A Novel Soluble Tissue Factor Variant with an Altered Factor VIIa Binding Interface*  

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Tissue factor (TF) residues Lys20 and Asp58 form part of a binding epitope previously shown by alanine scanning to be critical for high affinity interactions with factor VIIa (FVIIa). To explore the possibility of enhancing the affinity of a TF-based antagonist for FVIIa, we created libraries in which residues at 20, 58, and adjacent positions were varied in constructs containing the soluble extracellular domain of TF (sTF) fused to the bacteriophage M13 tail coat protein. TF variants monovalently displayed on phage were then sorted on the basis of binding to FVIIa. Sorting of preliminary libraries, in which position 58 and/or 20 and surrounding residues were randomized, led to the selection of TF proteins of essentially wild-type sequence. Therefore, we devised a strategy wherein TF position 20 was held fixed as alanine and 5 specific residues near to, and including, position 58 were randomized to effectively obtain alternative sequences at this interface. The consensus sequence reached with this library included wild-type residues at positions 61, 62, 65, and 66 but exclusively tryptophan at position 58. Analyses of the soluble TF20A,D58S (A20W58) TF protein indicated that it binds FVIIa with an affinity comparable with wild-type sTF but is defective as a cofactor for FVIIa-dependent factor X activation. Further experiments designed to elucidate the mechanism of binding suggest that the new binding interactions involve more than the simple addition of hydrophobic surface area.

The extrinsic blood coagulation cascade is triggered by the formation of a complex between factor VIIa (FVIIa) and its obligate, membrane-bound cofactor, tissue factor (TF). TF-FVIIa-mediated activation of factor X (FX) to factor Xa (FXa) and of factor IX to factor IXa leads to thrombin production and ultimately to fibrin clot formation at sites of vascular injury (1–3). Aberrant TF expression has been linked to the pathophysiological conditions associated with sepsis and injury (1–3). Aberrant TF expression has been linked to the pathophysiological conditions associated with sepsis and injury (1–3). Recent studies indicate this fact.

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The abbreviations used are: FVIIa, coagulation factor VIIa; TF, tissue factor; FX and FXa, zymogen and activated coagulation factor X, respectively; sTF, the soluble extracellular domain of TF, residues 1–219; hTFFA, the sTF variant containing Lys to Ala substitutions at positions 165 and 166; A20W58, the sTF variant containing K20A and D58S substitutions; BEGR-CK, biotinylated Glu-Gly-Arg chloromethyl ketone; BEGR-7a, biotinylated active site-inhibited FVIIa; EGF-1, the first epidermal growth factor-like domain of FVIIa.

We have recently tested the antithrombotic properties of a mutant of the soluble extracellular domain of TF (sTF, residues 1–219) (5). Incorporation of alanines in place of lysines at positions 165 and 166 (Fig. 1A) yields an sTF variant (hTFFA) that binds FVIIa with an affinity equivalent to wild-type sTF but is defective as a cofactor for FX activation (6–9). The hTFFA variant inhibits the extrinsic pathway of blood coagulation in vitro through competition with membrane TF for binding to FVIIa. In a rabbit model of arterial thrombosis, hTFFA partially blocks thrombus formation without increasing bleeding tendency. However, high doses of hTFFA are required for the antithrombotic effect, in part because FVIIa binds to cell surface TF approximately 1000-fold more tightly than to sTF (10). The greater apparent affinity is due to interaction of the FVIIa Glu-containing domain with phospholipid.

One approach to create a more potent version of hTFFA is to introduce amino acid substitutions at FVIIa-binding interfaces that increase the cofactor’s affinity for its ligand. The structure of FVIIa in complex with sTF has been recently determined at 2.0Å resolution by x-ray crystallography (11). An extensive interface is formed between sTF and FVIIa, resulting in the burial of 3620 Å2 of solvent-accessible surface area. Scanning mutagenesis of TF has shown that Lys20 and Asp58 make dominant contributions to FVIIa binding (9, 12), with alanine substitutions at these positions leading to 78- and 30-fold reductions in FVIIa affinity, respectively (9). Lys20 and Asp58 are on adjacent antiparallel β-strands (13, 14), forming part of a functional epitope that binds to the amino-terminal epidermal growth factor-like domain (EGF-1) of FVIIa via both hydrophobic and hydrogen-bonding interactions (Ref. 11; Fig. 1B). Previous work on the affinity maturation of human growth hormone (15) demonstrated that affinity improvements were obtained from changes at residues that were important for binding based on scanning mutagenesis studies. We reasoned, therefore, that randomizing codons for positions 20 and 58 and adjacent residues would allow us to remodel this interface with the goal of increasing TF affinity for FVIIa. Our implementation of this strategy involved the monovalent display of large combinatorial libraries of sTF variants as fusions to the gene III product of filamentous bacteriophage M13 and selection of tight binders by panning sTF-phage versus immobilized FVIIa.

