus-secreted vWbp to prothrombin derivatives, with tighter binding to prothrombin forms that have their active site labeled with $\alpha$-Phe-Pro-Arg-chloromethylketone (22). This tightening effect was previously also observed.
for binding of small peptides in the Ile^{16} pocket of trypsinogen with its specificity site occupied by pancreatic trypsin inhibitor (PTI) or covalently bound p-guanidinobenzoate (pGB) (23, 31). In the presence of PTI or pGB, the Ile^{16} pocket is fully formed, in contrast with free trypsinogen, which shows a disordered specificity pocket and Ile^{16}-binding pocket in the crystal structure. We found that the V1V2, I1A2, and L1V2 double mutants of SC(1–246) bind ProT{\textsuperscript{QQQ}} with similar affinities and show similar or higher potency in activating ProT{\textsuperscript{QQQ}}, and the I1I2V3 triple mutant binds and activates ProT{\textsuperscript{QQQ}} only slightly more weakly. In a study with isolated di- and oligopeptides, Bode (23) reported that the more effective peptides 11V2 and 11V2G3 used in activation studies of trypsinogen carrying a ligand in the specificity pocket (pGB or pancreatic trypsin inhibitor) were identical to the newly formed N-terminal sequence after cleavage of the activation peptide. However, the peptides V1V2, I1A2, and L1V2 bound to pGB-trypsinogen with affinities 30-, 160-, and 190-fold weaker than I1V2, respectively. In our studies, additional interactions of the D1 and the truncated D2 domains were shown to contribute to enhanced binding affinity and ProT{\textsuperscript{QQQ}} activation potential by SC(1–246) mutants with these N-terminal sequences. In the I1I2V3 triple mutant, Ile^{2} is similar in size and hydrophobicity to Val^{2} of the WT construct. Consequently, there was not much reduction in ProT{\textsuperscript{QQQ}} affinity and activation potency of this mutant. The equilibrium binding and kinetic data presented here indicate that small and nonpolar residues are preferred over bulky and charged ones for sufficient ProT binding and activation (Table 2), due to a better fit in the Ile^{16} pocket, even in the disordered state. The Val^{1}, Leu^{1}, and Ala^{2} residues of the double mutants are as functional as Ile^{1} and Val^{2} of WT SC(1–246). The presence of these residues in the pocket favors the packing and alignment of the side chains triggering conformational activation in a similar fashion as seen in the SC(1–325)-Pre2 complex, with the $\alpha$-ammonium group of Val^{1} possibly connecting through a salt bridge with Asp^{194}. Ala^{2} may be stabilized through the formation of a hydrogen bond with Asp^{189} in the ProT{\textsuperscript{QQQ}} specificity pocket, as observed for Val^{2} in the SC(1–325)-Pre2 crystal structure. In the crystal structure of SC(1–325)-Pre2, Ile^{1} is completely buried in the hydrophobic Ile^{16} pocket, whereas Val^{2} partially contacts the outer solvent. Replacement of valine with the bulky amino acid leucine showed a 15-fold decrease in affinity for pGB-trypsinogen (23). Even though binding and insertion of the first two N-terminal are additive, the Ile^{16} pocket can accommodate a less favorable residue at the second position.

Interestingly, the T1A2 mutant has the same T1A2T3 residues as those proteolytically generated in a ProT mutant upon cleavage at Arg{\textsuperscript{320}} (32). Substitution of IVE to TAT following ProT Arg{\textsuperscript{320}} did not prevent cleavage but ultimately generated a thrombin mutant Ila{TAT} with zymogen-like properties that bound the active site probe DAPA with $\sim$32,000-fold weaker affinity than WT thrombin ($K_D \sim 1\text{ nM}$) and only had 0.2% specific activity toward the thrombin-specific chromogenic substrate S2238. Our T1A2 mutant bound ProT{\textsuperscript{QOO}} with equilibrium $K_D$ of 100 $\pm$ 27 nM, and 5.2 $\pm$ 0.2 nM from ProT activation, in the presence of a chromogenic substrate and an activation potential 1.3 times that of WT SC(1–246). This functional mutant may attribute its potential of salt bridge formation with ProT{\textsuperscript{QQQ}} Asp{\textsuperscript{194}} and zymogen activation to cumulative N-terminal, D1 and D2 conformational interactions that shift the zymogen–protease equilibrium in Ila{TAT} toward the protease conformation.

