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Tissue Factor and Its Extracellular Soluble Domain: The Relationship between Intermolecular Association with Factor VIIa and Enzymatic Activity of the Complex†

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ABSTRACT: We find that the isolated, extracellular domain of tissue factor (TF_{1-218}; sTF) exhibits only 4% of the activity of wild-type transmembrane TF (TF_{1-263}) in an assay that measures the conversion of factor X to Xa by the TF:VIIa complex. Further, the activity of sTF is manifest only when vesicles consisting of phosphatidylserine and phosphatidylcholine (30/70 w/w) are present. To determine whether the decreased activity results from weakened affinity of sTF for VIIa, we studied their interaction using equilibrium ultracentrifugation, fluorescence anisotropy, and an activity titration. Ultracentrifugation of the sTF:VIIa complex established a stoichiometry of 1:1 and an upper limit of 1 nM for the equilibrium dissociation constant (K_d). This value is in agreement with titrations of dansyl-D-Phe-L-Phe-Arg chloromethyl ketone active site labeled VIIa (DF-VIIa) with sTF using dansyl fluorescence anisotropy as the observable. Pressure dissociation experiments were used to obtain quantitative values for the binding interaction. These experiments indicate that the K_d for the interaction of sTF with DF-VIIa is 0.59 nM (25 °C). This value may be compared to a K_d of 7.3 pM obtained by the same method for the interaction of DF-VIIa with TF_{1-263} reconstituted into phosphatidylcholine vesicles. The molar volume change of association was found to be 63 and 117 mL mol^{-1} for the interaction of DF-VIIa with sTF and TF_{1-263}, respectively. These binding data show that the sTF:VIIa complex is quantitatively and qualitatively different from the complex formed by TF_{1-263} and VIIa.

The complexation of factor VIIa (VIIa)[1] with tissue factor (TF) is widely believed to be a critical step in the initiation of blood coagulation. As it occurs in nature, TF is a transmembrane glycoprotein consisting of an extracellular domain (residues 1-219), a single transmembrane domain (residues 220-242), and a cytoplasmic domain (residues 243-263) with

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[1] Abbreviations: VIIa, factor VIIa; X, factor X; sTF, soluble tissue factor; TF_{1-263}, full-length tissue factor; PS, phosphatidylserine; PC, phosphatidylcholine; FFRCk, D-Phe-L-Phe-Arg chloromethyl ketone; DF-VIIa, dansyl-D-Phe-L-Phe-Arg-labeled VIIa.

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a sequence that contains a half-cysteine residue thioesterified to palmitate or stearate [for review see Bach (1988)]. When complexed with TF, the proteolytic activity of VIIa, a serine protease, toward its natural substrate, factor X (X), is increased by many orders of magnitude (Silverberg et al., 1977).

TF is frequently referred to as a "receptor" (Edgington et al., 1991), implying that docking of VIIa to transmembrane TF without concomitant conformational change in VIIa is sufficient to endow it with enhanced proteolytic activity. In this model, the interaction between VIIa and TF is passive, and the enhanced activity derives solely from facilitation of assembly of the cofactor–enzyme–substrate complex, TF:VIIa:X, on the membrane surface. However, detailed kinetic studies of the TF:VIIa complex indicate that TF is an essential enzyme activator (Nemerson & Gentry, 1986). This implies that structural alterations occur in VIIa upon formation of the binary complex with TF.

Studies of the TF:VIIa binding interaction are complicated by the requirement that full-length TF be reconstituted in phospholipid vesicles or solubilized in detergent. This makes the possible interactions of TF and VIIa which involve the lipid or detergent difficult to resolve from those due strictly to direct protein–protein interactions and complicates the thermodynamic description of the TF:VIIa interaction. To resolve these contributions, we have prepared a soluble TF (TF_{1-218}; sTF) which lacks the transmembrane and cytoplasmic domains.

