The Location of the Active Site of Blood Coagulation Factor VIIa above the Membrane Surface and Its Reorientation upon Association with Tissue Factor

A FLUORESCENCE ENERGY TRANSFER STUDY*

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The topography of membrane-bound blood coagulation factor VIIa (fVIIa) was examined by positioning a fluorescein dye in the active site of fVIIa via a tripeptide tether to yield fluorescein-t-phenylalanyl-l-prolyl-l-arginyl-fVIIa (Fl-FPR-fVIIa). The location of the active-site probe relative to the membrane surface was determined, both in the presence and absence of tissue factor (TF), using fluorescence energy transfer between the fluorescein dye and octadecylrhodamine (OR) at the phospholipid vesicle surface. When Fl-FPR-fVIIa was titrated with phospholipid vesicles containing OR, the magnitude of OR-, calcium ion-, and phosphatidylserine-dependent fluorescence energy transfer revealed that the average distance of closest approach between fluorescein in the active site of fVIIa and OR at the vesicle surface is 82 Å assuming a random orientation of donor and acceptor dyes (σ² = 2/3; the orientational uncertainty totals ~10%). The active site of fVIIa is therefore located far above the membrane surface, and the elongated fVIIa molecule must bind at one end to the membrane and project approximately perpendicularly out of the membrane.

When Fl-FPR-fVIIa was titrated with vesicles that contained TF, the efficiency of energy transfer was increased by a TF-dependent translational and/or rotational movement of the fVIIa protease domain relative to the membrane surface. If this movement was solely translational, the height of the active site of fVIIa was lowered by an average of 6 Å after binding to TF. The association of fVIIa with TF on the membrane surface therefore causes a significant reorientation of the active site relative to the membrane surface. This cofactor-dependent realignment of the active-site groove presumably facilitates and optimizes fVIIa cleavage of its membrane-bound substrates.

Blood coagulation is triggered, in both normal hemostasis and a variety of thrombotic diseases, by activation of the extrinsic pathway, which is initiated by the interaction of factor VIIa (fVIIa) with its essential protein cofactor, tissue factor (TF) (reviewed by Davie (1)). Human VIIa is a 50-kDa protein that requires proteolytic cleavage of a single arginine–isoleucine bond in the single chain precursor to generate the active serine protease, fVIIa (2). VIIa belongs to the family of vitamin K-dependent serine proteases in which N-terminal glutamic acid residues are modified to γ-carboxyglutamic acid (Gla) residues. The resulting Gla domain is required for calcium-dependent binding of fVIIa to membrane surfaces containing anionic phospholipids (3). TF, which does not require proteolytic activation, acts as a cell-surface receptor and cofactor for fVIIa. The catalytic activity of fVIIa toward its physiological substrates, fIX and fX, is increased 57,000-fold when fVIIa is complexed with TF, calcium ions, and phospholipid (4). The TF-fVIIa complex requires a negatively charged phospholipid surface for optimal proteolytic activity (4). The activation of fIX and fX by TF-fVIIa initiates the extrinsic pathway leading to the generation of thrombin and ultimately to the formation of a fibrin clot (reviewed in Ref. 1).

The solution structures of both fVIIa and sTF, the soluble extracellular domain of TF, have been shown, using various biophysical techniques, to be asymmetric or elongated (5, 6). However, the different techniques in these studies yielded different conclusions about the solution structure of the sTF-fVIIa complex. In one study, the proteins appeared to be positioned side-by-side in the complex (6), while in the other study, the complex appeared to be substantially more elongated than either of its components (5). Crystal structures of both sTF (7, 8) and sTF-fVIIa (9) have recently been determined, and in the crystals, sTF and fVIIa are elongated and aligned side-by-side in the sTF-fVIIa complex (7–9). However, none of these approaches has been used to investigate the topography of membrane-bound TF-fVIIa. Given the requirement of phospholipids and membrane-bound substrates for physiological activity, it is important to examine the orientation of fVIIa relative to the membrane surface and the possible existence of functionally

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1 The abbreviations used are: fVIIa, factor VIIa; TF, tissue factor; sTF, soluble domain of TF, consisting of amino acids 1–219; dcTF, des-cyttoplasmic TF; Gla, γ-carboxyglutamic acid; OR, octadecylrhodamin; PC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, 1-palmitoyl-2-oleoylphosphatidylserine; PE, phosphatidylethanolamine; Fl-FPR-fVIIa, fluorescein-t-phenylalanyl-l-prolyl-l-arginyl-fVIIa; dansyl, 5-dimethylamino naphtalene-1-sulfonyl.

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Tissue Factor Alters Membrane-bound Factor VIIa Topography

important topographical and/or conformational changes that occur when intact, membrane-integrated TF binds to fVIIa.