EXPERIMENTAL PROCEDURES

Materials—Coagulation factors VIIa, X, and Xa used in chromogenic assays, as well as biotinylated glutamyl-glycyl-arginine chloromethyl ketone (BEGR-CK), were purchased from Hematological Technologies. Chromogenic substrates Chromozym t-PA (N-methylsulfonyl-o-phenyl-l-glucyl-l-tyrosine-p-nitroanilide acetate) and Spectrozyme FXa (me-thoxyxycarbonyl-o-cyclohexylglycyl-l-tyrosine-p-nitroanilide acetate) were from Boehringer Mannheim and American Diagnostica, respectively. Substrates S-2266 (N-valyl-l-leucyl-l-arginine-p-nitroanilide dihydrochloride), S-2288 (N-isoleucyl-l-prolyl-l-arginine-p-nitroanilide dihydrochloride), and S-2366 (t-pyroglutamyl-l-prolyl-l-arginine-p-nitroanilide dihydrochloride) were from Pharmacia Hepar. Plasmids—Phagemids encoding sTF fused to the carboxyl-terminal domain (residues 249–406) of the M13 gene III product were con-

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structured using standard molecular biology techniques (16) from a vector, phGH-g3, previously developed for monovalent phage display (17, 18). One phagemid, called PTFAA-g3, encodes the sTF variant containing Lys to Ala substitutions at positions 165 and 166 and was used as the starting template for mutagenesis in preparation for library constructions. A second phagemid, pTF-g3, encodes wild-type sTF and was mutated to create the gene encoding the K20A and D58W substitutions in an otherwise wild-type protein.

Mutant and Library Construction—In preparation for library constructions, oligonucleotide-directed, site-specific mutagenesis (19) was performed on phagemid PTFAA-g3 to create DNA templates that encode hTFAA variants with markedly lower affinity for FVIIa. Mutant libraries were then created by substituting five TF codons simultaneously with NNS nucleotide sequences (where N represents G, A, T, or C and S represents G or C) via oligonucleotide-directed mutagenesis of the appropriately altered pTFAA-g3 template. This strategy ensured that phage incorporating TF encoded from the template DNA would be less likely to compete with library-derived phage for FVIIa binding should the mutagenesis efficiency be suboptimal. Specifically, for libraries 1 and 3 the template phagemid encoded a Lys to Ala substitution at position 20 (K20A) and an Asp to Glu substitution at position 58 (D58E), in addition to the Lys to Ala substitutions at positions 165 and 166. For library 2, the starting template encoded the three Lys to Ala substitutions but retained the wild-type Asp58 codon. For the construction of library 1, two primers were used to simultaneously mutate codons at positions 20 and 21 and at positions 54, 56, and 58, respectively, in the pTFAA (K20A,D58E-g3) template. Library 2 used pTFAA (K20A-g3) as template with randomization of codons 15, 17, 20, 22, and 24. A restriction site selection protocol was used to enrich for mutant sequences. In library 3, the mutation encoding K20A in the pTFAA (K20A,D58E-g3) template was left untouched, while five triplets encoding positions 58, 61, 62, 65, and 66 were altered. The preparation of filamentous phage displaying sTF variants, by electroporation of phagemid libraries into Escherichia coli strain XL1-Blue (Stratagene) and subsequent infection of bacteria with helper phage VCS M13 (Stratagene), was performed as described by Lowman and Wells (18). At least 10 clones from each of the unselected libraries were sequenced to ascertain the mutagenic efficiency. Library 1 contained 1 × 10⁶ transformants with about 10% of the clones having both sites mutated. Library 2 had only 1 × 10⁵ transformants but a 100% mutation frequency as a result of the restriction site selection. Library 3 comprised 1 × 10⁵ transformants with a 10% mutation frequency.

Binding Enrichments—Phage particles displaying TF variants were sorted on the basis of binding to biotinylated FVIIa (BEGR-7a). BEGR-7a was prepared using a biotinylated tripeptide chloromethyl ketone (BEGR-CMK) active site inhibitor as described elsewhere (9). Microtiter plate wells coated with streptavidin (Molecular Probes) and blocked with milk proteins were used to capture BEGR-7a. For selection experiments, phage displaying libraries of TF variants, in buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM CaCl₂ (TNC), were incubated in wells containing either streptavidin with BEGR-7a or BEGR-CK active site inhibitor as described elsewhere (9). The sTF variant, produced in nonsuppressor E. coli strain 27C7, was purified from periplasmic shockates using immunoaffinity chromatography (9). Purified preparations of A20W58 were subjected to SDS-polyacrylamide gel electrophoresis and Coomassie staining to quantify purity and subjected to electrospray mass spectrometry to verify appropriate molecular composition. The material routinely appeared to be ≤95% homogeneous (data not shown) and used as the correct mass for the fully oxidized species (predicted mass, 25027; observed mass, 25027 ± 3). The concentrations of sTF-A20W58 preparations were determined on the basis of initial rates of binding to monoclonal antibody D3, under mass transfer-limiting conditions, using a Pharmacia BIAcore instrument and a preparation of wild-type sTF of known concentration as a standard (21).