Equilibrium binding of various mutants involving Pro and Trp was moderate to weak (T1P2, M1W2, A1W2, and L1P2, ~60–200 nM), and their dramatically reduced activation potency suggests nonproductive interaction with the Ile^{16} pocket and binding through the partial D2 domain that may be perturbed by electrostatic or steric effects introduced by the mutations. Variations in the structural orientation of the partial D2 domain may alter binding and lead to weaker overall mutant affinity for ProT{\textsuperscript{QOO}}. Typically, these mutants were outliers in the correlation of predicted REU scores and measured $\Delta G$ values of equilibrium binding (Fig. S1), suggesting that the sampling protocol employed was not sufficient to overcome the major structural perturbation required to insert the steric bulk of a Trp residue or to accommodate the backbone angle restriction imposed by a Pro residue. The high energy penalty can be interpreted as a clear signal that the new sequence is incompatible with the native structure. Triple mutants containing bulky aromatic or charged residues as well as G1G2G3 bound $\sim$10-fold tighter than the G1G2 double mutant with $K_D$ of 48 $\pm$ 33 nM, respectively, suggesting that Gly at position 3 instead of native Thr is more conducive to steric complementarity. However, the low activation potencies of both the double and triple mutant indicated impaired salt bridge formation with ProT{\textsuperscript{QQQ}} Asp{\textsuperscript{194}}.

In conclusion, we have determined the affinities of a panel of 46 different SC N-terminal mutants for ProT{\textsuperscript{QOO}} and showed that the Ile^{16} pocket is specific for accommodating residues that are similar in size to I1V2, but improved fit for a variety of preferably noncharged position 2 residues except for proline can be induced by small substrate binding. This characterization of the SC N-terminal residues in the SC-ProT complex provides further information to better design antibodies as
drugs to target the SC N terminus. Our results suggest the distinct possibility that S. aureus may be capable of adapting to continuous use of antibiotics and selection pressure to escape the human immune response, by generating SC variants with similar or higher efficiency to activate ProT.

**Experimental procedures**

**Expression, purification, and labeling of proteins**

SC(1–246) was cloned into a modified pET30b(+) vector (Novagen) containing an N-terminal Hiso tag followed by a tobacco etch virus cleavage site (19, 30). The SC(1–246) N-terminal mutants were prepared through site-directed mutagenesis using degenerate and specific primers (Table S1), and mutations were confirmed by DNA sequencing. The mutants were expressed in Rosetta 2 (DE3) pLYS Escherichia coli in the presence of 100 μg/ml kanamycin, and expression was induced by 10 mg/ml lactose for 4 h. Mutants were purified from inclusion bodies, and the Hiso tag was removed as described (33, 34). The proteins were stored in 50 mM HEPES, 125 mM NaCl, pH 7.4, at −80 °C until use. The mutant concentrations were determined using the extinction coefficients and molecular mass calculated by the Expasy tool, RRID:SCR_018087 (Table S2). HEK293 cells expressing ProTQKO, in which the prothrombinase cleavage site Arg271 and the thrombin cleavage sites Arg155 and Arg284 were replaced by glutamine to prevent degradation, were a gift from Dr. Sriram Krishnaswamy (University of Pennsylvania School of Medicine) (20). ProTQKO was expressed, purified, and stored as described (35, 36). Four separate ProTQKO batches were prepared, and the concentrations were determined using \( E_{280\text{nm,0.1%}} = 1.47 \text{ml mg}^{-1} \text{cm}^{-1} \) and \( M_w = 72,000 \).

