Analysis of the Factor VIIa Binding Site on Human Tissue Factor: Effects of Tissue Factor Mutations on the Kinetics and Thermodynamics of Binding

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ABSTRACT: Surface plasmon resonance (SPR) measurements on a BIAcore instrument have been used to measure the effects of mutations in human tissue factor (TF), the initiator of blood coagulation, on the kinetics and affinity of binding to human FVIIa. TF mutant proteins were produced in soluble form by expression of the extracellular domain (sTF) in *Escherichia coli* followed by immunoaffinity purification. Mutants were designed and analyzed on the basis of the structure of sTF recently determined by X-ray crystallography [Muller et al. (1994) *Biochemistry 33,* 10864−10870]. Wild-type sTF binding to immobilized FVIIa has $k_{on} = 3.4 \pm 0.8 \times 10^5$ M^{-1} s^{-1} and $k_{off} = 2.1 \pm 0.1 \times 10^{-3}$ s^{-1} with a calculated K_D of 6.3 ± 1.2 nM and ΔG of −11.2 ± 0.1 kcal mol^{-1}. Calorimetric measurements indicate that binding occurs with a favorable ΔH of −32 kcal mol^{-1}, an unfavorable ΔS of −70 cal K^{-1} mol^{-1}, and a ΔC_p of −730 cal K mol^{-1}. The value of ΔC_p is consistent with burial of a large nonpolar surface area upon binding. Five residues on TF, Lys20, Trp45, Asp58, Tyr94, and Phe140, make a large contribution ($\Delta\Delta G$ = 1−2.5 kcal mol^{-1}) to FVIIa binding, a set of 17 mutations result in modest decreases in affinity ($\Delta\Delta G$ = 0.3−1 kcal mol^{-1}), and 40 mutations have $\Delta\Delta G$ smaller than the experimental uncertainty (±0.3 kcal mol^{-1}). Mutations at four sites result in small (0.3−0.5 kcal mol^{-1}) increases in affinity. Decreases in affinity result primarily from increased rates of dissociation. These data define a putative FVIIa binding site on one face of the TF structure with most of the contacts contributed by the N-terminal fibronectin type III domain. The critical binding residues are found on β-strands. An additional set of residues located on the surface of the C-terminal fibronectin type III domain opposite the FVIIa binding site have a role in the procoagulant activity of sTF but are not involved in FVIIa binding.

Tissue factor has a principle role in vertebrate hemostasis by serving as the initiator of the extrinsic pathway of blood coagulation (Davie et al., 1991; Ruf & Edgington, 1994). TF[1] is an integral membrane glycoprotein (263 residues) normally expressed in the adventitial cell layer surrounding blood vessels and on the surface of a variety of tissues (Drake et al., 1989; Wilcox et al., 1989). TF consists of an extracellular part (residues 1−219), a single membrane-spanning region (220−242) and a small cytoplasmic domain (243−263) (Morrissey et al., 1987; Scarpati et al., 1987; Spicer et al., 1987). Vascular damage exposes blood to TF, which forms a calcium-dependent, high-affinity complex with plasma coagulation factor VII (FVII). TF binding stimulates proteolytic activation of zymogen FVII to the serine protease FVIIa (Nemerson & Repke, 1985). TF also dramatically enhances the catalytic efficiency of FVIIa for both peptide (Lawson et al., 1992) and protein substrates (Bom & Bertina, 1990). The TF•FVIIa complex proteolytically activates factors IX and X, ultimately resulting in thrombin generation and the formation of a fibrin clot. Structure−function studies on TF and FVIIa are required to understand the mechanism of cofactor action.

Overexpression and/or aberrant utilization of TF have been linked to the pathophysiology of both thrombosis and sepsis. TF is expressed on cells found in atherosclerotic plaque (Wilcox et al., 1989) and can be induced on endothelial cells and monocytes by exposure to inflammatory cytokines or bacterial lipopolysaccharide (Gregory et al., 1989). Neutralizing anti-TF antibodies prevent death in a primate model of *Escherichia coli*-induced septic shock (Taylor et al., 1991), attenuate endotoxin-induced DIC in rabbits (Warr et al., 1990), and inhibit thrombus formation in a rabbit model of arterial thrombosis (Pawashe et al., 1994). Drugs that modulate the cofactor function of TF could have important therapeutic applications in the treatment of thrombosis and sepsis. Given that binding to TF is essential for the activation and catalytic function of FVIIa, one approach to develop inhibitors of the extrinsic pathway would be to target the interface between TF and FVIIa. Results of the structure−function analysis of TF•FVIIa should aid in the design of extrinsic pathway anticoagulants.

We describe here results of thermodynamic and kinetics measurements on the interaction between human FVIIa and the extracellular domain of human TF (sTF). Isothermal titration calorimetry has been used to measure the thermodynamics of binding, and surface plasmon resonance measurements on a Pharmacia BIAcore instrument have been used to determine the kinetics and affinity of binding to

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[1] Abbreviations: sTF, soluble tissue factor composed of the extracellular domain, residues 1−219; FVIIa, activated coagulation factor VII; SPR, surface plasmon resonance; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-[3-(diethylamino)propyl]carbodiimide; BEGR-ck, biotinylated glutamylglycinylarginyl chloromethyl ketone; BEGR-FVIIa, FVIIa modified with BEGR-ck. Mutants are named using the single-letter code for the amino acids with the wild-type residue first and then the sequence position, followed by the residue in the mutant. Colons separate multiple mutations.

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immobilized FVIIa for a panel of sTF mutants. Previous work (Ruf et al., 1994; Schullek et al., 1994; Gibbs et al., 1994) has used the TF-stimulated enzymatic activity of FVIIa to assay effects of TF mutations on the affinity for FVIIa. These data were interpreted on the basis of the proposed structural homology of TF to the cytokine receptor family (Bazan, 1990) to elucidate a putative binding site for FVIIa. Recently, the structure of the extracellular domain of TF has been determined by X-ray crystallography (Harlos et al., 1994; Muller et al., 1994). The structure shows sTF to be composed of two fibronectin type III domains[2] as observed for the human growth hormone receptor (DeVos et al., 1992). This structure allows us to make a more detailed analysis of the functional effects of TF mutations using the thermodynamic and kinetic data presented here.

EXPERIMENTAL PROCEDURES

Materials. Human FVIIa and BEGR-ck were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). The human FVIIa used in the calorimetry experiments was the kind gift of Dr. W. Kisiel (University of New Mexico). Streptavidin was purchased from Molecular Probes (Eugene, OR).

Oligonucleotide-Directed Mutagenesis. A phagemid vector designed for secretion of 1−219 human TF from *E. coli* under control of the alkaline phosphatase promoter and stII bacterial signal sequence was used as the template for oligonucleotide-directed mutagenesis. The procedure of Kunkel et al. (1987) was used to increase the efficiency of mutagenesis. Mutants selected by using this protocol were confirmed by dideoxynucleotide sequencing of the single-strand form of the phagemid. The DNA sequence was determined at the site of the mutation and for 30−50 bp on each side of this site.