In the presence of exogenous vesicles consisting of phosphatidylserine and phosphatidylcholine (30/70 w/w), the sTF:VIIa complex has about 4% of the activity of TF_{1-263}:VIIa. In this paper, we examine and compare the nature of the complex formed by XIIa and sTF with that formed with full-length TF (TF_{1-263}) inserted into phosphatidylcholine vesicles. We show that the reduced activity of sTF relative to full-length TF does not result from impaired VIIa-binding, as the assays were performed using conditions that would result in >90% of the TF molecules being complexed with VIIa. Furthermore, we have determined ΔG, the free energy of binding, and ΔV, the molar volume change, for association at 25 °C of sTF or TF_{1-263} to VIIa. In the case of TF_{1-263}, the K_d is 7.3 pM; with sTF it is 0.59 nM. Further, when factor VIIa binds to TF_{1-263}, there is a volume increase of 117 mL mol^{-1}; with sTF the volume increase is 63 mL mol^{-1}. Thus, we have demonstrated that the two binary complexes are structurally different. We propose that it is this structural difference and not binding per se that is reflected in the differences in activity of sTF and TF_{1-263}.

Experimental Procedures

Experimental Approach. Measurement of associations in the picomolar-to-nanomolar concentration range under equilibrium conditions is technically difficult. Earlier studies measured the degree of dissociation by separation of free from bound VIIa, which is not an equilibrium technique. To covercome the inherent difficulties in measuring tight binding, while still meeting the requirement of observing the system at equilibrium, we have utilized the techniques of high-pressure dissociation and fluorescence anisotropy. Fluorescence anisotropy studies were performed using fluorescent-labeled VIIa covalently modified at its active site with dansyl-D-Phe-L-Phe-Arg chloromethyl ketone (DF-VIIa).

Theoretical Considerations. The ability of pressure in the range of 0.1–3 kbar to dissociate protein complexes is well documented (Heremans, 1982; Weber & Drickamer, 1983). This effect can be understood by examining the thermodynamic expression for a simple dissociation $AB \rightleftharpoons A + B$. At equilibrium:

$$K_d = e^{-\Delta G^\circ/RT} = e^{-(\Delta E^\circ + P\Delta V^\circ - T\Delta S^\circ)/RT} \quad (1)$$

where K_d is the equilibrium dissociation constant and ΔG°, ΔE°, ΔV°, and ΔS° are the standard changes in free energy, internal energy, volume, and entropy, respectively. Consequently, the application of pressure drives the equilibrium toward association or dissociation, whichever condition takes up the least volume, according to the relationship

$$\delta(\ln K_d)/\delta P = -\Delta V^\circ/RT \quad (2)$$

Interactions between proteins result in the creation of small spaces at interfacial areas that cannot easily be filled by solvent. Some of these unfilled volumes will disappear upon dissociation due to better packing of the solvent at the former contact surfaces. For this reason, protein–protein associations are generally characterized by positive volume changes and application of pressure shifts the binding equilibrium toward dissociation.

The interaction of VIIa with sTF and TF_{1-263} was investigated by observing the change in the steady-state fluorescence anisotropy of a dansyl probe covalently attached to the active site of VIIa (see below). The anisotropy, r, is given by

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (3)$$

where I_{VV} and I_{VH} are respectively the emission intensities detected through vertical and horizontal polarizers, when vertical excitation is used and G is a factor which corrects for the transmissivity bias of the optics (Azumi & McGlynn, 1962; Paoletti & LePecq, 1969).

The anisotropy of light emitted by a collection of fluorophores free to rotate in solution is given by

$$r = r_0(1 + \tau/\phi)^{-1} \quad (4)$$

where τ is the fluorescence lifetime of the fluorophore and ϕ is the rotational correlation time, a measure of how fast the molecule is rotating. r_0 is the limiting or "frozen" anisotropy and accounts for depolarization due to factors other than molecular rotation. The rotational correlation time of a fluorescent molecule rigidly attached to a protein will depend on the solution viscosity, temperature, and the size and shape of the protein.

From these considerations it is clear that the rotational correlation time of a protein in solution is likely to increase when the protein is in a macromolecular complex. In general, the anisotropy increase results primarily from a decrease in the global rotational motion of the protein and secondarily from a decrease in local motions due to binding interactions.