One approach to investigating the mechanism by which a nonenzymatic cofactor stimulates enzyme activity is to position, covalently, a fluorescent reporter group in the active site of the enzyme. In so doing, information can be gained regarding two separate cofactor-dependent structural variables. Cofactor-dependent conformational changes in the active-site groove close to the probe can be detected and characterized, as well as cofactor-dependent changes in the location of the active site relative to the membrane surface. This approach has previously provided information on the structural ramifications of binding other blood coagulation cofactors to their cognate enzymes (10–13). The binding of fVα and thrombomodulin to fXa and thrombin, respectively, causes both a conformational change in the enzyme active site and a change in the location of the active site above the membrane surface (10, 12, 13). The binding of fVIIa to membrane-bound fXa does not detectably alter the location of the active site relative to the membrane surface, but does change the conformation of the active site of fXa (11). In contrast, protein S alters the location of the activated protein C active site relative to the membrane surface without detectably changing the active-site conformation near the P4 position. The same spectroscopic approach has now allowed us to detect and characterize TF-dependent alterations in fVIIa topography and in its active-site structure near P4 as well as to assess the extent to which the TF, fVα, fVIIa, thrombomodulin, and protein S cofactors utilize the same molecular mechanisms to stimulate the activities of their cognate enzymes.

This report describes the use of fluorescence energy transfer to determine the distance between donor dyes linked to the fVIIa active site and acceptor dyes located at the membrane surface and thereby to determine the orientation of the elongated fVIIa molecule when bound to the membrane. In addition, the effect of TF on the efficiency of this energy transfer has been measured, and TF-dependent changes in probe fluorescence have been examined in order to gain insight into the topographic and allosteric mechanisms by which TF enhances fVIIa proteolytic activity.

**EXPERIMENTAL PROCEDURES**

Reagents—n-Phenylalanil-1-prolyl-1-arginyl chloromethyl ketone was obtained from Calbiochem. Succinimidyl acetylthioacetate, octade- cylrhodamine (OR), and 5-(iodoacetamido)fluorescein were obtained from Molecular Probes, Inc. Dioleoylphosphatidylcholine, dioleoylphosphatidylserine, 1-palmitoyl-2-oleoylphosphatidylcholine (PC), 1-palmitoyl-2-oleoylphosphatidylserine, 1-butyryl-2-oleoylphosphatidylcholine (BC), and bovine liver phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids. Chromozym t-PA was obtained from Boehringer Mannheim. 1-Octyl-3-(4-nitrophenyl)boronic acid (40 mM in buffer A) was added to parallel OR-free and OR-containing lipid films with vigorous mixing, followed by the addition of 5 µg of dcTF to yield a final volume of 0.5 ml. Each mixture was dialyzed for a minimum of 72 h at ambient temperature against 3 × 2 liters of buffer A, with changes after 24 and 36 h. The amount of dcTF exposed on the outside of the vesicles was quantified by measuring the TF-dependent enhancement of fluorescence intensity of the fVIIa active site in a chromatographic assay (20). TF-containing vesicles were stored at 4°C and used within 1 week. The effect of OR on the ability of TF-reconstituted vesicles to promote fX activation was determined using a two-stage assay. Samples containing 10 µM fVIIa, 0.2–1.0 µM dcTF reconstituted in PC, and 0.05% (w/v) bovine serum albumin in buffer A plus 2.5 mM CaCl2 were added to equilibrate at 37°C for 10 min, and then fX was added to 1.0 µM. The activation of fX was allowed to proceed for 10 min, at which point an aliquot was removed and added to 2 volumes of 10 mM EDTA. After the synthetic fXa substrate S-2222 was added to a final concentration of 333 µM to the EDTA-quenched activation aliquots, the absorbance at 405 nm was monitored for 10 min on a Molecular Devices Vmax instrument. Equivalent rates of fXa activation were observed in samples that either contained or lacked OR (data not shown). Furthermore, the ability of TF-containing vesicles to promote fVIIa cleavage of the synthetic substrate Chromozym t-PA was not detectably different for vesicles lacking or containing OR.

**Spectral Measurements**—Steady-state fluorescence intensity and anisotropy measurements were made using the same instrumentation and procedures as described previously (11). Spectral measurements were made at 25°C in buffer A plus 2.0 mM CaCl2, using 4 × 4-mm quartz microcells. The excitation and emission wavelengths were 490 and 520 nm, respectively, with 4-nm band-pass widths. To reduce fVIIa adsorption to the walls of the microcells, each was coated for a minimum of 4 h with 400 µl sonicated PC vesicles in buffer A (13). Samples in the microcells were mixed as described (21). The quantum yield, the spectral overlap integral, and the anisotropy were determined as described previously (11).