**TF-dependent FVIIa Amidolytic Activity—**For kinetic determinations, peptide hydrolysis by TF-FVIIa was monitored by incubating 100 nM sTF with 100 nM FVIIa in 20 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM CaCl₂ (HNC) plus 0.01% Tween 20 for 0.5 h at ambient temperature and then adding chromogenic substrate to final concentrations in the range of 0.03–4 μM. The rate of change of absorbance at 405 nm was converted to mol of substrate cleaved using the equation *v* = 10⁻⁵ mol·cm⁻²·s⁻¹ from p-nitroaniline. Apparent equilibrium inhibition constants (*Kₐ*) of hTFAA domains, TF1-7, C, and IV-49C were determined by performing assays in which 20 nM sTF was incubated with a 5-fold excess of FVIIa and varying inhibitor concentrations in HNC plus Tween and then adding substrate Chromozym t-PA to a 2 mM final concentration. *Kₐ* values were determined by nonlinear regression fitting of the relative initial velocity versus inhibitor concentration data to an equation developed for tightly binding inhibitors (24, 25).

**FX Activation Assay—**Conversion of zymogen to activated factor X by TF-FVIIa was measured in a two-stage chromogenic assay. In one microtiter plate, 1 nM sTF was pre-equilibrated at ambient temperature with 50 nM FVIIa and 500 μM vesicles composed of 70% phosphatidylcholine, 30% phosphatidylserine (26) in HNC containing 0.1% PEG-8000. FX was then added to concentrations ranging from 0.01 to 5 μM. At intervals after the FX addition, samples of the reaction mixture were quenched with an equal volume of 50 mM EDTA, pH 8, in a second plate containing 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM CaCl₂ (TNC), were incubated in wells containing either streptavidin with BEGR-7a or streptavidin alone. After 1–2 h of incubation at ambient temperature, unbound phage were removed, and the wells were washed extensively with TNC buffer containing 0.05% Tween 20. Bound phage were eluted by using 50 mM EDTA in a 10-min incubation at 37 °C. The titer of infective TF-containing phage particles eluted from the wells was determined by infecting XL1 cells with eluted phage, streaking dilutions to LB plates containing ampicillin (to select for cells bearing TF-encoding phagemids), and counting colony-forming units. The ratio of the phage titer (colony-forming units/ml of elution buffer) from wells containing FVIIa to the titer eluted from wells containing streptavidin alone was calculated to monitor per round enrichments in specific binding.

**Production of sTF-A20W58—**The gene encoding the A20W58 sTF variant was produced by oligonucleotide-directed mutagenesis on pTF-g3, using separate primers to effect mutations leading to the K20A and D58W substitutions. The introduction of appropriate mutations was confirmed by dideoxynucleotide sequencing (22). The sTF variant, produced in nonsuppressor E. coli strain 27C7, was purified from periplasmic shockates using immunoaffinity chromatography (9). Purified preparations of A20W58 were subjected to SDS-polyacrylamide gel electrophoresis and Coomassie staining to quantify purity and subjected to electrospray mass spectrometry to verify appropriate molecular composition. The material routinely appeared to be ≤95% homogeneous (data not shown) and used as the correct mass for the fully oxidized species (predicted mass, 25027; observed mass, 25027 ± 3). The concentrations of sTF-A20W58 preparations were determined on the basis of initial rates of binding to monoclonal antibody D3, under mass transfer-limiting conditions, using a Pharmacia BIAcore instrument and a preparation of wild-type sTF of known concentration as a standard (21).

**TF-FVIIa Interface**

![Ribbon diagrams of the sTF-FVIIa complex (A) and an enlargement of the TF-FVIIa interface that includes TF residues Lys30 and Asp50 (B). These images are based on a 2-A resolution crystallographic structure of the complex (11). In A, TF is in yellow, and the light and heavy chains of FVIIa are in green and cyan, respectively. The two Lys to Ala substitutions of hTFAA are shown in white. B, an enlargement of the interface boxed in A. Here, TF is yellow, and the EGF-1 domain of FVIIa is in green. In red are key interfacial residues. TF Lys30 has hydrophobic interactions with FVIIa Ile69 and forms hydrogen bonds to the backbone carbonyls of FVIIa Cys70 and Gly78. TF Asp50 forms a hydrogen bond with the backbone amide of FVIIa Gly78.](http://www.jbc.org/)

2 The D58E change leads to an sTF protein with 10-fold lower affinity for FVIIa compared with the wild-type cofactor (9). Additionally, the sTF variant bearing both K20A and D58E substitutions has no detectable binding to BEGR7a immobilized on a BIAcore flow cell (R. Kelley, unpublished observation).
and then assayed for FXa activity in the buffer above, containing 25 mM EDTA instead of CaCl₂, by adding Spectrozyme-FXa to a 0.5 mM concentration. Reactions performed as above, but in the absence of FXa, were used to define background proteolytic rates. Substrate cleavage rates were converted to mol of FXa produced via standard curves prepared using purified FXa. Rates of FXa activation were determined by linear regression analysis from plots of mol of FXa produced over the assay time course.