For a sample containing a mixture of free and bound labeled protein, the measured anisotropy, r_m, is

$$r_m = (r_f I_f + r_b I_b)/(I_f + I_b) \quad (5)$$

where r_f, r_b, I_f, and I_b are the anisotropies and intensities of free and bound labeled protein. Defining Δr as the difference between the measured anisotropy and the anisotropy of the free protein ($\Delta r = r_m - r_f$), Δr_{max} as the difference between the anisotropy of the bound protein and that of the free protein, and α as the degree of association, we have

$$\alpha = \Delta r/\Delta r_{max} \quad (6)$$

Measurement of anisotropy at high pressures is complicated by the pressure-dependent birefringence of the quartz windows. A method for correcting for this effect has been described by Paladini and Weber (1981). The correction factors involved, however, are difficult to obtain and may change as the windows age. We circumvented this by taking advantage of our ability to measure DF-VIIa anisotropy in the presence and absence

of TF. At each pressure we determined $\Delta r_{pressure}$, the difference between the anisotropy of DF-VIIa alone and that in the presence of an equimolar quantity of TF. Δr_{max} was determined from the titration of DF-VIIa with TF at atmospheric pressure. Because the absolute anisotropies of the TF:DF-VIIa complex and DF-VIIa alone are similar, their birefringence corrections will be essentially the same. In this situation, the correction to $\Delta r_{pressure}$ becomes negligible. We tested this approximation by comparing the observed maximum change in Δr for a titration at 1 atm to the maximum change in Δr obtained from fitting the pressure dissociation data.

The data are analyzed according to eq 2. The quantity ln K_d is calculated from the fraction bound at each pressure and then plotted against pressure. The best straight line through those data representing >10% dissociation is determined. The ordinate intercept of the line yields the K_d at atmospheric pressure, and its slope yields ΔV, the molar volume change for the association.

Apparatus for Atmospheric and High-Pressure Anisotropy Experiments. Fluorescence anisotropy measurements were performed on an SLM 4800 fluorometer modified in our laboratory for photon counting and computer control. The excitation monochromator bandwidth was 16 nm, and a 340-nm bandpass filter with a bandwidth of 10 nm was used to eliminate second-order light, and a polarization scrambler was used to make the vertical and horizontal intensities equal. The sample emission was detected through a 535-nm bandpass filter with a bandwidth of 10 nm. Measurements were repeated and averaged until the standard error of the anisotropy was 0.002 or smaller. Measured anisotropies were blank subtracted according to the equation

$$r_{corr} = (I_{sample}r_{sample} - I_{blank}r_{blank})/(I_{sample} - I_{blank}) \quad (7)$$

High-pressure experiments were performed in a sample chamber (Paladini & Weber, 1981) built for us by the Physics Department machine shop at the University of Illinois, Urbana.

Equilibrium Ultracentrifugation Experiments. Sedimentation equilibrium studies of the sTF:VIIa complex were performed at 23 °C in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control, RTIC temperature controller, Rayleigh interference optics, and a pulsed laser diode light source (670 nm). Data were acquired using a television camera-based, on-line data acquisition and analysis system (Laue, 1981, 1992). Samples were loaded into short column cells (0.7 mm) (Yphantis, 1960) at several concentrations up to 1 mg/mL total protein. Data were collected at invervals after the estimated equilibrium time and tested for equilibrium by subtracting successive scans (Yphantis, 1964). Blank correction was performed as described previously (Laue et al., 1984). The data from experiments at all concentrations were analyzed globally according to a model for an associating heterodimer (Luckow et al., 1989; Olsen et al., 1992) using the nonlinear least squares analysis package NONLIN (Johnson et al., 1981).