Fluorescence lifetime measurements were performed using an ISS Model K2 multifrequency phase fluorometer. The fluorescent samples were excited with the 488-nm line of a Coherent Innova 400-15/3-15-15-watt argon-ion laser. Fluorescence emission was collected through a CVI 515-mm cutoff filter. The samples were corrected for background contribution as described by Reinhart et al. (22). Phase and modulation data were analyzed using GLOBALS UNLIMITED from the University of Illinois, and the time-resolved data were fit best (lowest χ²) by a single exponential decay.

**Energy Transfer Measurements**—Energy transfer experiments were performed as described (11) with samples containing 10 mM FI-FFR-

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fluorescein or unmodified fVIIa. At the end of a titration, an excess of EDTA (3 mM more than the CaCl₂ concentration) was added to each sample to chelate the calcium and to eliminate the calcium-dependent binding of Fl-FPR-fVIIa to the PC/PS surface in the samples. The spectral measurements were then repeated to determine what fraction of the acceptor-dependent reduction in donor emission intensity was due to Fl-FPR-fVIIa binding to the membrane. In most experiments, a second addition of EDTA increased its concentration by 50%, and another spectral measurement was made to ensure that all of the labeled fVIIa had dissociated from the vesicles. The normalized Q̄D/Q̄DA value used to calculate L was obtained by dividing the Q̄D/Q̄DA value before EDTA addition by the Q̄D/Q̄DA value after EDTA addition. The phospholipid dependence of the resonance energy transfer was examined by titrating samples with vesicles (≥ OR) composed of 100% PC or PC/PS/PE rather than PC/PS.

To determine the cofactor dependence of the resonance energy transfer, dTF was reconstituted into PC/PS or 100% PC vesicles and used as the titrant. The magnitude of the membrane binding-dependent energy transfer when Fl-FPR-fVIIa was bound to dTF in phospholipid vesicles was determined by adding an excess of EDTA to each sample and calculating the normalized Q̄D/Q̄DA value as described above.

**RESULTS**

**Fluorescent Labeling of the Active Site of fVIIa**—A fluorescein derivative of fVIIa was prepared for this study because fluorescein has a much greater spectroscopic sensitivity than dansyl, thereby allowing lower concentrations of fVIIa and TF to be used in our experiments. Human fVIIa was inactivated with an active site-directed reagent, N-acetylthioacetylthiofumarate, followed by reaction with 5-(iodoacetamido)fluorescein to yield, after purification, Fl-FPR-fVIIa. This derivative of fVIIa has one end of the FPR tripeptide covalently attached to the active-site histidine and serine (9) and a fluorescein dye attached to the other end of the tripeptide tether. Although >99% of the fVIIa molecules reacted covalently with the FPR reagent, the number of dyes per protein molecule in our Fl-FPR-fVIIa preparations was typically 0.8 when calculated as described by Bock (17), presumably because the reaction with the iodoacetamido fluorescein was incomplete. For the experiments discussed in this paper, the presence of non-fluorescein-labeled fVIIa molecules in a sample does not interfere with the interpretation of the spectroscopic data.

**Spectral Properties of Fl-FPR-fVIIa**—The wavelength of maximum emission (corrected) and the average values for the quantum yield (Q̄), steady-state anisotropy (τ), and fluorescence lifetime of the fluorescein dye in Fl-FPR-fVIIa were found to be 520 nm, 0.40, 0.21, and 4.2 ns, respectively, in buffer A plus 2 mM CaCl₂. The presence or absence of either PC/PS or PC/PS/PE vesicles had no significant effect on any of the spectral properties of Fl-FPR-fVIIa (− OR data in Fig. 1 and data not shown). Thus, the binding of Fl-FVIIa to a membrane surface elicited no detectable alteration in the environment of the fluorescein dye, which is presumed to be located near P₂ in the Fl-FPR-fVIIa active-site groove.

**Fluorescence Energy Transfer: Active Site to Membrane Surface**—The fluorescein covalently attached to the active-site histidine of Fl-FPR-fVIIa was used as the donor dye in our fluorescence energy transfer experiments, while the rhodamine in OR was the acceptor dye. OR possesses a long octadecyl hydrocarbon chain that partitions into the nonpolar core of a phospholipid bilayer in aqueous solution, thereby anchoring the rhodamine moiety at the phospholipid surface because the dye is charged at pH 7.5 and remains in the aqueous phase (23).

When Fl-FPR-fVIIa was titrated with PC/PS vesicles (the donor-containing D sample), there was no change in fluorescein emission intensity (Fig. 1). However, when Fl-FPR-fVIIa was titrated with PC/PS vesicles containing OR (the donor- and acceptor-containing DA sample), the fluorescein intensity decreased until sufficient vesicles were present to bind all of the protein (Fig. 1). The phospholipid concentration dependence of the DA spectral change indicates that human fVIIa binds to PC/PS vesicles with greater affinity than does bovine fVIIa (3). The association of all of the Fl-FPR-fVIIa molecules in our sample with vesicles was confirmed by gel filtration (see below).