Expression and Purification of sTF Mutants. E. coli strain 27C7 transformed with the expression plasmid was grown overnight at 30 °C in 250 mL of low phosphate containing media. Cells were harvested by centrifugation, frozen at −20 °C for 1 h, and then subjected to osmotic shock to release periplasmic contents by resuspension in 20 mL of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, and 1 mM benzamidine. After incubation on ice for 1 h, the suspension was centrifuged at 10000*g* for 20 min, and then the sTF in the supernate was purified by immunoaffinity chromatography. Briefly, the osmotic shock supernate was loaded onto a column (1 × 20 cm) having the D3 monoclonal antibody (Paborsky et al., 1989) immobilized onto controlled pore glass and previously equilibrated with 50 mM Tris-HCl, pH 8, and 0.5 M NaCl. Proteins bound nonspecifically were removed by washing with 3 column volumes of 50 mM Tris-HCl, pH 8, 1.0 M NaCl, and 0.5 M tetramethylammonium chloride, and then the sTF was eluted with 0.1 M sodium acetate, pH 3, and 0.2 M NaCl. Peak fractions were pooled and neutralized. Concentrations of purified sTF were determined by absorbance measurements using an ϵ_{280} of 29.4 mM^{-1} cm^{-1} calculated from quantitative amino acid analysis data. An ϵ_{280} of 24 mM^{-1} cm^{-1} was used for the Trp to Phe mutants of sTF.

[2] The extracellular portion of TF consists of two domains, residues 1−103 and 104−219, with topology of a fibronectin type III unit. β-Strands are labeled A, B, C, C′, E, F, and G according to the fibronectin type III nomenclature.

Circular Dichroic Spectroscopy. Far-UV CD spectra were recorded on a Cary/AVIV 60 DS spectropolarimeter. Quartz, cylindrical cuvettes having a path length of 0.1 cm were used, and 10 spectra were collected and averaged. Spectra were collected at 25 °C using 4 μM protein solutions containing 10 mM sodium phosphate, pH 7.0.

BIAcore Measurement of sTF Binding to Immobilized FVIIa. The effects of mutations in sTF upon FVIIa binding were determined by surface plasmon resonance measurements on a Pharmacia BIAcore instrument (Karlsson et al., 1991). FVIIa was immobilized by first reacting with a biotinylated inhibitor (BEGR-ck) followed by capture of the covalently modified protein (BEGR-FVIIa) on a sensor chip surface derivatized with streptavidin. BEGR-FVIIa was prepared by adding BEGR-ck (final concentration of 100 μM) to a solution containing 19 μM FVIIa, 300 μM sTF, 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5 mM $CaCl_2$. After incubation at ambient temperature for 2 h, the solution was desalted on a PD-10 column equilibrated with buffer containing 50 mM EDTA. Streptavidin was immobilized on the sensor chip surface by coupling through free amino groups. The carboxylated dextran matrix was first activated with a mixture of NHS and EDC using a protocol supplied by the manufacturer. An aliquot of a 1 mg/mL solution of streptavidin in 10 mM sodium acetate, pH 5, was injected at a flow rate of 2 μL min^{-1}. Injections were repeated until the SPR signal was increased by 5000−10 000 resonance units above the original baseline. Unreacted NHS was blocked by injection of an aliquot of 1 M ethanolamine. An aliquot of a solution of BEGR-FVIIa was then injected to obtain an additional increase in SPR signal of 2000−3000 resonance units. Any residual sTF bound to BEGR-FVIIa was eluted by injection of an aliquot of 50 mM EDTA.

The affinity of sTF variants for FVIIa was calculated from the kinetics of binding to immobilized FVIIa. For a 1:1 binding interaction

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} A{\cdot}B \quad (1)$$

$$K_D = k_{off}/k_{on} \quad (2)$$

We determined k_{off} by analyzing the response curve observed upon return to buffer flow after saturation with a high concentration (usually ≥1 μM) of sTF. Association rate constants (k_s) were calculated by using a series of six sTF concentrations ranging from 31.25 nM to 1 μM in 2-fold increments, and k_{on} was determined from the concentration dependence of k_s. Kinetics measurements commonly employed a flow rate of 10 μL min^{-1}, and the sensor chip surface was regenerated by elution of bound sTF with an injection of 50 mM EDTA. Kinetic constants were determined by nonlinear regression analysis as described by O'Shannessy et al. (1993) using software supplied by the manufacturer.

Isothermal Titration Calorimetry. Measurements of the enthalpy change (ΔH) for wild-type sTF binding to factor VIIa were performed on a Microcal, Inc., OMEGA titration calorimeter as previously described (Kelley & O'Connell, 1993). Protein solutions were prepared by exhaustive dialysis versus a buffer solution of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5 mM $CaCl_2$. FVIIa at a concentration of 5.8 μM was loaded into the 1.4 mL calorimeter cell

and titrated by injection of aliquots of a solution of 480 μM sTF. Heats of binding were calculated by subtracting heats of sTF dilution measured in separate experiments. These data were analyzed by using software supplied by the manufacturer. Enthalpy measurements were made at 25 and 37 °C to obtain an estimate of ΔC_p from the relationship

$$\Delta C_p = \frac{\Delta H(T_2) - \Delta H(T_1)}{T_2 - T_1} \quad (3)$$

Coagulation Measurements. The procoagulant function of wild-type and mutant sTF was measured on an ACL300 coagulometer operated in the research mode. sTF was added to pooled, platelet-depleted human plasma that had been anticoagulated by addition of citrate. A sTF concentration of 500 nM was used. Coagulation was initiated by mixing equal volumes of this solution and a solution of 25 mM calcium chloride, and the clotting time was measured.

RESULTS

SPR Measurements of sTF Binding to Immobilized FVIIa. Initial experiments indicated that amine coupling of FVIIa to the sensor chip yielded a surface with weakened and poorly reproducible affinity for sTF (Kelley, 1994). Since modification of the active site of FVIIa has been shown to have little impact on binding to TF (Bach et al., 1986), we chose to immobilize FVIIa through the protease active site. FVIIa was modified with a biotinylated peptide chloromethyl ketone (Williams et al., 1989) followed by capture of the biotinylated FVIIa (BEGR-FVIIa) on a sensor chip having amine-coupled streptavidin. The rate constant for dissociation of BEGR-FVIIa from streptavidin was less than 1×10^{-5} s^{-1} (data not shown). As shown in Figure 1, sensorgrams observed for injection of sTF onto this surface gave a good signal to noise ratio with the rate and extent of binding increasing with sTF concentration. No binding was detected for a sensor chip derivatized with streptavidin alone (data not shown). As expected since the TF·FVIIa interaction is calcium dependent, bound sTF could be eluted by injection of an aliquot of 50 mM EDTA. The BEGR-FVIIa–streptavidin surface showed good stability toward this regeneration procedure and only slowly lost sTF binding capacity after repeated experiments.