Proteins. Human recombinant VIIa, used for the dissociation studies, was a generous gift of Dr. Ulla Hedner, Novo Nordisk, Copenhagen. For activity assays, factors X and VIIa were purified from human plasma by the methods of Miletich et al. (1981) and Broze and Majeris (1980), respectively. The active site probe, dansyl-FFRcK, was prepared by reacting dansyl chloride (Sigma Chemical Co., St. Louis) with D-Phe-L-Phe-Arg chloromethyl ketone (FFRcK) (Calbiochem, La Jolla) (C. Kettner, personal communication). VIIa was active-site-labeled using 10 μM dansyl-FFRcK in 50 mM Tris buffer (pH 7.5, 0.1 M NaCl). The extent of reaction with VIIa was assessed by measurement of coagulant activity in a two-stage assay (Bach et al., 1981). The reaction was complete after 3 h. DF-VIIa was separated from reagents on a G-15 Sephadex column. Incorporation of the dansyl label was determined by absorbance spectroscopy using molar extinctions of 58 400 M^{-1} cm^{-1} at 280 nm for human VIIa and 1580 and 3940 M^{-1} cm^{-1} at 280 and 340 nm for the dansyl group.[2] The labeling ratio observed was 1 mol of dansyl to 1 mol of VIIa when VIIa was fully inhibited. An SDS gel run under reducing conditions showed that the fluorescent label was bound exclusively to the heavy chain of VIIa.

Recombinant human tissue factor (TF_{1-263}), with Cys_{245} replaced by Ser, was a generous gift of Dr. G. Vehar and Dr. D. Higgins, Genentech Inc., South San Francisco. TF_{1-263} was reconstituted into egg lecithin vesicles (Avanti, Alabaster, AL) by the procedure of Mimms et al. (1981). The lipid:protein molar ratio was 5000:1. The activity of this protein is identical with that of native TF purified from human brain [see Bach (1988)].

Cloning and Expression of sTF. The soluble extracellular domain of TF (TF_{1-218}; sTF) was obtained by recombinant DNA techniques, the details of which will be reported elsewhere. Briefly, to prepare the expression vector containing the extracellular domain of the human TF structural gene, a 2147 bp DNA insert comprising the human TF coding region was obtained from a λgt11 recombinant phage (Spicer et al., 1987). This DNA insert was transferred first into plasmid pUC19 (Messing, 1983), and then into the replicative form of phage mp 19 (Yanis-Perron et al., 1985) to provide the ssDNA for oligonucleotide-directed mutagenesis. Thus, the TF coding sequence was truncated to include only the extracellular domain. For expression and export of sTF, this DNA fragment was positioned just 3′ to a segment of DNA encoding the bacteriophage M13 gene VIII leader sequence and its corresponding ribosome binding site. This insert was placed downstream from the *ptac* promoter in a plasmid that allowed expression of sTF in BL-21(DE3) *Escherichia coli* cells after induction with isopropyl thiogalactoside. After maintaining the cells at 25 °C for 24 h, the medium (6 L) was clarified and concentrated to about 300 mL.

Purification of sTF. The concentrated media was centrifuged at 12800*g* for 1 h, and $(NH_4)_2SO_4$ was added to the supernatant to 25% saturation; the precipitate was pelleted after 1 h, and the supernatant was brought to 65% saturation. After 1 h, the resulting precipitate was pelleted and dissolved in 50 mL of 25 mM sodium acetate buffer, pH 5.2. This solution was dialyzed against the buffer (2 × 2 L; 8 h) and clarified by centrifugation. The supernatant was applied to a 75-mL S-Sepharose column, equilibrated with the buffer. The column was first washed with 250 mL of the same buffer, and then the protein was eluted with a 500-mL linear gradient of NaCl (0 to 0.5 M). sTF eluted at 160–208 mL. Following dialysis versus 25 mM Tris-Cl, pH 7.5, containing 0.2 mM EDTA (2 × 2 L, overnight), the sample was applied to a 75-mL Q–Sepharose column equilibrated with this buffer. The column was washed with 250 mL of the same buffer; the proteins were then eluted with a 500-mL linear gradient of NaCl (0–0.4 M). sTF eluted at 186–240 mL. The purity of the fractions was determined by SDS–PAGE, and the fractions

[2] The extinction of human VIIa at 280 nm was based on the number of tryptophan and trysosine residues in recombinant VIIa (Thims et al., 1988) using average extinction coefficients for tryptophan (5500 M^{-1} cm^{-1}) and tyrosine (1200 M^{-1} cm^{-1}) residues in a protein (Wetlaufer, 1962). The extinction of DF-VIIa was calculated using the extinction coefficients for ε-danyllysine in water (J. B. A. Ross, unpublished data) and our calculated value for the extinction of VIIa at 280 nm, assuming additivity of the absorptions.