The OR-dependent decrease in fluorescein intensity results from singlet-singlet energy transfer from the donor dyes to the acceptor dyes in the DA sample.

If the decrease in fluorescein intensity (Fig. 1) was due solely to energy transfer that occurs when Fl-FPR-fVIIa binds to the OR-containing membrane, then the dissociation of Fl-FPR-fVIIa from the membrane surface should eliminate the OR-dependent spectral change because the average separation between free Fl-FPR-fVIIa molecules and vesicle-bound OR would then be too large for significant energy transfer to occur. Since fVIIa binding to membranes is calcium-dependent (3), excess EDTA was added to the samples at the end of the titration to chelate the calcium ions and to dissociate fVIIa from the PC/PS surface. Although this released Fl-FPR-fVIIa from the vesicle surface (see gel filtration data below), the dissociation of Fl-FPR-fVIIa from the surface did not return the donor intensity to its original value in the DA sample. This is shown in Fig. 2, where the data of Fig. 1 were normalized and expressed as the ratio of the donor quantum yields in the presence and absence of acceptor in order to facilitate analysis. Instead of the Q̄D/Q̄R of 1.0 expected in the absence of energy transfer, the Q̄D/Q̄R was only 0.91 after Fl-FPR-fVIIa was released from the vesicle surface (Fig. 2). Thus, a portion of the observed reduction in donor intensity in the DA sample (Fig. 1) was not due to membrane binding-dependent energy transfer, but rather to some combination of an OR-dependent inner filter
The presence and absence of the acceptor (OR) in PC/PS or PC vesicles, respectively. The ratio of the donor quantum yields in the PC/PS or PC vesicles that contained or lacked the OR dye (DA and D, respectively). The ratio of the donor quantum yields in the presence and absence of the acceptor ($Q_{DA}/Q_D$) was determined for the PC (●) and PC/PS (○) titrations. Acceptor densities were $1.9 \times 10^{-4}$ OR molecules/Å$^2$ in this PC/PS experiment and $2.0 \times 10^{-4}$ OR molecules/Å$^2$ in this PC experiment. At the end of each titration, EDTA was added to 5 mM, and the resultant $Q_{DA}/Q_D$ values were shown (●, PC; ○, PC/PS).

The existence of a distance-dependent decrease in donor intensity that is not related to energy transfer between membrane-bound donor and acceptor dyes was confirmed by titrating with OR at 100% PC (●) vesicles. Since PC vesicles lack the negatively charged phospholipids required to bind vitamin K-dependent proteins (24), any reduction in the observed $Q_{DA}/Q_D$ value would have to result from something other than Gla-dependent energy transfer between membrane-bound FL-FPR-FVIIa and OR. When FL-FPR-FVIIa was titrated with PC vesicles in the presence or absence of OR, the $Q_{DA}/Q_D$ value decreased steadily, and the $Q_{DA}/Q_D$ value of 0.92 obtained at 307 μM PC was similar to the residual $Q_{DA}/Q_D$ value of 0.91 observed after PC/PS samples with a similar OR surface density (σ) were diluted with excess EDTA to 304 μM (Fig. 2).

Two independent approaches therefore reveal that part of the observed reduction in $Q_{DA}/Q_D$ occurs even when FL-FPR-FVIIa does not bind to the membrane surface. This membrane binding-independent reduction was caused primarily by an inner filter effect because the addition of 170 μM PC vesicles containing OR at σ = $2.2 \times 10^{-4}$ OR molecules/Å$^2$ to a solution of 5 mM disodium fluorescein reduced its emission intensity by 6% (data not shown), an amount similar to that observed in the experiments of Figs. 1 and 2 at the same phospholipid concentration. A similar reduction in $Q_{DA}/Q_D$ was also observed in our previous studies (10, 11, 19, 25), although the magnitude of the effect was much smaller in those cases because less phospholipid (and hence OR) was required to bind those proteins to vesicles.

The extent of energy transfer from membrane-bound FL-FPR-FVIIa to OR at the vesicle surface was therefore given by the EDTA-reversible portion of the decrease in $Q_{DA}/Q_D$ observed at the end of the titration (Fig. 2). The EDTA-dependent increase in $Q_{DA}/Q_D$ is equivalent to the decrease in donor intensity that is dependent upon FL-FPR-FVIIa binding to PC/PS vesicles that contain OR.