An apparent k_{on} of $3.4 \pm 0.8 \times 10^5$ M^{-1} s^{-1} was calculated from the concentration dependence of association shown in Figure 1B. Analysis of the sensorgram observed upon return to buffer flow after injection of 3 μM sTF (Figure 1C) yielded an apparent k_{off} of $2.1 \pm 0.1 \times 10^{-3}$ s^{-1}. The ratio of the kinetic constants gives a K_D of 6.3 ± 1.2 nM and a calculated ΔG of -11.2 ± 0.1 kcal mol^{-1}. These values represent the mean of triplicate measurements made on the same sensor chip over a period of a few days. A comparison between sensor chips and preparations of BEGR-FVIIa gives similar mean values but a 3-fold larger standard deviation. The affinity determined by SPR measurements is within the range of K_D values (0.6–20 nM) reported for sTF binding to FVIIa from a variety of experimental methods (Ruf et al., 1991; Waxman et al., 1992; Neuenschwander & Morrissey, 1994; Schullek et al., 1994).

Dissociation of bound sTF was more precisely described by two exponentials rather than a single kinetic phase. This result could be explained by heterogeneity in the FVIIa preparation, by significant rebinding during dissociation (Karlsson et al., 1991), or by a more complicated binding scheme than mechanism 1. No evidence for heterogeneity is observed in the association kinetics, suggesting that the FVIIa is homogeneous. Two alternative binding schemes are

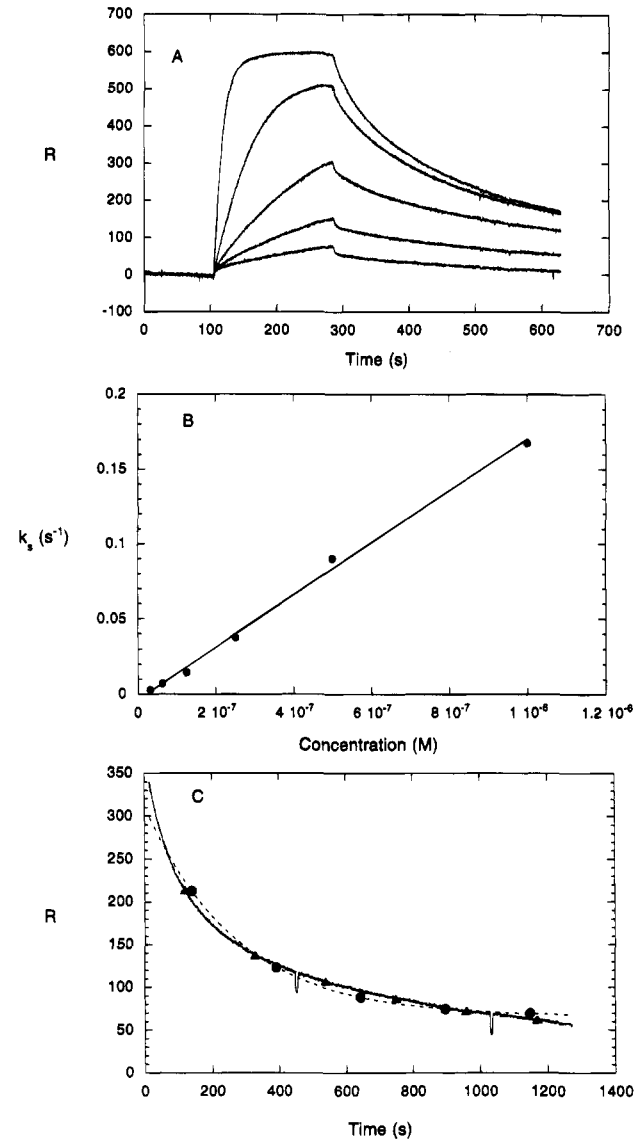

FIGURE 1: Kinetics of sTF binding to immobilized FVIIa measured by surface plasmon resonance on a Pharmacia BIAcore instrument. (A) Sensorgrams observed for injection of solutions of wild-type sTF, at concentrations ranging from 62.5 nM to 1 μM (in 2-fold increments), onto a BEGR-FVIIa–streptavidin surface at a flow rate of 10 μL min^{-1}. The solutions also contained 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5 mM $CaCl_2$, and the temperature was 25 °C. The sensor chip surface was regenerated by injection of an aliquot of 50 mM EDTA (not shown). (B) Concentration dependence of k_s values determined by nonlinear regression analysis of the association data of panel A (100–260 s) as described by O'Shannessy et al. (1993). The solid line represents a linear least squares analysis by using the equation $k_s = k_{on}C + k_{off}$, where C is the concentration of free sTF. (C) Sensorgram (solid line) observed for dissociation following injection of 3 μM sTF. The dashed line represents the result of nonlinear regression analysis using a single-exponential decay.

$$A + B \overset{\text{fast}}{\rightleftharpoons} AB \overset{\text{slow}}{\rightleftharpoons} AB^* \quad (4)$$

$$A \overset{\text{slow}}{\rightleftharpoons} A^* + B \overset{\text{fast}}{\rightleftharpoons} A^*B \quad (5)$$

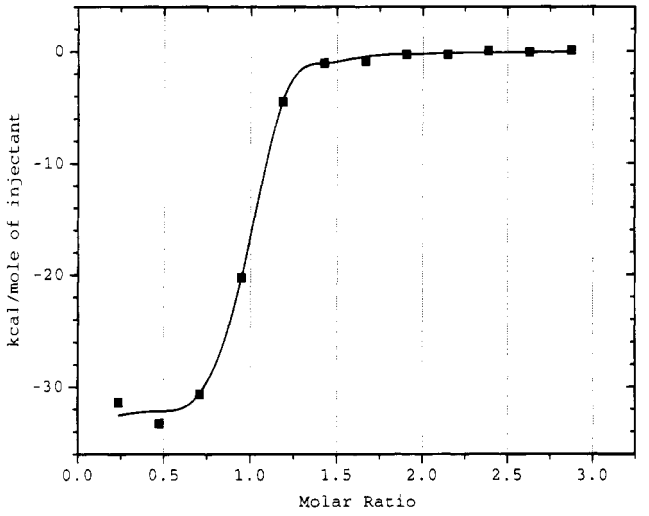

FIGURE 2: Heats of binding measured by isothermal calorimetry for titration of 5.8 μM FVIIa by addition of 4 μL aliquots of a solution of 480 μM wild-type sTF at 25.5 °C. The solid line is the result of nonlinear regression analysis yielding an n of 0.9 and ΔH of -32 kcal mol^{-1}.

Mechanism 4 predicts a hyperbolic dependence of k_s on free ligand concentration which is inconsistent with the linear dependence observed (Figure 1B). Mechanism 5 is also inconsistent with the data because the slow isomerization of A limits the rate of binding, and thus k_s should be fairly independent of free ligand concentration. The fit to a single exponential was not significantly improved by changes in flow rate in the range of 10−50 μL min^{-1}, by increasing the sTF concentration to 300 μM, or by decreasing the amount of immobilized BEGR-FVIIa. Although these results suggest that the deviation from single-exponential behavior does not originate from rebinding of sTF during the dissociation experiment, the relatively large k_{on} could make it difficult to prevent rebinding. We conclude that mechanism 1 is most consistent with the data. This conclusion is supported by the agreement in the K_D value calculated from the SPR data by using mechanism 1 with the results of other measurements.