**Real Time Kinetic Measurement of sTF/FVIIa Interactions**—The affinities of wild-type and A20W58 sTF for immobilized FVIIa were determined by surface plasmon resonance measurements on a Pharmacia BIAcore instrument as described previously (9), with the exception of using Neutravidin (Pierce) in place of streptavidin as the reagent for binding of sTF proteins to a flow cell coated with Neutravidin alone.

**Calorimetric Measurements**—Isothermal titration calorimetry was performed to determine enthalpy changes and dissociation constants for wild-type and A20W58 sTF binding to FVIIa (9), using FVIIa at a 5 μM concentration in the calorimeter cell and titrating by injecting aliquots of 92 μM sTF. Enthalpy measurements made at 25, 30, and 35 °C were used to obtain an estimate for ΔCP (9). The thermal stability of sTF proteins, exhaustively diazoylised into TNC, was monitored by differential scanning calorimetry between 15 and 95 °C using a Microcal MC-2 scanning calorimeter. Melting temperatures of 54 and 49 °C were determined for the wild-type and A20W58 sTF proteins, respectively, indicating that the introduced substitutions do not substantially destabilize the TF variant.

**RESULTS**

**Sorting Phage Monovalently Displaying Altered sTF Proteins**—We wanted to test whether monovalent phage display of sTF would allow us to remodel a FVIIa binding epitope with the goal of increasing TF affinity for FVIIa. We chose to construct libraries in which 5 residues were randomized, with a potential diversity of $3.2 \times 10^{10}$ amino acid sequences, since this library size can be fully represented using current methods for transformation of E. coli. Positions 20 and 58 were included in the initial libraries because previous work with human growth hormone showed that affinity improvements resulted from changes at sites that had significant roles in binding based on alanine scanning (15). Other residues were chosen for randomization because mutations at these sites result in modest effects on FVIIa binding (9) and because these residues are found within or near the Lys23/Asp58 interface with FVIIa in the sTF/FVIIa complex structure (11).

In library 1, we used two mutagenic primers simultaneously to randomize codons at residues at position 20 and 21 and at positions 54, 56 and 58, respectively. Position 21 was chosen for randomization because the T21A mutant has a slightly increased affinity for FVIIa (9). The affinity of the double mutant sTF D54A,E56A for FVIIa is decreased by 2-fold from wild-type (9), and Glu24 interacts with Arg79 from the light chain of FVIIa in the sTF-FVIIa complex structure (11). The R79A mutant of FVIIa has non-wild-type residues at key positions (see “Experimental Procedures”). Conservation of Thr21 was surprising given the effect of T21A on FVIIa affinity. Thr21 may be important for proper folding of sTF, since this residue is fully buried in the structure of free sTF (13, 14), and the single-site T21A mutant is poorly expressed in E. coli.

To further probe affinity determinants near position 20, we constructed a second library in which residue 58 was held fixed as the wild-type Asp, while positions 15, 17, 20, 22, and 24 were randomized. Alanine substitutions at sites 15 and 17 result in small changes in FVIIa affinity; I22A and E24A mutants have slightly larger affinity defects of about 3-fold each (9). Both Ile22 and Glu24 are in contact with Arg79 from FVIIa, while Thr17 interacts with FVIIa residue I69 (11). After six rounds of sorting this library (~10²²-fold overall binding enrichment), sequencing of DNA from 12 phage revealed a consensus of wild-type residues at all positions tested except for position 54 (Table I), where variations were well tolerated, including some substitutions (Lys, Asn, and Ser) that led to slight improvements in FVIIa affinity. Conservation of Thr21 was surprising given the effect of T21A on FVIIa affinity. Thr21 may be important for proper folding of sTF, since this residue is fully buried in the structure of free sTF (13, 14), and the single-site T21A mutant is poorly expressed in E. coli.