Distance of Closest Approach of Active-site Probe to Membrane Surface—The spectral overlap integral ($J_{DA}$) was $3.47 \times 10^{15}$ M$^{-1}$ cm$^{-1}$ nm$^4$ for the fluorescein-rhodamine donor-acceptor pair. If the dyes are totally randomized in orientation during the lifetime of the excited state of the donor dye, then $\kappa^2$, the geometric factor that depends on the relative orientation of the donor and acceptor transition dipoles, is equal to 2/3. Assuming that $\kappa^2 = 2/3$ and that the index of refraction (n) = 1.4, the value of $R_o$, the distance between donor and acceptor dyes at which singlet-singlet energy transfer is 50% efficient, was calculated to be 53.6 Å. $L$, the distance of closest approach between the fluorescein dye in the active site of membrane-bound FVIIa and rhodamine dyes localized at the phospholipid surface, was calculated using data such as those shown in Fig. 2 and Equation 1 (26).

$$Q_D/Q_{DA} = 1 + (\pi\sigma R_o^2)(R/L)^4$$

This approach is justified here because $L > 1.5 R_o$ (27), as was true in our previous coagulation protein studies (10–12, 19, 25).

Since the magnitude of the observed energy transfer depends upon σ, the density of the OR molecules on the surface of the vesicles, it is important to measure the energy transfer at different σ values. Fig. 3 (open circles) shows the results of 19 different FL-FPR-FVIIa to PC/PS-OR energy transfer experiments, where the net $Q_D/Q_{DA}$ value is plotted as a function of the product between $R_o$ and the acceptor density. As expected (26), a linear plot fit the data well, and the combined data yielded an average value of 82 ± 3 Å for $L$ (Table I), where the uncertainty shown represents the standard deviation for the 19 separate measurements. Although PE probably increases the affinity of the membrane surface for FVIIa (15), the presence of PE in the vesicle did not alter the measured value of $L$ and hence the orientation of membrane-bound FVIIa (Table I).

The calculation of $L$ assumes that the acceptor dyes are randomly and uniformly distributed on the membrane surface. Since it is conceivable that OR at the membrane surface associates with FVIIa, thereby increasing the local density of OR, an excess of unmodified FVIIa was added to a sample of PC/PS-OR vesicles to determine whether binding of OR to FVIIa could be detected by a change in either the absorbance or the fluorescence emission of OR. No spectral changes were observed, which indicates that the acceptor dyes do not associate with membrane-bound FVIIa. Furthermore, the presence of OR in
PC/PS vesicles had no effect on the TF-dependent activation of FX by fVIIa, as detected by a two-stage activity assay, or on TF-dependent enhancement of fVIIa hydrolysis of the chromogenic substrate Chromozym-t-PA (data not shown). Finally, the uniform distribution of OR in the vesicles is confirmed by the linear dependence of $Q_{DA}/Q_O$ on $\sigma$ in our experiments (Fig. 3). Any non-uniform distribution of OR, such as a concentration of OR near fVIIa, would be revealed by a nonlinear dependence of $Q_{DA}/Q_O$ on $\sigma$ in our experiments. We therefore conclude that the OR dyes were distributed uniformly and randomly on the phospholipid surface in our experiments.

To determine whether every Fl-FPR-fVIIa molecule binds to a PC/PS vesicle in our samples, we examined Fl-FPR-fVIIa-PC/PS samples by gel filtration. Only one fluorescent peak eluted from the column near the void volume with the vesicles (Fig. 4A). In contrast, very little Fl-FPR-fVIIa eluted with the vesicles when an excess of EDTA was added to a separate aliquot of the same sample (Fig. 4B). These results indicate that all Fl-FPR-fVIIa molecules were bound to PC/PS in our samples in the presence of Ca$^{2+}$ ions. The slight retardation of the fluorescent peak relative to the radioactive peak in Fig. 4A results from Fl-FPR-fVIIa dissociation from PC/PS during the chromatography. Complete separation of Fl-FPR-fVIIa from PC/PS was not observed because the dissociated fVIIa was more likely to rebind to another PC/PS vesicle than to enter a pore of a gel filtration resin bead at the high concentrations of PC/PS used in our fluorescence experiments. Consistent with this interpretation, $\sim 60\%$ of the Fl-FPR-fVIIa eluted as free protein in a single peak when the experiment was repeated using 5-fold less PC/PS (data not shown). We conclude that all of the Fl-FPR-fVIIa molecules bound to the PC/PS surface in our experiments.

The values for $L$ (Table I) were calculated assuming that the $\kappa^2$ orientation factor equals 2/3 and hence that the transition dipoles of the donor and acceptor dyes are oriented randomly during the lifetime of the donor excited state. Since the fluorescein dyes in the active site of fVIIa and the rhodamine dyes at the membrane surface do not rotate with complete freedom, there is uncertainty in the relative orientation of the donor and acceptor transition dipoles during the excited state lifetime of the donor dye. From the measured steady-state anisotropies of the fluorescein in Fl-FPR-fVIIa ($r = 0.21$) and the membrane-bound rhodamine dyes in PC/PS-OR ($r = 0.22$) (10), the maximum uncertainty in $R_\circ$ due to orientation effects ($\kappa^2$) is calculated (28) to range from $-26\%$ to $+28\%$ of the $R_\circ$ ($\kappa^2 = 2/3$) for Fl-FPR-fVIIa and OR bound to PC/PS vesicles. However, a more reasonable estimate of the uncertainty in $R_\circ$ is $\pm 10\%$ (23, 29, 30), both because the OR acceptors are oriented randomly in the plane of the membrane (29, 30) and because the separation between the donor and acceptor dyes is significantly greater than $R_\circ$ (31). Thus, although the uncertainty in $\kappa^2$ prevents us from determining $L$ to two significant figures, the energy transfer data clearly demonstrate that the active site of membrane-bound fVIIa is located far above the phospholipid surface. Thus, one end (the Gla domain) of the elongated fVIIa molecule (9) binds to the membrane, while the other end contains the active site and projects away from the surface.