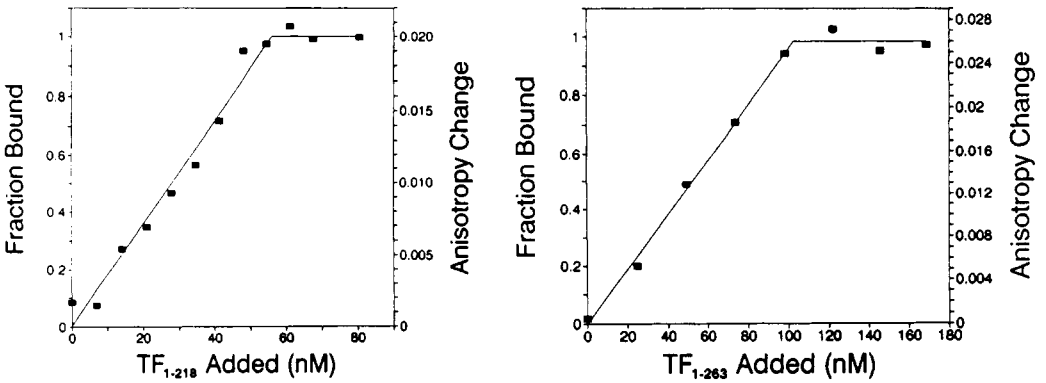

FIGURE 1: Fluorescence anisotropy titration of DF-VIIa with sTF and TF_{1-263}. See Results for conditions. The solid line in the left panel (sTF) and in the right panel (TF_{1-263}) represents the theoretical curve for a site titration. Examination of the residuals for fitting the data to a bimolecular association gave an *upper limit* of 1 nM for the K_d in each case.

containing the homogeneous protein were pooled and concentrated. The typical yield was 15 mg of sTF from 6 L of culture medium. The authenticity of the expressed protein was established by amino acid composition, by NH_2-terminal sequence analysis (10 residues), and by carboxypeptidase P digestion. The amino-terminal sequence was that expected for human TF, and the carboxyl-terminal digestion yielded exclusively Arg (residue 218) rather than the expected Glu, indicating that a single residue was removed by *E. coli* proteases. sTF concentrations were determined from the absorbance at 280 nM using an $A^{1\%}_{1cm}$ of 14.9.

Assay of TF and sTF. TF was assayed by adding an appropriate amount of TF to a solution of VIIa (10 nM), X (250 nM), and Ca^{2+} (5 mM), in 10 mM Hepes, 0.14 M NaCl, and 0.1% BSA, pH 7.5. The concentration of X was determined using an $A^{1\%}_{1cm}$ of 11.6 (Di Scipio et al., 1977). At intervals, 25-μL aliquots were withdrawn and added to 125 μL of 50 mM EDTA and 0.05% BSA, pH 7.5. Spectrozyme Xa (American Diagnostica, Greenwich, CT) was added to a final concentration 0.5 mM. The change in absorbance at 405 nm was monitored continuously for 10 min. Slopes were calculated from a least squares fit to the data. sTF was assayed in an identical manner except that the incubation mixture contained 10 μM PS/PC (30/70 w/w).

RESULTS

Comparison of the Activities of sTF and TF_{1-263}. The activities of sTF and TF_{1-263} reconstituted in PS/PC (30/70 w/w) vesicles were compared over a range of concentrations between 0.1 and 1 nM for sTF and between 0.01 and 0.1 nM for TF_{1-263}. These assays were performed in the presence of 10 nM VIIa and 250 nM X. Whereas sTF was found to be inactive in the absence of lipid, it exhibited activity in the presence of 10 μM PS/PC vesicles, corresponding to the lipid concentration when TF_{1-263} was present at 133 pM. Under these conditions, the activities were directly proportional to TF concentration and the ratio of the activity of TF_{1-263}:VIIa to that of sTF:VIIa was approximately 25:1, assuming random orientation of TF_{1-263} in the vesicles (Bach et al., 1986). To determine whether the decreased activity of sTF:VIIa results from an altered interaction of sTF with VIIa, we compared the stoichiometry and dissociation constants for the sTF:VIIa association using ultracentrifugation and fluorescence anisotropy.