### Table I

| Residue position | Kd (hTFAA) | Kd (mut) |
|------------------|------------|----------|
| Wild type       | Lys Thr Asp Glu Asp | 1 |
| Selectants      | Lys Thr Lys Glu Asp (2) | 1.4 |
|                  | Lys Thr Asn Glu Asp | 1.8 |
|                  | Lys Thr Glu His Asp (3) | 1.0 |
|                  | Lys Thr Ser Glu Asp | 2.3 |
|                  | Lys Thr Asp Glu Asp | 0.9 |
|                  | Lys Thr Ala Glu Asp (3) | 1.1 |
|                  | Lys Thr Asp Trp Asp | 0.9 |
| Consensus        | Lys Thr Var Glu Asp | ND |

### Table II

| Residue position | Kd (hTFAA) | Kd (mut) |
|------------------|------------|----------|
| Wild type        | Lys Thr Lys Ile Glu | 1 |
| Selectants       | Lys Val Lys Ile Ser (7) | 2.0 |
|                  | Lys Thr Lys Ile Glu (2) | 1.0 |
|                  | Arg Thr Lys Ile Glu (5) | 4.5 |
|                  | Arg Thr Lys Ile Glu | 1.0 |
|                  | Arg Ser Lys Ile Thr | 1.9 |
|                  | Trp Ile Lys Val Lys | 1.4 |
|                  | Lys Thr Lys Ile Glu | 2.5 |
| Consensus        | Lys Thr Lys Ile Glu | ND |

### Table III

| Residue position | Kd (hTFAA) | Kd (mut) |
|------------------|------------|----------|
| Wild type        | Lys Thr Lys Ile Glu | 1 |
| Selectants       | Lys Val Lys Ile Ser (7) | 2.0 |
|                  | Lys Thr Lys Ile Glu (2) | 1.0 |
|                  | Arg Thr Lys Ile Glu (5) | 4.5 |
|                  | Arg Thr Lys Ile Glu | 1.0 |
|                  | Arg Ser Lys Ile Thr | 1.9 |
|                  | Trp Ile Lys Val Lys | 1.4 |
|                  | Lys Thr Lys Ile Glu | 2.5 |
| Consensus        | Lys Thr Lys Ile Glu | ND |

### Table III

| Residue position | Kd (hTFAA) | Kd (mut) |
|------------------|------------|----------|
| Wild type        | Lys Thr Lys Ile Glu | 1 |
| Selectants       | Lys Val Lys Ile Ser (7) | 2.0 |
|                  | Lys Thr Lys Ile Glu (2) | 1.0 |
|                  | Arg Thr Lys Ile Glu (5) | 4.5 |
|                  | Arg Thr Lys Ile Glu | 1.0 |
|                  | Arg Ser Lys Ile Thr | 1.9 |
|                  | Trp Ile Lys Val Lys | 1.4 |
|                  | Lys Thr Lys Ile Glu | 2.5 |
| Consensus        | Lys Thr Lys Ile Glu | ND |
The hTFAA template for library 3 coded for K20A and D58E substitutions. The retention of the Ala50 codon in library 3 selectants was confirmed by DNA sequencing. The number in parentheses indicates the number of times the given variant appeared among the selected clones. The consensus sequence reflects those residues selected at each position that were significantly enriched (>4-fold) above their expected random frequency in an NNS-based library (10).

![Table III](image)

Identity of TF variants selected on the basis of binding immobilized BEGR-7a: library 3, after six rounds of sorting

The hTFAA template for library 3 coded for K20A and D58E substitutions. The retention of the Ala50 codon in library 3 selectants was confirmed by DNA sequencing. The number in parentheses indicates the number of times the given variant appeared among the selected clones. The consensus sequence reflects those residues selected at each position that were significantly enriched (>4-fold) above their expected random frequency in an NNS-based library (10).

| Residue position | Kd (hTFAA) | Kd (mutant) |
|------------------|------------|-------------|
| 56               | 81         | 2           |
| 62               | 68         | 0.8         |
| 65               | 66         | 0.8         |

* Dissociation constants for hTFAA and its variants were determined from kinetic parameters for binding immobilized FVIIa using a BIAcore instrument.

** Not determined.

As well as silent mutational changes at many positions that coded for the wild-type residue after sorting (data not shown). These results, therefore, strongly reinforced the findings from the scanning mutagenesis studies that Lys50 and Asp58 are very important for TF interactions with FVIIa. Furthermore, the results dictated a change in strategy to circumvent the problem of returning wild-type residues at randomized positions.

We theorized that the dominant contribution to binding from Lys50 and Asp58 may have masked more subtle interactions from other sites. Consequently, in a third library, the template K20A substitution was left unperturbed, and a single primer was used to randomize codons for residues at positions 58, 61, 62, 65, and 66. The latter four positions were chosen because double alanine substitutions at positions 61 and 62 and positions 65 and 66, had the effects of modestly decreasing and increasing sTF affinity for FVIIa, respectively (9). Although this library did not bear a complete complement of randomized sequences (we estimated that 1 × 10^6 of the 3.4 × 10^7 independent transmutants necessary for completeness were present in the starting library), six rounds of sorting nevertheless led to selection of phase specifically binding to FVIIa (−10^11 fold overall binding enrichment). Sequences from 12 plasmid templates (Table III) indicated a strong consensus developed in which positions 61, 62, 65, and 66 returned as wild type. Interestingly, position 58 returned exclusively as a tryptophan in place of the wild-type Asp or the template Glu. To fully characterize the variant TF containing the K20A and D58W substitutions (A20W58), oligonucleotide-directed mutagenesis was used to place the wild-type Asp codon in library 3 selectants was confirmed by DNA sequencing. The number in parentheses indicates the number of times the given variant appeared among the selected clones. The consensus sequence reflects those residues selected at each position that were significantly enriched (>4-fold) above their expected random frequency in an NNS-based library (10).