**Cofactor Dependence of Fl-FPR-fVIIa Fluorescence**—The sensitivity of probe emission to cofactor was examined by titrating Fl-FPR-fVIIa with dcTF reconstituted into either 100% PC or PC/PS vesicles. This membrane-anchored form of TF contains both the extracellular and transmembrane domains of TF, but lacks most of the cytoplasmic C terminus. The cytoplasmic domain has previously been shown to be unimportant for TF activity (32). When an excess of dcTF in PC (dcTF/PC) was added to a sample of Fl-FPR-fVIIa, the fluorescein intensity did not change significantly (−OR data in Fig. 5), its lifetime did not change (data not shown), and its anisotropy decreased only from 0.21 to 0.20 (data not shown). Similar results were obtained when an excess of dcTF in PC/PS vesicles (dcTF-PC/PS) was added to samples of Fl-FPR-fVIIa. Since the binding of
FI-FPR-fVIIa to membrane-anchored TF elicited little change in fluorescein emission, it appears that the active-site conformation near $P_3$ is altered only slightly, if at all, by the association of TF with fVIIa. This enzyme-cofactor couple therefore differs markedly in this respect from others involved in hemostasis (see “Discussion”).

Cofactor Dependence of $L$—To determine whether the height and/or orientation of the fVIIa active site above the membrane surface is altered when fVIIa binds to TF, FI-FPR-fVIIa was titrated with dcTF-PC vesicles that either contained or lacked OR. As shown in Fig. 5, there was an OR-dependent decrease in fluorescein intensity. At the end of the titration, FI-FPR-fVIIa was released from the vesicle surface by the addition of an excess of EDTA, and this eliminated the energy transfer (Fig. 6). As before, the OR inner filter effect caused the $Q_{DA}/Q_D$ value to be <1.0 after fVIIa was released from the membrane surface. Equivalent results were obtained with dcTF-PC/PS and dcTF-PC/PS/PE (data not shown).

It is important to note that the energy transfer depicted in Fig. 6 was observed with PC vesicles because they also contained TF. As shown in Fig. 2, FI-FPR-fVIIa does not bind to PC vesicles, and hence, no EDTA-sensitive energy transfer is observed. Thus, the energy transfer shown in Fig. 6 occurs only because FI-FPR-fVIIa is binding tightly to dcTF, not because FI-FPR-fVIIa is binding directly to the vesicle surface. Furthermore, the elimination of this energy transfer by EDTA addition shows that fVIIa association with TF at these concentrations is calcium ion-dependent, consistent with the results of previous studies (33–35).

As expected for a titration of two proteins that bind very tightly to each other, the $Q_{DA}/Q_D$ value decreased almost linearly as the TF concentration increased until all of the enzyme had bound to TF on the phospholipid surface and the sample contained equimolar amounts of fVIIa and surface-exposed dcTF (Fig. 6). Further addition of dcTF-PC then caused a small decrease in $Q_{DA}/Q_D$ because of the inner filter effect (Fig. 6). The magnitude of the membrane binding-dependent (EDTA-dependent) energy transfer was used to calculate $L$ using Equation 1. Since the fluorescein intensity and lifetime were not altered when FI-FPR-fVIIa bound to dcTF-PC or dcTF-PC/PS, the quantum yield of the fluorescein was unchanged by association with the cofactor. Similarly, $J_{DA}$ was unaltered by the presence of TF. Although FI-FPR-fVIIa association with TF may have altered the $x^2$ value, we initially assumed that $x^2 = 2/3$. Thus, using the same $R_o$ value that was used in the −TF experiments, the distance of closest approach between the active-site probe and the membrane surface was calculated to be $76 \pm 2 \, \text{Å}$ (again, the uncertainty shown is the standard deviation for the averaged value of 16 total determinations). The same value for $L$ was obtained when FI-FPR-fVIIa was titrated with dcTF-PC, dcTF-PC/PS, or dcTF-PC/PS/PE (Table I), so the phospholipid composition does not affect the topography of the membrane-bound TF-fVIIa complex. The active-site probe in membrane-bound fVIIa therefore changes its location when fVIIa binds to TF. This is shown by the increased efficiency of resonance energy transfer that results from a TF-dependent reorientation, either translational and/or rotational, of the active-site groove.