Ultracentrifugation Studies. Analysis of the equilibrium ultracentrifuge data shows that sTF and VIIa have molecular weights of 27 700 and 49 000, respectively. Neither protein shows a tendency toward reversible self-association at the concentrations examined. Analysis of the data obtained from

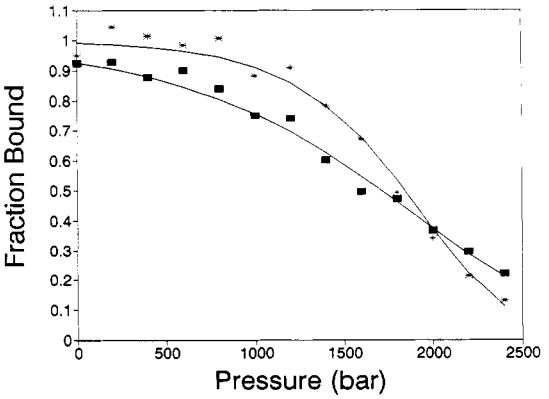

FIGURE 2: Pressure dissociation of sTF:DF-VIIa and TF_{1-263}:DF-VIIa complexes. Data for sTF:DF-VIIa (■) and TF_{1-263}:DF-VIIa (*) plotted as fraction bound versus pressure. The solid line represents the best fit theoretical curve calculated as described in Theoretical Considerations.

studying an equimolar mixture of sTF and VIIa indicates that they form a complex with a molecular weight of 73 800, indicating a one-to-one stoichiometry. Analysis of the fringe displacement data according to a heterodimeric association model yielded an upper limit of 1 nM for the K_d. Determination of the K_d to greater accuracy and precision requires a more sensitive approach.

Fluorescence Anisotropy Studies. Titration experiments at atmospheric pressure were performed by the addition of concentrated aliquots of sTF or TF_{1-263} reconstituted in PC vesicles to samples containing 60 nM DF-VIIa in 50 mM Tris buffer, pH 7.5, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.1 mg/mL ovalbumin. The sample was maintained at 25 ± 0.1 °C. The results are shown in Figure 1. The graphs for both sTF and TF_{1-263} titrations show a linear increase in anisotropy up to approximately equimolar concentrations of TF followed by a sharp break, indicative of tight binding and saturation. An upper limit of approximately 1 nM for the K_d's for both sTF and TF_{1-263} with DF-VIIa was established by nonlinear least squares fitting of these data to a bimolecular association model. The maximum change in anisotropy observed for association of TF_{1-263} and DF-VIIa was 0.027 while that observed for the association of sTF and DF-VIIa was 0.020. The most likely explanation for this difference is the dissimilarity in size of the membrane-bound and soluble complexes.

The pressure dependence of the anisotropy change of 100 nM DF-VIIa in the presence of either 100 nM sTF or TF_{1-263} is shown in Figure 2. The solid lines represent the best fits to the data. For TF_{1-263}, the free energy, ΔG, for complex formation is -15.1 ± 0.3 kcal mol^{-1}. This ΔG corresponds to