| Substrate | Wild-type sTF | sTF-A20W58 |
|-----------|---------------|------------|
|         | kcat (μmol/min) | Km (μM) | kcat (μmol/min) | Km (μM) |
| Chromozym | 7.48 ± 0.22 | 1.22 ± 0.01 | 6.31 ± 0.17 | 1.08 ± 0.03 |
| t-PA     | 5.02 ± 0.05 | 1.52 ± 0.07 | 4.33 ± 0.03 | 1.48 ± 0.05 |
| S-2286   | 5.61 ± 0.18 | 1.48 ± 0.02 | 4.68 ± 0.02 | 1.39 ± 0.05 |
| S-2286*  | 4.25 ± 0.13 | 3.54 ± 0.04 | 8.15 ± 0.20 | 3.88 ± 0.11 |

| Substrate | Wild-type sTF | sTF-A20W58 |
|-----------|---------------|------------|
|         | kcat (μmol/min) | Km (μM) | kcat (μmol/min) | Km (μM) |
| Chromozym | 7.48 ± 0.22 | 1.22 ± 0.01 | 6.31 ± 0.17 | 1.08 ± 0.03 |
| t-PA     | 5.02 ± 0.05 | 1.52 ± 0.07 | 4.33 ± 0.03 | 1.48 ± 0.05 |
| S-2286   | 5.61 ± 0.18 | 1.48 ± 0.02 | 4.68 ± 0.02 | 1.39 ± 0.05 |
| S-2286*  | 4.25 ± 0.13 | 3.54 ± 0.04 | 8.15 ± 0.20 | 3.88 ± 0.11 |

![Table IV](image)

TF binding affinity for FVIIa

BIAcore surface plasmon resonance measurements assayed sTF binding to Neutravidin-immobilized BEGR-7a; Isothermal titration calorimetry (ITC) experiments monitored sTF binding to FVIIa in solution at 25 °C.

| sTF species | kcat | Kd | Kd |
|-------------|------|----|----|
| Wild type   | 3.04 ± 0.33 | 1.36 ± 0.49 | 4.4 ± 1.2 |
| A20W58     | 3.84 ± 0.42 | 2.19 ± 0.30 | 5.7 ± 0.2 |

![Table V](image)

Soluble TF cofactor function for FVIIa amidolysis of p-nitroanilide substrates

Reactions of 100 nM TF · FVIIa with varying concentrations of the listed chromogenic substrates were performed. Kinetic parameters were determined by fitting initial velocity versus substrate concentration data to the Michaelis-Menten equation.

| Substrate | Wild-type sTF | sTF-A20W58 |
|-----------|---------------|------------|
| Chromozym | 7.48 ± 0.22 | 1.22 ± 0.01 | 6.31 ± 0.17 | 1.08 ± 0.03 |
| t-PA     | 5.02 ± 0.05 | 1.52 ± 0.07 | 4.33 ± 0.03 | 1.48 ± 0.05 |
| S-2286   | 5.61 ± 0.18 | 1.48 ± 0.02 | 4.68 ± 0.02 | 1.39 ± 0.05 |
| S-2286*  | 4.25 ± 0.13 | 3.54 ± 0.04 | 8.15 ± 0.20 | 3.88 ± 0.11 |

![Table VI](image)

Altered TF-FVIIa Interface

Affinity of sTF-A20W58 for Factor VIIa—We compared the affinity of the wild-type and A20W58 sTF proteins to bind to FVIIa using two assay systems. In the first, surface plasmon resonance measurements were used to monitor sTF binding kinetics to biotinylated, active site-inhibited FVIIa captured on a BIAcore flow cell coated with a streptavidin analog. The other assay monitored the thermodynamics of sTF proteins binding to FVIIa in solution via isothermal titration calorimetry. In both systems, the dissociation constants calculated from the measured parameters indicated that the A20W58 protein had an affinity for FVIIa comparable with that of wild-type sTF (Table IV), although the former assay showed that the A20W58 sTF has somewhat faster association and dissociation rates compared with those of the wild-type protein.