Calorimetric Measurement of sTF Binding to FVIIa. Heats of sTF binding to FVIIa determined by using isothermal titration calorimetry are shown in Figure 2. Analysis of these data using a single class of binding sites model yields an n of 0.90 ± 0.01, consistent with the 1:1 stoichiometry observed by other methods (Waxman et al., 1992), and a ΔH of 32.0 ± 0.5 kcal mol^{-1}. The affinity of the interaction is too high to determine the binding constant by calorimetry.[3] Since the binding constant determined by SPR indicates a ΔG of -11.2 kcal mol^{-1}, the entropy change upon binding is large and unfavorable ($\Delta S = -70$ cal mol^{-1} K^{-1}). Although the limited availability of purified FVIIa precluded extensive calorimetric experiments, the ΔH was also determined at 37 °C ($\Delta H = -40.8$ kcal mol^{-1}) to give an estimated ΔC_p of -730 cal mol^{-1} K^{-1}. The sign and magnitude of ΔCp are consistent with burial of a large nonpolar surface upon binding (Livingstone et al., 1991; Connelly & Thomson, 1992).

[3] For accurate determination of the binding constant K, the c value for the measurements ($c = K$[M], where [M] is the concentration of the titrant) needs to be between 1 and 1000 (Wiseman et al., 1989). If the K_D for sTF•FVIIa is 5 nM, then the concentration required to give a c value of 1000 is 5 μM. At this concentration, however, the total heat change would be small, resulting in an imprecise binding isotherm.

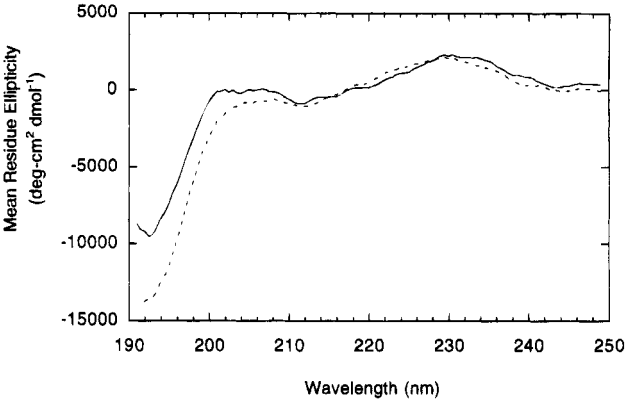

FIGURE 3: Far-UV CD spectra recorded for wild type (solid line) and the W45F (dashed line) mutant of sTF.

sTF Mutant Production. sTF residues were initially selected for mutagenesis by choosing segments of the TF extracellular domain proposed (Bazan, 1990) to be structurally homologous to the loops of the growth hormone receptor that are involved in hormone binding (Bass et al., 1991; DeVos et al., 1992; Clackson & Wells, 1995). Double (e.g., K65A:D66A) and triple (e.g., R136A:N137A:N138A) mutants of clustered charged or polar residues were constructed in order to speed identification of the binding site for FVIIa. The four tryptophans of sTF were individually mutated to phenylalanine since there is a change in Trp fluorescence upon sTF binding to FVIIa (R. Kelley, unpublished results). In addition, Ruf et al. (1994) had identified Lys20, Asp58, and Phe140 as important residues for binding to FVIIa. Once the crystal structure of the TF extracellular domain became available (Harlos et al., 1994; Muller et al., 1994), the mutagenesis strategy was altered to emphasize solvent-accessible residues that are spatially close to these sites. For example, an inspection of the structure for residues close to Lys20 identifies Leu133. Similarly, Gln37 is observed to pack against Trp45, and Lys48 is detected as a close neighbor to Asp58.

As indicated by SDS−PAGE (not shown), sTF mutants could be obtained in high purity by secretion from *E. coli* and immunoaffinity purification. Most mutant proteins could be obtained in 0.2−0.5 mg quantities from a 250 mL culture. Electrospray−ionization mass spectroscopy (Bourell et al., 1994) confirmed the molecular weights expected for these mutants. Wild-type sTF isolated in this fashion stimulated the activity of FVIIa on peptide and macromolecular substrates as previously described (Neuenschwander & Morrissey, 1994). A few sTF mutants, i.e., I152A, N199A: R200A, failed to bind to the antibody column and were purified by a combination of anion-exchange and hydrophobic interaction chromatography. Alanine substitutions of residues Ser16, Phe19, Thr60, Arg74, Tyr103, Ser142, Phe147, and Asp178:Asp180 resulted in very poor expression, and the mutant proteins could not be purified.

The global conformation of a few of the mutant proteins was compared to wild-type sTF by using CD spectroscopy. sTF displays an unusual far-UV CD spectrum that is only weakly dichroic as shown in Figure 3. A small and positive peak of ellipticity at 230 nm, probably arising from the aromatic chromophores, is detected. The broad peak of negative ellipticity centered at 217 nm commonly observed

in β-sheet proteins (Doi & Jirgensens, 1970) is absent in sTF. The negative ellipticity below 200 nm is more typical of random coil conformation than a folded protein. Since sTF purified from *E. coli* was used to determine the X-ray structure (Harlos et al., 1994; Muller et al., 1994), we must conclude that sTF is properly folded but the information content of the CD spectrum is low. The mutant sTF proteins, as represented by the spectrum of W45F shown in Figure 3, gave spectra similar to those of the wild-type protein, suggesting that the mutations do not perturb the overall conformation of the molecule. A more detailed analysis of the conformation by using NMR is precluded because sTF gives broad resonances (W. Fairbrother, unpublished results) presumably due to oligomerization at high concentration (2 mM).

Effect of sTF Mutations on FVIIa Binding. K_D values for binding of sTF mutants to BEGR-FVIIa were determined as described above for the wild-type protein. Wild-type and mutant values were compared using data collected on the same sensor chip because this gives less error than a comparison of K_D values measured on different sensor chips. Given the errors in measuring the kinetic constants for the wild-type protein, changes in kinetic constants with mutation must be greater than 2-fold to be considered significant. Affinities were compared relative to the wild-type protein by calculating the effect of the mutation on the change in free energy of binding

$$\Delta\Delta G = RT \ln \left(\frac{K_D(\text{mut})}{K_D(\text{wt})} \right) \qquad (6)$$

The estimated standard error in $\Delta\Delta G$ values calculated from data collected on one sensor chip is 0.3 kcal mol^{-1}. As shown in Figure 4, single-site substitutions of 14 residues in the N-terminal domain—Trp14, Lys20, Ile22, Glu24, Trp25, Gln37, Lys 41, Asp44, Trp45, Lys48, Phe50, Thr52, Asp58, and Tyr94—and two residues in the C-terminal domain—Arg135 and Phe140—result in decreases in affinity (positive $\Delta\Delta G$) that are greater than the experimental uncertainty (0.3 kcal mol^{-1}). Five of the single-site mutants, K20A, W45F, D58A, Y94A, and F140A, gave $\Delta\Delta G$ values of >1 kcal mol^{-1}. Double mutants D54A:E56A and D61A:E62A in the N-terminal domain and N107A:Q110A, S195A:R196A, and N199A:R200A in the C-terminal domain also gave small decreases in affinity. Except for the S195A:R196A mutant, these effects have not been further defined with single-site substitutions; the S195A mutant has no effect on binding. Mutants K15A, K65A:D66A, L104A:E105A, and Y185A bind BEGR-FVIIa with an affinity 1.5−2-fold higher than that of wild-type sTF.