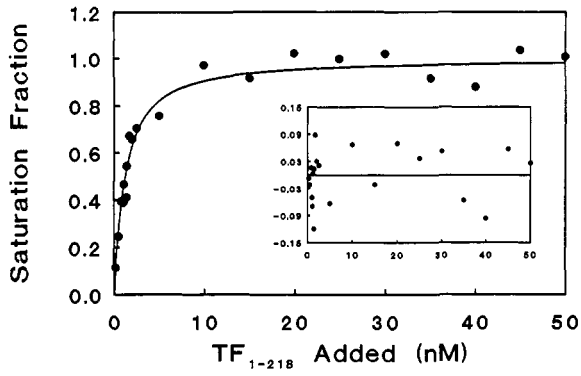

FIGURE 3: Titration of VIIa with sTF. VIIa (0.5 nM) was incubated for 15 min at 25 °C with the indicated concentrations of sTF, factor X (250 nM), and Ca^{2+} (5 mM) in 10 mM HEPES, 0.14 M NaCl, and 0.1% bovine serum albumin (BSA), pH 7.5. The reaction was initiated by the addition of 10 μM PS/PC (30/70 w/w). Aliquots (25 μL) were withdrawn every 0.5 min and added to 125 μL of 50 mM EDTA and 0.05% BSA, pH 7.5. A chromogenic substrate (Spectrozyme Xa, American Diagnostica, Greenwich, CT) was added (0.5 mM final concentration). The rate of generation of the free chromophore was monitored at 405 nm.

a K_d of 7.3 pM. The ΔV for the association is 117 ± 9 mL mol^{-1}. By contrast, for sTF, ΔG is −12.5 ± 0.1 kcal mol^{-1}, corresponding to a K_d of 0.59 nM. The ΔV for this association was 63 ± 4 mL mol^{-1}.

The maximum anisotropy change used to determine these thermodynamic parameters was obtained from the titrations of DF-VIIa carried out at 1 atm. Other values for Δr_{max} were evaluated for their ability to fit the data. For TF_{1-263}, any change in the value for Δr_{max} decreased the goodness of fit as measured by the residuals. This is strong evidence that our procedure for correcting for the birefringence-induced decrease in anisotropy as a function of pressure is correct. For sTF, Δr_{max} could not be fit for a unique value as the full dissociation curve was not achieved over a range from 1 bar to 2.4 kbar. Whereas 95% dissociation was observed for TF_{1-263} at 2.4 kbar, 80% dissociation was observed for sTF. Larger values of Δr_{max} could fit the data while smaller values resulted in statistically worse fits. However, it must be emphasized that the Δr_{max} determined by the value obtained from the titration at 1 atm is an *upper limit*. The birefringence effects depolarize the emission, driving all anisotropies toward zero. Values of Δr_{max} larger than that obtained at 1-atm pressure are therefore physically unreasonable. Thus, the pressure data can be interpreted unambiguously.

Titration of VIIa with sTF. As yet another way of estimating the association of sTF and VIIa, we measured the ability of the complex to activate factor X, making the assumption that the fractional occupancy of VIIa with sTF is proportional to the reaction velocity. Because no reaction is observed in the absence of lipids, we allowed sTF, VIIa, X, and Ca^{2+} to equilibrate for 15 min before initiating the reaction with 10 μM PS/PC (30/70 w/w) vesicles. This procedure reduced the noise in the assay. The results (Figure 3) fit by nonlinear least squares (Bevington, 1969) indicate that 0.5 nM VIIa was 50% saturated with 1.1 nM sTF. This experiment indicates that the K_d for the sTF:VIIa complex is 1 nM.

Discussion

Coagulation reactions are known to proceed much more rapidly on membrane surfaces than free in solution [see Mann et al. (1990) for a review]. The mechanism of this acceleration has not yet been fully elucidated. In this regard, we and others have found that sTF, a soluble TF construct lacking the transmembrane domain, is much less active than TF_{1-263}, the full-length membrane-spanning form. In the present study, we have compared the binding of human VIIa to sTF and to TF_{1-263} reconstituted in PC vesicles to determine whether this reduced activity can be attributed to impaired binding of VIIa to sTF. This comparison required the development of a true equilibrium technique to measure the TF:VIIa association.