Cofactor Function of sTF-A20W58—To characterize the cofactor function of the altered sTF for FVIIa-dependent enzymatic activity, we tested the ability of the A20W58 sTF-FVIIa complex to cleave peptidic pseudosubstrates, to be inhibited by Kunitz-type protease inhibitors, and to activate a macromolecular substrate, factor X. Comparison of A20W58 and wild-type sTF as cofactors for FVIIa amidolytic activity revealed only subtle defects in function, independent of p-nitroanilide substrate used (Table V). The observed defects ranged from 83 to 88% of wild type for kcat values and 89–104% of wild type for kcat/Km values. Similarly, comparison of inhibition by Kunitz domains, which vary principally in residues at the P2–P4’ sites,3 showed only minor differences in Kcat values. Specifically, Kunitz domain TF7I-C inhibited the A20W58 enzyme complex with a Ks of 56.7 ± 3.0 nM (104.8 ± 5.5% of wild type), while IV-49C blocked the altered complex with a Ks of 76.5 ± 2.2 nM (133.0 ± 3.8% of wild type). A more severe defect, however, was observed in the ability of A20W58 to act as a cofactor in the FVIIa-dependent activation of factor X. These experiments used a 50-fold excess of FVIIa over sTF (1 nM) to ensure that the small differences in dissociation constant between the mutant and wild-type proteins resulted in negligible (less than 10%) differences in enzyme (sTF-FVIIa) concentration. Here we found that the A20W58 and wild-type sTF enzyme complexes had similar Kcat values for substrate binding, yet the mutant complex had only 30% of the catalytic activity of the wild type (Fig. 2). By comparison, hTFAA produces a 12-fold reduced kcat as a cofactor for FVIIa-mediated FX activation (5). Consistent with these FX activation data, the A20W58 sTF variant has a procoagulant activity intermediate to those of wild-type sTF (high activity) and hTFAA (low to zero activity).

3 The primary sequences (in single-letter code) from the P5 to P4’ site of Kunitz domains are as follows: PGPRCAAILL for TF7I-C and PGPCRAMMK for IV-49C. The boldface letters denote the Arg at P1.
Fig. 2. Activation of factor X by 50 nM FVIIa in complex with 1 nM wild-type (■) or A20W58 (▲) sTF, in the presence of vesicles containing a 7:3 mixture of phosphatidylcholine/phosphatidylserine. Kinetic parameters determined by fitting data to the Michaelis-Menten equation were as follows: for the wild-type sTF as cofactor, $k_{\text{cat}} = 783 \pm 126 \text{ nM mol}^{-1} \text{ min}^{-1}$ and $K_m = 20.0 \pm 1.1 \text{ mol of FX/min/mol of TF-FVIIa}$; for sTF-A20W58, $K_m = 522 \pm 105 \text{ nM}$, $k_{\text{cat}} = 6.1 \pm 0.4 \text{ mol of FX/min/mol of TF-FVIIa}$. The $k_{\text{cat}}$ calculation, we presumed that the limiting concentration of sTF is saturated and equals the concentration of enzymatically active TF-FVIIa complexes.

Thermodynamics of sTF-A20W58 Interaction with Factor VIIa—The replacement of charged Lys and Asp residues by nonpolar Ala and Trp side chains at positions 20 and 58, respectively, led us to hypothesize that high affinity FVIIa binding by sTF-A20W58 is driven by the burial of a more hydrophobic surface. This “hydrophobic effect” model predicted a more favorable entropic change and predicted a more negative change in heat capacity when the variant, as opposed to the wild-type, sTF binds to FVIIa (27). To test these predictions, we used titration calorimetry experiments to obtain estimates of $\Delta S$ and $\Delta C_p$ for the sTF proteins binding to FVIIa. Directly contrary to the predictions, we determined from the measured enthalpic changes and dissociation constants that the entropic term is less favorable for the variant compared with that for the wild-type TF (Table VI). Similarly, slopes of the enthalpic change as a function of temperature revealed a less negative $\Delta C_p$ for A20W58 (~712 calmol$^{-1}$K$^{-1}$) compared with the wild-type cofactor (~1051 calmol$^{-1}$K$^{-1}$).

**DISCUSSION**

Remodeling a TF Binding Interface for FVIIa—We initiated monovalent phage display experiments with the hope of identifying changes at a TF interface for FVIIa binding that would allow us to create sTF variants that bind FVIIa more tightly than the wild-type. The interface that was targeted for remodeling is dominated by two sequentially distant residues (Lys$^{20}$ and Asp$^{58}$) that come together to form a nonlinear FVII binding epitope in the folded protein (Fig. 1). Our initial goal went unrealized when we found that simultaneously randomizing the side chains displayed at and around the two key positions led to the selection of an interface of essentially wild-type sequence. In light of these results, we proceeded to devise a phage display-based method to select for a remodeled interface that retains high affinity receptor binding characteristics. To perform the remodelling, we first generated an sTF variant containing an alanine substitution, known to substantially disrupt TF-FVIIa interactions, at one of the key sites (K20A). We then sorted a phage library of sTF proteins that retained the crippling substitution but also contained variations at and near to the other key position of the epitope. Selection of variants on the basis of FVIIa binding led to a solution in which a Trp replaced the Asp at position 58. We found that the A20W58 variant binds FVIIa with wild-type sTF affinity. Thus, this phage-based selection strategy identified a D58W change that effectively reduced a 78-fold decrease in affinity imposed by the K20A substitution. The tactic of introducing an alanine substitution at a key site to reduce target affinity and then to select for alternate complexes at other functionally important epitope residues by sorting phage for tight binding to its receptor appears to be a powerful way to remodel an interface while maintaining high affinity binding characteristics. This strategy should be generally applicable to the remodeling of any protein-protein interface so long as detailed structural and functional studies of the interface are available to guide the selection of appropriate residues to vary.