**Preparation and characterization of SC(1–246)-BODIPY**

To create a labeled SC construct, Ser7 of SC(1–246) was converted to cysteine through site-directed mutagenesis (Agilent Technologies) and confirmed by DNA sequencing. Purified SC(1–246)-S7C was reduced with 2 mM DTT and dialyzed against 5 mM MES, 125 mM NaCl, 1 mg/ml PEG, 10 mM EDTA, pH 7.4, buffer to remove free DTT. Approximately 5–10 mg of protein was incubated for 1 h at 25 °C with a 10-fold molar excess of BODIPY-FL-iodoacetamide (Thermo Fisher Scientific) to label the free S7C thiol. The excess probe was removed by Sephadex G-25 chromatography in 50 mM HEPES, 125 mM NaCl, 0.1 mM EDTA, pH 7.4, buffer. Labeled SC(1–246)-S7C-BODIPY-FL (SC(1–246)-BODIPY) was dialyzed against storage buffer (50 mM HEPES, 125 mM NaCl, pH 7.4) and stored at −80 °C. The concentration and labeling ratio were determined using \( E_{280\text{nm,0.1%}} = 0.936 \text{ml mg}^{-1} \text{cm}^{-1} \) and \( M_w = 29,150 \) for WT SC(1–246) and SC(1–246)-S7C, and \( E_{505\text{nm}} = 63,771 \text{cm}^{-1} \text{M}^{-1} \) for BODIPY-FL-iodoacetamide. An absorbance ratio (\( A_{280\text{nm}} / A_{505\text{nm}} \)) of 0.03 was used to correct for the probe contribution to absorbance at 280 nm. The purity of SC(1–246)-BODIPY was established by 4–20% polyacrylamide SDS-PAGE under reduced and nonreduced conditions. The fluorescence of the labeled protein was imaged under UV light, and proteins were then stained with colloidal Coomassie Blue G-250. To determine whether SC(1–246)-BODIPY forms a binary complex with ProTQKO, a fixed concentration (2.5 μM) of ProTQKO was incubated with different concentrations of SC(1–246)-BODIPY (0, 0.5, 1.0, and 1.5 μM) at 25 °C for 15–30 min. The samples were run on a 6% polyacrylamide gel under native conditions (Tris-glycine buffer, pH 8.3, no SDS) at 4 °C. The fluorescence was imaged, and the proteins were stained as described above.

**Prothrombin activation assay**

Activity titrations of ProT complexes with WT or mutant SC(1–246) were performed in 50 mM HEPES, 110 mM NaCl, 5 mM CaCl2, 1 mg/ml PEG 8000, pH 7.4, buffer, in PEG 20,000-coated 96-well plates (Nunc). Varying concentrations of WT or mutant SC(1–246) were incubated with 1, 10, or 20 nM ProT for 10 min at 25 °C. The reaction was initiated by the addition of 600 μM chromogenic substrate S-2238 (Diapharma), and the rate was measured in a ThermoMax plate reader (Thermo Fisher Scientific) for 10 min at 405 nm until the absorbance reached 0.1. Initial rates (mAbs/min) for the mutants were normalized to that of WT SC(1–246). The normalized rate dependences as a function of the SC(1–246) concentration were fitted by the quadratic binding equation using SCIENTIST (MicroMath) to obtain the \( V_{lim} \) and \( K_D \) (19).