Previous studies by other groups have determined dissociation constants ranging from 82 pM to 9 nM for the interaction of VIIa with TF present on a variety of human cell lines (Broze, 1982; Rodgers et al., 1984; Ploplis et al., 1987; Fair & MacDonald, 1987). Each of these studies depended on the separation of bound VIIa from free VIIa. Accordingly, none were true equilibrium measurements. Similarly, in a study of the binding of bovine VII and VIIa to bovine TF in our laboratory (Bach et al., 1986), we obtained K_d values in the nanomolar range. That study also employed a nonequilibrium technique. A study by Warn-Cramer and Bajaj (1986), using a human brain TF extract and a purely kinetic approach, yielded a K_d ranging from 110 to 460 pM. More recently, a study by Ruf et al. (1991b) measured the binding of VIIa to detergent-solubilized TF_{1-263} and to their own recombinant soluble TF using a technique in which TF was immobilized on a microtiter plate by a monoclonal antibody, previously shown to inhibit VIIa binding by 20% (Morrissey et al., 1988). This group determined dissociation constants of 4.2 nM and 90.3 nM for detergent-solubilized TF_{1-263} and the soluble TF construct, respectively. The wide range of values reported by these various studies points to the need for a study of the VIIa:TF interaction using both a well-defined TF preparation and an equilibrium binding technique capable of measuring tight associations.

We examined the interaction of sTF with VIIa using three independent approaches: (1) sedimentation equilibrium, (2) pressure-dependent fluorescence anisotropy, and (3) enzymatic titration. Pressure-dependent fluorescence anisotropy is a well-established technique for studying tight protein–protein interactions under equilibrium conditions. The three techniques yielded consistent results for sTF: a K_d of 0.6–1 nM. In addition, we examined the interaction of VIIa with full-length TF reconstituted in pure PC vesicles. The tighter association of VIIa with the full-length reconstituted TF precluded quantitation of the K_d for this interaction by any means other than pressure-dependent fluorescence anisotropy. The K_d obtained by this method was 7.3 pM, indicating a 2.6 kcal mol^{-1} difference in the ΔG of these interactions.

Under the conditions we used to measure the activity of sTF, it is clear that at least 90% of sTF is complexed with VIIa. Consequently, the observed difference in its activity compared to TF_{1-263} cannot be the result of decreased complex formation. The dissociation constants determined in this study are markedly different from the values of 90.3 and 4.2 nM reported for sTF and TF_{1-263}, respectively, in a study by Ruf et al. (1991b). If these higher values were correct, the titration experiment (Figure 3) would have shown increasing sTF:VIIa activity over all concentrations used. Instead, our data showed no increase in activity past 10 nM sTF, indicating that saturation of VIIa was achieved. We attribute these differences to differences in TF preparations and in methodology.

The 25-fold difference in activities of the sTF:VIIa and TF:VIIa complexes might conceivably be accounted for by an ordered addition of X to sTF:VIIa according to a model similar to that proposed for bovine TF:VIIa (Nemerson & Gentry, 1986). The pressure–dissociation and sedimentation equilibrium studies, however, did *not* contain X or lipid. Therefore, these experiments could not be affected by an ordered addition

reaction mechanism. Furthermore, it is unlikely that the difference in the measured dissociation constants results from an affinity of the DF-VIIa for the lipid as there is no evidence that VIIa binds to pure PC vesicles (Bach et al., 1986). The 2.6 kcal mol^{-1} difference in ΔG and the 2-fold difference in ΔV clearly indicate that the two complexes are structurally different. This lipid-dependent structural change may be common to clotting enzymes. For example, the k_{cat} for the activation of prothrombin by factor Xa increases by an order of magnitude upon addition of acidic phospholipids (Rosing et al., 1980).

In accord with Ruf et al. (1991a), we found that the sTF:VIIa complex does not generate Xa in the absence of acidic phospholipids. Interestingly, TF_{1-263} is functional in PC vesicles whereas no activity was detected with sTF in the presence of PC alone (data not shown). The mechanism by which acidic vesicles function in the sTF-dependent reaction is unknown. While it is possible that these vesicles complex with substrate to form the true substrate for sTF:VIIa, as suggested by Ruf et al. (1991a), it is also possible that sTF:VIIa forms a nonproductive ternary complex with X in the absence of lipids. In this model, interaction of the acidic phospholipid with *ternary complex* is required for substrate turnover.

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