Effect of Mutations on Kinetics of sTF·FVIIa Interaction. Alanine substitution at only one solvent-accessible site, K20A, resulted in a significant decrease (12.5-fold) in the apparent association rate constant (k_{on}) (Figure 5). Other substitutions effecting decreased on-rates involved mutation of buried residues (e.g., W14F, W25F). None of the mutants studied here resulted in increased rates of association. The association curves for K20A were only poorly described by a single kinetic component, and a much better fit was obtained by using two phases. Biphasic association kinetics were not observed for any other sTF mutants. This result implies that the K20A mutation changes the mechanism of

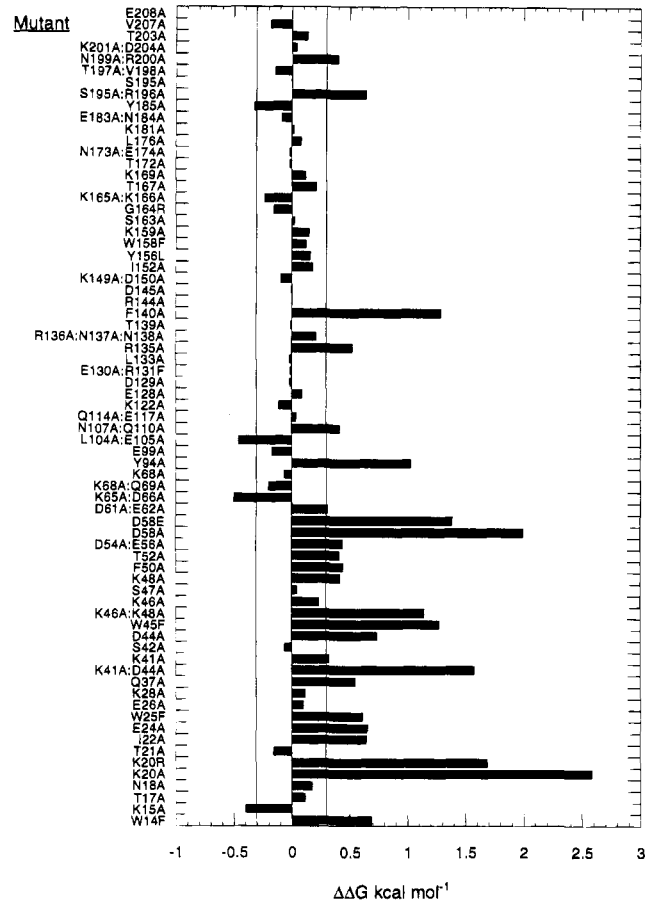

FIGURE 4: Effects of sTF mutations on the affinity for FVIIa binding reported as $\Delta\Delta G$ values. Dissociation constants were determined as described in the text and used to calculate $\Delta\Delta G$ values by using eq 6. Vertical lines at 0.3 and −0.3 kcal mol^{-1} denote the standard error in the measurements.

binding, with mechanism 5 a likely candidate. In contrast, the association kinetics measured for the K20R mutant could be adequately described by a single exponential with no change in the on-rate compared with the wild-type protein. These results suggest that a positively charged side chain at residue 20 is important for orienting FVIIa as it approaches the binding site or for maintenance of a binding competent state of sTF.

Substitutions of solvent-accessible residues Lys20, Ile22, Glu24, Gln37, Asp44, Trp45, Asp58, Tyr94, Asn107:Gln110, and Phe140 resulted in significant increases in the off-rate for dissociation of sTF from immobilized FVIIa (Figure 5). Increases in off-rate tend to be larger than on-rate effects. Indeed, since the accuracy of the off-rate determinations on the BIAcore is reduced when $k_{off} > 10^{-2}$ M^{-1}, and because some of the mutants (e.g., K41A:D44A, W45F, D58A) have $k_{off} > 10^{-2}$ M^{-1}, the relative effects (Figure 5) on the apparent off-rate may only represent a lower limit on the increase in dissociation rate. Thus, for mutants with k_{off}(mut)/k_{off}(wt) > 10, the $\Delta\Delta G$ value may be underestimated.

The double mutant K46A:K48A also had an increased off-rate although no effect was observed for the single mutants K46A or K48A. Similarly, the K41A mutant had no effect by itself, but the double mutant K41A:D44A had a faster off-rate than measured for the single substitution, D44A. For this set of mutations, the $\Delta\Delta G$ of the double mutant is greater than the sum of the $\Delta\Delta G$ values measured for the single mutants. This result suggests that the residues cooperate in

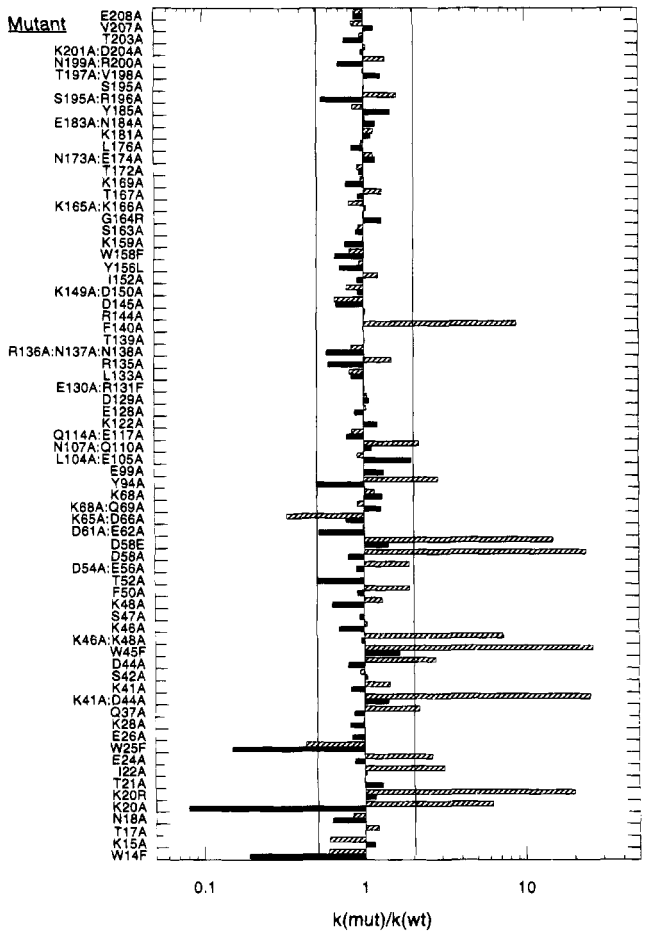

FIGURE 5: Effects of sTF mutations on the kinetics of sTF·FVIIa interaction reported relative to the value measured for the wild-type protein. Filled bars represent k_{on} values, and k_{off} effects are shown as hatched bars. Vertical lines denote the standard error.