A Defective Cofactor—Characterization of the A20W58 sTF variant revealed only subtle defects in its ability to act as a cofactor for FVIIa processing of peptidic substrates and for FVIIa inhibition by Kunitz domains. The observation of no substantial effect either with peptide substrates that vary at P2 and P3 or with Kunitz inhibitors that vary at P2$, P3$, and P4$ suggests that the altered cofactor allowed FVIIa to bind in an enzymatically active conformation. TF binding enhances the catalytic efficiency of FVIIa on peptide substrates through effects on both $k_{\text{cat}}$ and $K_m$ (28). Allosteric effects are responsible for the increase in catalytic efficiency because residues involved in catalysis are distant from the interface with TF (11). This allosteric linkage is retained in the A20W58 mutant protein, which is functional for organizing the active site of FVIIa. The A20W58-sTF protein, however, has a more pronounced defect as a cofactor for FVIIa proteolysis of a macromolecular substrate. Notably, comparison of kinetic analyses indicated that the deficit in FX activation by the A20W58 binary complex stems from a >3-fold reduction in $k_{\text{cat}}$ (with only minor perturbations of $K_m$) compared with the wild-type enzyme. Previously, Dickinson et al. (29) had shown that substitutions in the EGF-1 domain of FVIIa, at its interface with TF, led to decreased TF binding but had no effect on proteolytic function. In contrast, we found that mutations in TF, at its interface with the FVIIa EGF-1 domain, can result in reduced catalytic efficiency on macromolecular, but not peptide, substrates.

Proper cleavage of macromolecular substrates appears to involve multiple contact sites on the TF-FVIIa binary complex. For example, the complex of FVIIa with the K165A,K166A double mutant of sTF displays a 12.4-fold reduced $k_{\text{cat}}$ and 2.7-fold increased $K_m$ for FX activation relative to wild-type sTF-FVIIa (5). These lysine residues are on the surface of the C-terminal domain of TF, far from the active site of FVIIa in the binary complex and have been proposed to interact with the Gla-containing domain of FX (30). Our results with the A20W58 mutant sTF suggest that the altered TF-FVIIa complex is fully capable of binding substrate factor X and imply that the defect in macromolecular substrate processing is due to a misalignment of substrate with respect to the active site of
FVIIa during the formation of the ternary TF-FVIIa-FX complex. We propose that FVIIa is bound to A20W58 sTF in an altered conformation, thus perturbing interactions of the macromolecular substrates with the multiple contact points in the ternary complex. The interface between TF and the EGF-1 domain of FVIIa appears important for enzymatic function through the specific organization of the binary complex as well as through providing a driving force for binding. Our suggestion that the TF-FVIIa EGF-1 interface may be important for macromolecular substrate positioning during ternary complex formation parallels a similar proposal (31) made based on changes in TF-FVIIa catalytic efficiency observed when FVIIa EGF-1 calcium binding residues were changed to alanine.

**Mechanism of High Affinity FVIIa Binding**—In the complex formed between wild-type sTF and FVIIa, both Lys20 and Asp58 make important side chain hydrogen bonds to the backbone of FVIIa EGF-1. These residues were presumed to be critical for binding, since even conservative Arg and Glu replacements showed large decreases in binding (9). Nonetheless, we were able to select a tight binding variant that replaced these residues with nonpolar side chains. Modeling experiments suggest that the Trp30 residue can be accommodated in the sTF-FVIIa complex with only small adjustments in the interface. In one rotamer (32), the Trp side chain is positioned to displace a cluster of bound water molecules. We expected that Ala30 and Trp58 would contribute to binding through hydrophobic interactions, but the changes in binding thermodynamics are inconsistent with the features expected for the hydrophobic effect (27). The A20W58 sTF binds FVIIa with a more favorable ΔH, a less favorable entropy change, and a less negative ΔCp than measured for the wild-type protein. The changes in ΔS and ΔCp are opposite to what is expected for an increase in burial of nonpolar surface. However, this analysis ignores conformational changes that occur upon binding (33) and that may be altered when comparing the mutant and wild-type complexes. These conformational changes may be related to the defect in FX activation observed for the mutant complex. In addition, the removal of the specific hydrogen bonds supplied by Lys20 and Asp58 may result in a looser packing of the interface and thus an unexpected trend in the binding thermodynamics. Clearly, structure determination both on the A20W58 sTF-FVIIa complex and on FVIIa alone is needed to better understand these results.

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