To ascertain whether every FI-FPR-fVIIa molecule could bind to dcTF in our samples, gel filtration was used to quantify the fraction of FI-FPR-fVIIa that eluted with dcTF-PC vesicles in the void volume. As before, all fluorescein-labeled material eluted with dcTF-PC vesicles near the void volume, while no FI-FPR-fVIIa eluted in the void volume in samples that contained PC and lacked dcTF (data not shown). Thus, all of the FI-FPR-fVIIa was able to form an enzyme-cofactor complex with dcTF-PC.

The effect of TF association on the active-site location of fVIIa is shown in Fig. 3, where the results of 14 different titrations of FI-FPR-fVIIa with dcTF-PC ($n = 8$) or dcTF-PC/PS ($n = 6$) vesicles containing different amounts of OR are compiled. The depiction of the energy transfer results in Fig. 3 clearly demonstrates the marked effect that TF has on the efficiency of energy transfer between active-site probes and acceptors at the surface of the membrane. Although small, the difference in the mean $Q_{DA}/Q_D$ values obtained in the absence and presence of TF is significant at the 99% confidence level (unpaired $t$ test, $p < 0.01$). The observed change in energy transfer is not due to any effect of the transmembrane domain of TF on $\sigma$ because the small OR-excluded area occupied by TF would decrease, not increase, the efficiency of energy transfer.

The change in energy transfer efficiency caused by TF could
result from a change in $L$ and/or a change in $R_o$. Any change in $R_o$ would arise from a change in $\kappa^2$ since $Q_D$ and $J_{DA}$ are the same in the presence and absence of TF. Changes in $\kappa^2$ could originate either from a rotation of the entire active-site groove of the protease domain and/or from a change in the local environment of the dye. If the latter were the case, then the anisotropy of the dye would change. Since we observed only a very small change in the anisotropy ($\Delta r = 0.01$) of the fVIIa dye upon binding to TF, we conclude that any changes in orientation of the donor dipole would have to result from the rotational movement of the entire active-site groove relative to the membrane surface rather than from a local perturbation of dye environment. Alternatively, the TF-dependent increase in energy transfer could result solely from a decrease in the separation between the donor and acceptor dyes. The magnitude of such a movement can be determined by calculating $L$ and assuming that $\kappa^2 = 2/3$ both in the presence and absence of TF (i.e., assuming that the relative orientation of the donor and acceptor dyes does not change when TF binds to fVIIa). If this assumption is correct, then the binding of TF to fVIIa moves its active site 6 Å closer to the membrane surface (Table 1). Finally, $Q_{DA}/Q_D$ could have been altered by a combination of rotational and translational movements of the active-site groove when Fl-FPR-fVIIa associated with TF on the membrane surface.

**DISCUSSION**

Three primary conclusions can be drawn from the data presented here. First, we have determined that the active site of membrane-bound fVIIa is located far above the membrane surface. Second, we have demonstrated that association of fVIIa with TF on the membrane surface alters the location of the fVIIa active site relative to the membrane. Third, we have shown that binding of TF to fVIIa does not detectably alter its active-site conformation near $P_4$, in contrast to what has been observed with other cofactor-enzyme complexes (10, 11, 13).

The fluorescence energy transfer measurements show that the active-site groove of membrane-bound fVIIa is positioned high above the membrane surface. The Fl-FPR-fVIIa to OR energy transfer data yielded an average value of 82 Å ($\kappa^2 = 2/3$) for the distance of closest approach between fluorescein dyes in the active site of fVIIa and rhodamine dyes at the surface of the phospholipid bilayer. Even considering the orientational uncertainty in this measured value ($\pm 10\%$; see “Results”), the large distance reveals that the elongated fVIIa molecule must bind to the membrane surface at one end, as originally suggested for the homologous prothrombin and IXa molecules by Lim et al. (36). Furthermore, the energy transfer results show that the protease domain is located farthest from the membrane when fVIIa is bound to the bilayer, that the elongated protein must project from the surface in order to position its active site ~80 Å above the membrane, and that membrane-bound fVIIa forms a stable extended tertiary conformation in the absence of TF (Table 1). Finally, the topography of membrane-bound fVIIa is not detectably different whether or not PE is present in the phospholipid bilayer.

The above structural conclusions, based on a fluorescence examination of membrane-bound fVIIa, are consistent with the recent crystal structure data obtained in the absence of phospholipid. In particular, the sTF-fVIIa complex in the crystal has an overall length of ~115 Å, with the protease domain of fVIIa located distal to the Gla domain that interacts with the bilayer (9).