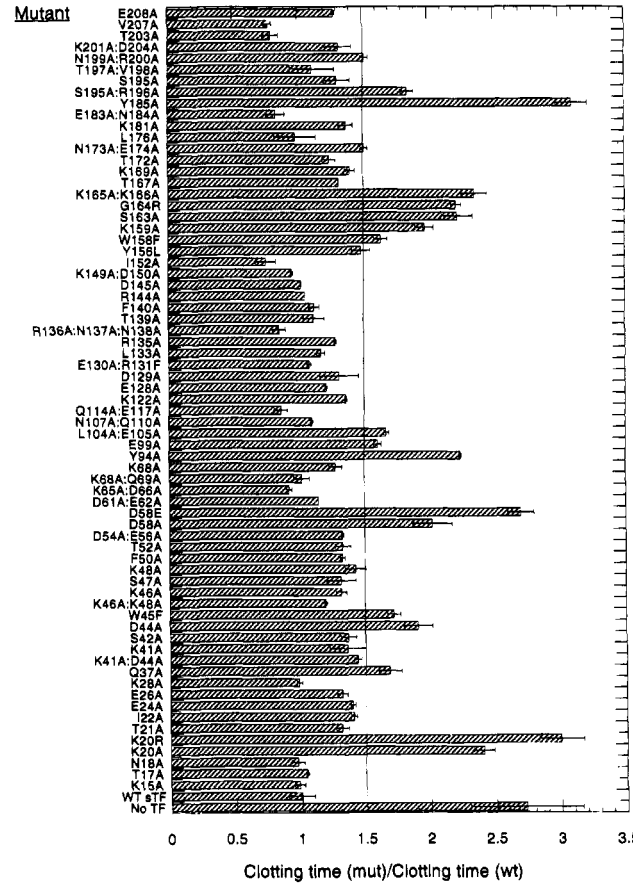

FIGURE 6: Relative effects of mutations on the procoagulant activity of sTF measured with human plasma. Values shown with error bars represent the mean of triplicate measurements. A clotting time 1.5-fold that of wild type is considered to be a significant difference.

binding ligand, or the double mutant has an altered conformation (Wells, 1990). Two mutants, K65A:D66A and W25F, had decreased off-rates. Lys65 and Asp66 are solvent-accessible residues, and thus the effect of alanine substitutions for these residues may indicate that the charged side chains make unfavorable interactions with FVIIa. Trp25 is >90% buried, and the W25F mutation could perturb the conformation of sTF, resulting in increased nonspecific binding.

Effect of sTF Mutations on Coagulation. The effect of mutations on the procoagulant function of sTF was examined by using a one-stage clotting assay. Wild-type sTF diluted to 500 nM in plasma gave a clotting time of 112 ± 11 s upon mixing with an equal volume of 25 mM calcium chloride. Addition of calcium alone gave a clotting time of 303 ± 50 s. As reported by Neuenschwander and Morrissey (1992), sTF gives a longer clotting time than membrane-bound TF, probably as a result of the slower autoactivation of FVII in the presence of sTF. Increasing the concentration of wild-type sTF above 500 nM did not result in a shorter clotting time, suggesting that this concentration is sufficient to rapidly saturate the FVII present in plasma. Since the mutations that have an effect on FVIIa binding usually increase the dissociation rate, the time to reach equilibrium should be shorter with the mutants than for wild-type sTF. For the weaker binding mutants, however, 500 nM sTF may be insufficient to saturate the FVII present, and a prolonged clotting time will result. Limited amounts of some of the mutants prevented the use of higher sTF concentrations in the coagulation assay. In addition, this assay displayed some batch-to-batch variation and is presumably sensitive to differences in the salt, pH, or contaminant level of the sTF preparation. Therefore, only clotting times increased by at least 1.5-fold from wild type are considered to be significant. Given the clotting time measured for addition of calcium alone (2.7-fold longer than wild-type sTF), the range of the assay for measuring defects in cofactor function is quite limited.

The clotting times measured for 500 nM sTF mutants in plasma are shown relative to wild-type sTF in Figure 6. As was expected, mutants having large reductions in affinity for FVIIa—K20A, K20R, D44A, W45F, D58A, D58E, Y94A—gave prolonged clotting times. An additional set of mutations, E99A, L104A:E105A, W158F, K159A, S163A, G164R, K165A:K166A, and Y185A, did not weaken FVIIa binding but are observed to have prolonged clotting times. Addition of 10 nM FVIIa to the citrated plasma reduced the clotting times measured for this set of mutants and also for wild-type sTF by about 3-fold (data not shown). Since these mutants gave longer clotting times than wild-type sTF even in the presence of activated enzyme, these mutants cannot be solely defective in FVII autoactivation but must also be impaired for FXa generation. A few sTF mutants appear to have shorter clotting times than the wild-type protein, but it is unclear whether this reflects improved cofactor function or the purity level of the mutant.

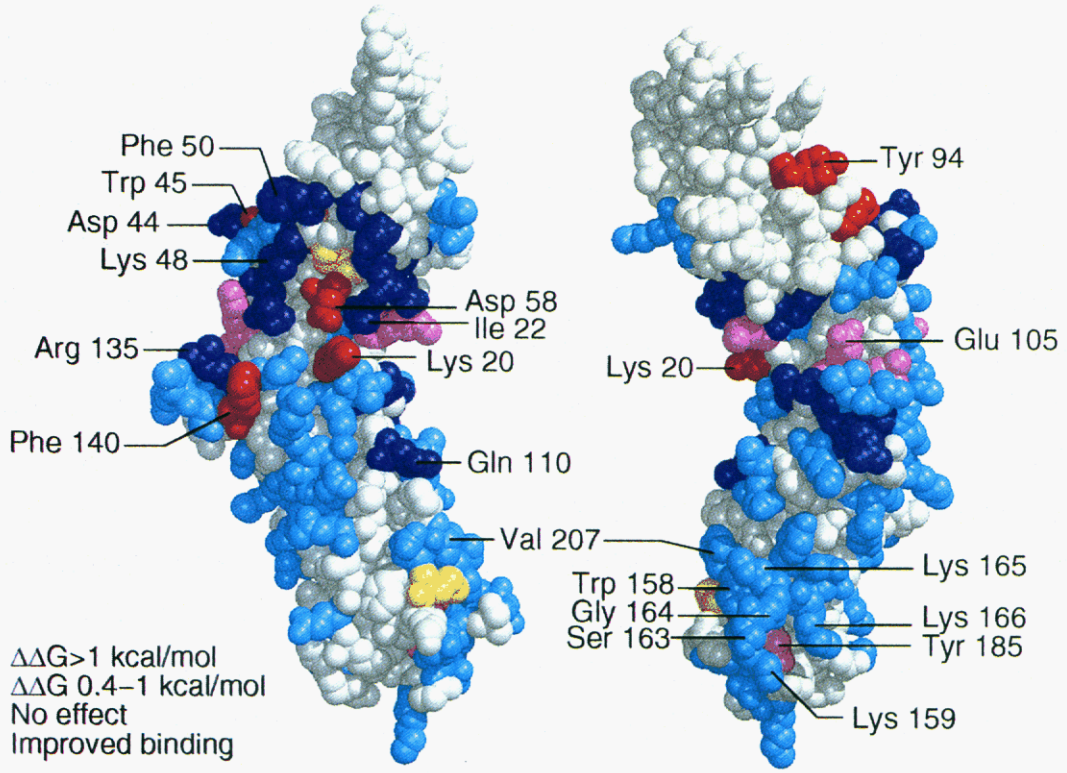

FIGURE 7: Space-filling representation of the structure of sTF (Muller et al., 1994) with residues colored according to the magnitude of the $\Delta\Delta G$ effect for FVIIa binding. Residues that have not been mutated are colored white with the exception of the cysteine residues of the two disulfide bonds which are colored yellow. Two views related by a 90° rotation are shown. This figure was generated by using the MidasPlus program (Ferrin et al., 1988).