**Direct and competitive fluorescence equilibrium binding**

Fluorescence measurements were performed with a PTI QuantaMaster 30 spectrofluorometer at 25 °C using acrylic cuvettes coated with PEG 20,000. Titrations were performed in 50 mM HEPES, 110 mM NaCl, 5 mM CaCl2, 1 mg/ml PEG 8000, pH 7.4, buffer with 1 mg/ml ovalbumin, and fluorescence was measured at \( \lambda_{em} = 496 \text{nm} \) (3–6-nm band pass) and \( \lambda_{ex} = 535 \text{nm} \) (4–6-nm band pass). Two fixed concentrations of SC(1–246)-BODIPY were titrated with the ligand, ProTQKO. The competitive binding assays were performed with one fixed concentration of SC(1–246)-BODIPY in the presence of a single fixed concentration of unlabeled WT or mutant SC(1–246) competitor, titrated with the ligand, ProTQKO. The SC(1–246)-BODIPY control titrations with ProTQKO were performed to obtain the stoichiometry and \( K_c \) for SC(1–246)-BODIPY. The titrations of SC(1–246)-BODIPY in the presence of competing WT or mutant SC(1–246) were performed to obtain the stoichiometry and dissociation constant \( K_c \) for the competitors. The fractional change in fluorescence was calculated as \( (F_{obs} - F_o) / F_o = \Delta F/F_o \), and the data were fit by the quadratic binding equation (37) using SCIENTIST (MicroMath) software. For the competition experiments, titrations in the absence and presence of competitor were fit simultaneously by the cubic binding equation (37). Nonlinear least-squares fitting was performed using SCIENTIST (MicroMath) either with or without fixed stoichiometry for the competitive data to obtain the dissociation constants \( K_O \) and \( K_C \), maximum fluorescence intensities \( (F_{max} - F_o)/F_o = \Delta F_{max}/F_o \), and stoichiometric factors \( n \) for SC(1–246)-BODIPY and \( m \) for unlabeled WT and mutant SC(1–246). The error estimates represent the 95% confidence interval. The Gibbs free energy (\( \Delta G \)) values for WT and mutant SC(1–246) binding to ProTQKO were calculated using the equation, \( \Delta G = RT \ln K_{eq} \) where \( R = 1.987 \times 10^{-3} \text{ kcal mol}^{-1} \text{ degree}^{-1} \) and \( T = 298.15 \text{ K (25 °C)} \), and \( K_{eq} \) is expressed in m.
Binding of the staphylocoagulase N terminus to prothrombin

Computational modeling

To analyze the energetic effects of the N-terminal mutations of SC(1–246) on the binding with ProT$^{5562}$, the mutations were performed in silico using the Rosetta software suite (RRID: SCR_015701) (38). The X-ray crystal structure of the complex 1nu9 (19) was relaxed using Rosetta3 (August 2016 build 58479), using constraints to maintain atomic positions close to the experimental input structure. The relaxation was performed 100 times, and the lowest-energy structure was selected for mutation (/rosetta-3.9/main/source/bin/relax.default.linuxgccrelease -s 1nu9_cleaned.pdb -in:file:fullatom -nustuct 100 -relax:fast -relax:constrain:relax_to_start_coords). Mutations were introduced into the structure using Rosetta’s fixed backbone design (fixxbb) application (39) and a resfile specifying the identities of residues at positions 1, 2, and 3. All 20 amino acids were tested at each of the three positions, resulting in 8,000 output structures, representing putative single, double, and triple mutants (/rosetta-3.9/main/source/bin/fixxbb.default.linuxgccrelease -s relaxed_with_constraints.pdb -resfile resfiles/$key -in:file:fullatom -use_input_sc -ex1 -ex2 -out:path:pdb pdbfiles -out:path:score scorefiles -out:path:scorefile scored_interface_energies.list). The predicted change in the interaction energy between the mutated SC(1–246) and Pre-2 was calculated using Rosetta’s interface analyzer application, to evaluate the resulting changes in the energetic binding contribution of the N-terminal segment of SC(1–246). (/rosetta-3.9/main/source/bin/InterfaceAnalyzer.default.linuxgccrelease -in:path ../pdbfiles -in:file:structure_batch.list -add_regular_scores_to-source/bin/relax.default.linuxgccrelease -srelaxed_with_constraints.pdb -resfile resfiles/$key -in:file:fullatom -use_input_sc -ex1 -ex2 -out:path:pdb pdbfiles -out:path:score scorefiles -out:path:scorefile scored_interface_energies.list). The sixth column “dG_separated” was extracted using awk. (% cat ../scorefiles/*.fasc | awk '{print $NF " \n"}'} grep -v SEQUENCE | sort -nrk2 > sorted_interface_energies.list.)

Data availability

The structure of the SC(1–325)-Pre2 complex 1NU9 is available in the Protein Data Bank. All remaining data are contained within the article and supporting information.

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