The topography of membrane-bound fVIIa is very similar to that of the other vitamin K-dependent enzymes. In each case, the fluorescence energy transfer results show that the active site is located far above the membrane surface, necessitating an approximately perpendicular orientation of the elongated protein with respect to the membrane. Factor Xa (10), the prothrombin derivative meizothrombin (19), factor IXa (11), activated protein C (2) and thrombin bound to thrombomodulin (12) each have active sites located >60 Å above the phospholipid bilayer. In particular, energy transfer measurements between fluorescein in the enzyme active site and OR at the membrane surface yielded average distances of 84 Å ($\kappa^2 = 2/3$) for fXa and 89 Å ($\kappa^2 = 2/3$) for IXa (11). The very similar domain structures identified by primary sequence analysis therefore translate to very similar membrane-bound tertiary structures for the vitamin K-dependent enzymes.

We also show here that the binding of TF to fVIIa alters the location of the fVIIa active site. The observed TF-dependent increase in the efficiency of energy transfer could result either from a translational or a rotational movement of the fVIIa protease domain or from some combination of these. If the reorientation was caused solely by a translational movement of the protease domain, the active site of fVIIa is lowered by 6 Å upon binding to TF. Whatever the case, it is clear that the active site in the TF-fVIIa complex has a unique location above the membrane that differs from that of fVIIa. Since the substrates of the TF-fVIIa complex are also bound to the membrane, the enzyme active site and the substrate cleavage site must be aligned if activation is to occur at an optimum rate. Thus, it is reasonable to conclude that the TF-dependent positioning of the fVIIa protease domain is functionally significant.

In the recently solved crystal structure of the complex of fVIIa with proteolytically digested sTF (9), the tripeptide active-site inhibitor was found to be oriented parallel to the portion of the fVIIa Gla domain thought to interact with the membrane. The distance between the tripeptide in the active site and the putative membrane surface on the Gla domain of fVIIa was estimated to be ~80 Å (9). The energy transfer and crystallography data are therefore in excellent agreement. Assuming that the solution and crystal structures of fVIIa and sTF are very similar, then the elongated TF-fVIIa complex is oriented approximately perpendicularly when bound to the membrane.

A cofactor-dependent movement of an active-site groove has also been observed previously with other blood coagulation proteins. For example, fVIIIa increases the height of the active site of fXa above the membrane by 8 Å (assuming the movement is exclusively translational) (10). Factor Va also reorients the protease domain of its prothrombin substrate (10). Similarly, protein S has been observed to alter the location of the active site of activated protein C relative to the membrane surface. In addition, the binding of thrombin to thrombomodulin fixes the active site of the otherwise soluble enzyme at a specific height above the membrane surface (12). In contrast, no significant alteration in energy transfer efficiency was observed when fVIIIa bound to fIXa, suggesting that in this instance either the cofactor does not significantly reorient the active site of the enzyme or that the combined rotational and translational movements yield no net change in energy transfer (11). Since the cofactor dictates the active-site location in four of the five membrane-bound coagulant and anticoagulant enzyme complexes examined to date, it seems likely that cofactor-dependent changes in enzyme topography constitute one mechanism for regulating activation rates.

Since there is almost no change in fluorescein emission when Fl-FPR-fVIIa binds to TF, very little alteration in active-site structure near $P_4$ occurs upon fVIIa association with its cofactor. These data contrast sharply with the results obtained with other coagulation proteases labeled near $P_4$ via tripeptide linkages. For example, fluorescent probes in the active sites of IXa...
(10), thrombin (13), and fIXa (11, 37) were each found to exhibit spectral changes upon binding to fVIIa, thrombomodulin, and fVIIIa, respectively, indicating that conformational changes are elicited near P4 in the active sites of these enzymes when they associate with their cofactors. Thus, the allosteric linkages between cofactor-binding sites and various active-site locations differ for different enzyme-cofactor complexes. For example, the binding of thrombomodulin to thrombin elicits conformational changes in its active site near both P4 and P1, although only the change at P4 correlates with a change in substrate specificity (13). fVIIa is allosterically linked to the P4-binding site in the active sites of fXa and fIXa, respectively, indicating that conformational changes upon binding to fVa, thrombomodulin, and TF or sTF (38–43) are altered substantially by association with TF or sTF (38–43). Thus, cofactor-dependent conformational changes near P1 in the active-site groove have so far been observed only with the largest of the coagulation cofactors (fVIIIa, fIXa, and thrombomodulin).

We have shown here that TF alters the active-site location of fVIIa above the membrane. Since the cofactor dictates the positioning of the enzyme active site in four out of the five coagulation complexes examined as well as the location of one substrate protease domain (19), it is clear that cofactor alteration of enzyme (and probably substrate) topography is a general phenomenon. These results strongly suggest that one mechanism by which cofactors regulate the activity of their cognate enzymes is to properly align enzyme active sites and substrate cleavage sites above the membrane surface, the plane of reference that is common for these enzymes, substrates, and cofactors.

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