DISCUSSION

We have described here a detailed study of the FVIIa binding site on human sTF. A majority of the solvent-accessible residues, and almost all of the residues neighboring sites with $\Delta\Delta G > 1$ kcal mol^{-1}, have been mutated. Our results using direct binding methods confirm those of others (Ruf et al., 1994; Schullek et al., 1994; Gibbs et al., 1994) showing that Lys20, Trp45, Asp58, and Phe140 make important contributions to FVIIa binding. In addition, we identify Tyr94 as an important residue for FVIIa binding. In contrast to Gibbs et al. (1994), we find that the V207A mutation has no effect on FVIIa binding or cofactor function. These other studies measured changes in TF·FVIIa affinity by assay of the TF stimulation of FVIIa enzymatic activity. It is difficult to compare our results with those of Ruf et al. (1994) because these authors produced TF mutants in full-length form and assayed FVIIa affinity by measurements of factor X activation using detergent-solubilized TF. The $\Delta\Delta G$ values we measure tend to be smaller than those calculated from the data of Schullek et al. (1994) and Gibbs et al. (1994) for sTF binding to FVIIa. This difference could reflect the presence of substrate in the amidolytic assays. Indeed, the data of Butenas et al. (1994) suggest that the K_D determined for TF·FVIIa in an amidolytic assay is somewhat dependent on the substrate used. Our data obtained in the absence of substrate suggest that Ile22 and Arg135 make less important contributions to binding than described previously.

Structure of the FVIIa Binding Site on sTF. As shown in Figure 7, residues implicated in FVIIa binding form a convex surface extending from Tyr94 of the N-terminal domain into the domain−domain interface. The putative FVIIa binding site is centered on the B (Lys20), C′ (Asp58), and E (Trp45) strands of the N-terminal domain, but FVIIa also contacts residues from the C-terminal domain (Asn107 or Gln110, Arg135, Phe140) at the domain−domain interface. Both the ΔC_p value and mutagenesis results are consistent with a large contact surface on sTF for binding FVIIa. Given that the molecular weight of FVIIa is about twice that of sTF, an extended binding site is not unreasonable. The total accessible surface of TF residues with $|\Delta\Delta G| > 0.3$ kcal mol^{-1} is large (>2000 Å^2). Although it is unlikely that all of the accessible surface of these residues will become buried upon binding to FVIIa, other residues may become buried without actively contributing to the ΔG of binding. For example, Leu133 lies between Lys20 and Phe140 and is likely to become buried on binding FVIIa, and yet the L133A mutant has negligible $\Delta\Delta G$. Some residues that contact ligand may make favorable contributions to ΔH but not to ΔG because of a compensating unfavorable ΔS (Novotny et al., 1989; Kelley & O'Connell, 1993).

Solvent-accessibility calculations[4] indicate that Trp14, Ser16, Phe19, Thr21, Trp25, Thr60, Glu62, Tyr103, Phe147, Tyr185, and Arg196 are each >90% buried, and thus any effects from mutation of these residues are likely to result from changes in the protein conformation. Mutation of Thr21 and Glu62 was tolerated with little impact on ligand binding whereas the sTF mutants W14F, S16A, F19A, W25F, T60A, Y103A, and F147A were expressed very poorly in *E. coli*. Trp25 is conserved in all fibronectin type

[4] Solvent accessibilities were calculated from the 1.7 Å structure of sTF (Muller et al., unpublished results) by using the method of Lee and Richards (1971) with a 1.4 Å radius probe. The fraction buried was calculated using a reference tripeptide and includes side-chain and main-chain contributions.

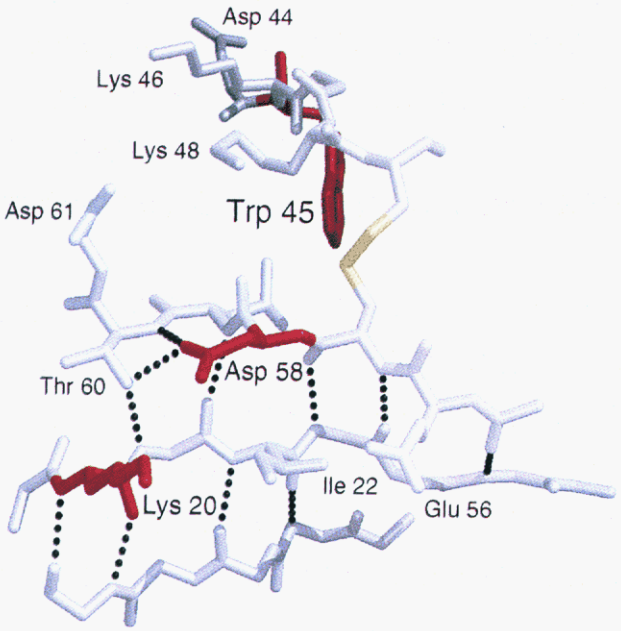

FIGURE 8: Hydrogen bonding within a portion of the FVIIa binding site on sTF. Backbone atoms of the A (13−17), B (19−26), C′ (45−48), and E (55−58) strands of the N-terminal domain and selected side chains are shown. Potential hydrogen bonds are shown as dotted lines. Side chains of Lys20, Trp45, and Asp58 are colored red, and the Cys49−Cys57 disulfide bond is shown in yellow. This figure was produced using the MidasPlus program (Ferrin et al., 1988).

III domains (Bazan, 1990) and is presumed to play an important structural role. Arg196 is buried in the interface between the two domains and the side chain is hydrogen bonded to backbone atoms of residues 103, 104, 106, and 199. The side chains of Phe19, Tyr103, and Phe147 pack together to form an aromatic cluster at the interface between the two domains. Asn18, Leu104, Glu105, Asn107, and Asn199, although less than 90% buried, contribute to the accessible surface area buried in the interface between the two domains. Effects on FVIIa binding or cofactor function from mutations of these residues could reflect a change in the relative orientation of the two domains.

Adding together all of the $\Delta\Delta G$ values determined for residues that are <90% buried yields slightly more free energy (14.5 kcal mol^{-1}) than determined for wild-type sTF binding to FVIIa ($\Delta G = -11.3$ kcal mol^{-1}).[5] Most of this binding free energy is provided by contacts with residues from the N-terminal domain. With the exception of Asp44, all of the key residues for FVIIa binding reside on β-strands (Muller et al., 1994; Figure 8). Lys20 and Asp58 are contained on adjacent antiparallel β-strands formed from discontiguous polypeptide segments. The critical importance of Lys20 and Asp58 for binding to FVIIa is supported by the observation of decreased binding for the conservative Arg and Glu replacements at these sites, respectively. Thr 60 appears to play an important role in maintenance of this conformation. The Oγ of Thr60 makes hydrogen bonds to the side chain of Asp58 and also to the backbone of the E strand (Figure 8). Substitution with alanine would be expected to disrupt the conformation of this region, consistent with the failure of the T60A mutant to express in *E. coli*.

[5] The summation assumes the sites act independently, with mutations not affecting the conformation, and any contribution from the backbone or β-carbon is ignored.

A comparison with members of the cytokine receptor family suggests that Trp45 of sTF is not a contact residue for FVIIa binding. Trp45 has a fraction buried of 0.71 and the side chain packs against the side chains of Gln37 and Arg74 and also against the backbone of the C and F strands. A Trp is found at the same sequence position in the INF-α, INF-γ, and IL-2 receptors (Bazan, 1990) and at a structurally equivalent position in the hGH receptor (DeVos et al., 1992). Trp45 is probably important for maintaining the fibronectin type III fold, and the W45F mutation could influence the contribution to binding from nearby residues Gln37, Asp44, Lys46, and Lys48.

Thermodynamics of the sTF·FVIIa Interaction. The large and negative ΔC_p value associated with binding suggests that burial of nonpolar surface provides a large driving force for the interaction; however, the unfavorable ΔS is inconsistent with the hydrophobic effect (Kauzmann, 1959; Tanford, 1980). As noted by Sturtevant (1977), this discrepancy could be accounted for by a "stiffening" of vibrational modes upon binding which will contribute negative values to ΔH, ΔS, and ΔC_p. By using this model, the hydrophobic portion of ΔC_p is calculated to be −540 cal mol^{-1} K^{-1}, which suggests that about 1600 Å^2 of nonpolar surface area becomes buried upon binding (Livingstone et al., 1991). Fixing vibrational modes would provide a favorable ΔH of −30 kcal mol^{-1} but a negative ΔS that opposes the positive ΔS from the hydrophobic effect. Alternatively, the unfavorable ΔS could be the result of conformational change concomitant with binding as previously proposed on the basis of results of hydrodynamic measurements (Waxman et al., 1993).

In contrast to the expectation based on the ΔC_p value, many of the mutations that have an impact on FVIIa binding involve changes in charged or polar residues. For most of the residues mutated, however, we do not know whether the polar or nonpolar parts of the side chain are important for binding. We conclude that the driving force for sTF·FVIIa binding is burial of a large amount of nonpolar surface area. Single-site alanine substitutions probably result in only small changes in the amount of nonpolar surface available for binding. Within this desolvated binding interface, charged or polar groups on sTF are necessary to complement residues on FVIIa through hydrogen-bonding or ionic interactions. A positively charged residue at position 20 seems to be important in determining the on-rate for FVIIa binding. The C′ strand appears to supply a positive charge for binding, either from Lys46 or Lys48, since the $\Delta\Delta G$ effect of single mutants K46A or K48A is small but the double mutant K46A:K48A has a $\Delta\Delta G$ of 1.2 kcal mol^{-1}. Mutations of some charged residues, i.e., K15A, K65A:D66A, E105A, may improve binding because the polar interactions at these sites are not as well satisfied in the complex as in the unbound state. Individually, polar interactions contribute little to ΔG because the bonding can be satisfied by water in the free components. Collectively, the polar interactions may determine specificity by preventing binding to the incorrect hydrophobic surface.

Comparison to Other Studies of Protein−Protein Interaction. On the basis of a homology-modeled structure of sTF, Gibbs et al. (1994) concluded that sTF conserved the mode of ligand binding observed for the hGH receptor (DeVos et al., 1992). The crystal structure of sTF, however, shows that the FVIIa binding site is located on the opposite side of the cytokine receptor fold relative to the hGH binding site

on the hGH receptor (Muller et al., 1994). Most of the residues important for binding hGH in both the hGH receptor (DeVos et al., 1992) and the prolactin receptor (Somers et al., 1994) are provided by loop regions whereas β-strand residues in sTF are used to bind FVIIa. Residues of the AB loop in the N-terminal domain of both the hGH and prolactin receptors are important for hormone binding; in sTF these residues (17−19) are buried in the domain−domain interface.

As with similar studies of antibody−antigen (Jin et al., 1992; Kelley & O'Connell, 1993) and hormone−receptor (Cunningham & Wells, 1993; Clackson & Wells, 1995) interaction, a few residues make a dominant contribution to the free energy of binding. Lys20, Trp45, Asp58, Tyr94, and Phe140 together account for >70% of the ΔG of binding. Similarly, most of the changes in affinity result from increases in the rate of dissociation. Protein−protein association has been proposed to involve a diffusive-entrapment mechanism (Northrup & Erickson, 1992), and single point mutations may not have a large effect on the association step of this mechanism. Stabilization of the protein−protein complex requires precise docking of the two surfaces, and thus defects in this process could be manifested as increased dissociation rates.

The magnitude of the $\Delta\Delta G$ values observed for the key residues of the FVIIa binding site on sTF are smaller than the changes measured for mutants of the growth hormone receptor (Clackson & Wells, 1995) or the anti-p185^{HER2} antibody (Kelley & O'Connell, 1993). In these systems key residues for ligand binding are contributed by loop regions of the protein. Substitutions in loop regions may result in larger local conformational changes than replacements of residues presented on a more rigid, hydrogen-bonded scaffold. In addition, the FVIIa binding site on sTF is more extended than the ligand binding sites in these other systems. Within this extended binding site, the energetic importance of individual contacts is smaller than in the more compact binding sites. In both the hGH receptor and the anti-p185^{HER2} antibody the largest $\Delta\Delta G$ effects are for replacement of hydrophobic residues. In contrast, mutation of two charged residues in sTF, Lys20 and Asp58, have the greatest impact on FVIIa binding.

Effects of Mutations on the Procoagulant Function of sTF. A set of residues that are less than 90% buried, and do not contribute to the interface between the two domains (Glu99, Trp158, Lys159, Ser163, Gly164, Lys165, Lys166), have a role in the coagulant function of sTF but are not involved in FVIIa binding. These residues, excluding Glu99, are contained in the C-terminal domain and are found on the opposite surface of the molecule from the putative FVIIa binding site. Residues from the 157−174 segment of TF had previously been shown to be important for factor X activation by the TF·FVIIa complex (Ruf et al., 1992b). Lys165 and Lys166 have been implicated in macromolecular substrate recognition (Roy et al., 1991) and have been proposed to have a role in substrate (FX) binding (Ruf et al., 1992a). Alanine substitution of Tyr185, a buried residue, also results in a large decrease in the procoagulant function of sTF. The side chain of Tyr185 contacts both Lys159 and Lys166 and thus is probably important for supporting the conformation of this region. Further work is required to determine whether the C-terminal domain provides a secondary substrate binding site or functions indirectly to maintain FVIIa in a catalytically active conformation.

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