FRET studies with Factor X mutants provide insight into the topography of the membrane-bound Factor X/Xa

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FRET (fluorescence resonance energy transfer) studies have shown that the vitamin K-dependent coagulation proteases bind to membrane surfaces perpendicularly, positioning their active sites above the membrane surfaces. To investigate whether EGF (epidermal growth factor) domains of these proteases play a spacer function in this model of the membrane interaction, we used FRET to measure the distance between the donor fluorescein dye in the active sites of FI–FPR (fluorescein–D-Phe-Pro-Arg-chloromethane)-inhibited fXa (activated Factor Xa) and its N-terminal EGF deletion mutant (fXa-desEGF1), and the acceptor OR (octadecylrhodamine) dye incorporated into phospholipid vesicles composed of 80% phosphatidylcholine and 20% phosphatidylserine. The average distance of closest approach (L) between fluorescein in the active site and OR at the vesicle surface was determined to be $56 \pm 1 \text{ Å}$ ($1 \text{ Å} = 0.1 \text{ nm}$) and $63 \pm 1 \text{ Å}$ for fXa-desEGF1 compared with $72 \pm 2 \text{ Å}$ and $75 \pm 1 \text{ Å}$ for fXa, in

the absence and presence of fVa (activated Factor V) respectively, assuming $\kappa^2 = 2/3$. In comparison, an L value of $95 \pm 6 \text{ Å}$ was obtained for a S195C mutant of fXa in the absence of fVa in which fluorescein was attached directly to Cys¹⁹⁵ of fXa. These results suggest that (i) EGF1 plays a spacer function in holding the active site of fXa above the membrane surface, (ii) the average distance between fluorescein attached to FI–FPR in the active site of fXa and OR at the vesicle surface may not reflect the actual distance of the active-site residue relative to the membrane surface, and (iii) fVa alters the orientation and/or the height of residue 195 above the membrane surface.

Key words: γ -carboxyglutamic acid, epidermal growth factor (EGF), Factor Xa, fluorescence resonance energy transfer (FRET), prothrombinase, vitamin K.

INTRODUCTION

fX (Factor X) is a vitamin K-dependent coagulation protease zymogen that, upon activation to fXa (activated fX) forms a complex with fVa (activated Factor V) on the negatively charged membrane surfaces in the presence of Ca^{2+} (prothrombinase) to convert prothrombin into thrombin at the final step of the blood-clotting cascade [1–5]. The complex formation improves the catalytic efficiency of fXa by more than five orders of magnitude by a not completely understood mechanism [1,6,7]. Similarly to other vitamin K-dependent coagulation proteases, the structure of fXa from its N-terminal end includes a Gla (γ -carboxyglutamic acid) domain, two EGF (epidermal growth factor)-like domains and a trypsin-like protease domain that is located at the C-terminal end of the molecule [3]. The dramatic enhancement in the catalytic activity of fXa in the prothrombinase complex appears to arise from an approx. two orders of magnitude improvement in the K_m of prothrombin activation due to the Ca^{2+} -dependent interaction of the protease and the substrate via their Gla domains on the same negatively charged membrane surfaces [1,7]. The remaining three orders of magnitude improvement in the catalytic efficiency of fXa is attributed to the cofactor function of fVa which improves the k_{cat} of the activation reaction by interacting with the protease and/or the substrate on the membrane surface [1,2,7].

The function of EGF-like domains in fXa is less clear, although it is known that the N-terminal EGF domain (EGF1) has a Ca^{2+} -binding site and that the occupancy of this site by the metal ion is

required for the catalytic function of the enzyme [8]. A structural role for the EGF1 domain can be inferred from the observation that the isolated EGF1 domain and Gla domain bind Ca^{2+} with at least 10-fold lower affinity than when they are covalently linked, suggesting that the domain–domain interaction is required for the proper folding and function of fXa on the membrane surface [9,10]. Recent results have excluded any interaction between either Gla or EGF1 of fXa with fVa in the prothrombinase complex [11,12]. However, the EGF1 domain of fXa may serve an essential spacer function between the Gla domain and the serine protease domain to maintain the active site at an appropriate height above the membrane surface in order to cleave the membrane-bound substrate [12]. In support of this hypothesis, light-scattering studies have demonstrated that the interaction of fXa and prothrombin with membrane surfaces via their Gla domains extends both molecules radially out of the membrane surface [13]. Further support for an elongated conformation for fXa on the membrane surface is provided by FRET (fluorescence resonance energy transfer) studies which have demonstrated that the binding of fXa to a negatively charged membrane surface via its Gla domain maintains the height of the active site of the protease $\sim 61 \text{ Å}$ ($1 \text{ Å} = 0.1 \text{ nm}$) above the membrane surface in the absence of fVa and $\sim 69 \text{ Å}$ above the membrane surface in the presence of fVa in the prothrombinase complex [14]. On the basis of these results, it has been hypothesized that a cofactor function for fVa may involve changing the distance and/or topographical orientation of the active site of fXa in the activation complex [14].

Abbreviations used: EGF, epidermal growth factor; FI, fluorescein; FPR, D-Phe-Pro-Arg-chloromethane; FRET, fluorescence resonance energy transfer; fX, Factor X; fXa, activated Factor X; fVa, activated Factor V; fX-desEGF1, fX with the N-terminal EGF domain deleted; fX-Cys¹⁹⁵, fX with a S195C mutation; fXa-Cys¹⁹⁵, fXa with a S195C mutation; Gla, γ -carboxyglutamic acid; OR, octadecylrhodamine; PC, dioleoylphosphatidylcholine; PS, dioleoylphosphatidylserine; PC/PS vesicles, phospholipid vesicles containing 80% PC and 20% PS; RVV-X, fX activator from Russell's viper venom; SpFXa, Spectrozyme FXa.

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The extent to which this mechanism of fVa cofactor function contributes to the catalytic efficiency of fXa in prothrombinase is not known.

To investigate a spacer function for EGF1 of fXa, in the present study, we prepared a recombinant fXa mutant in which EGF1 of the protease was deleted (fXa-desEGF1). We labelled the active sites of both this mutant and wild-type fXa with the FI (fluorescein)-labelled tripeptide inhibitor FPR (D-Phe-Pro-Arg-chloromethane) and determined the distances of closest approach between the donor FI in the active sites of these proteases and the acceptor OR (octadecylrhodamine) dye incorporated into phospholipid vesicles composed of 80 % PC (dioleoylphosphatidylcholine) and 20 % PS (dioleoylphosphatidylserine) (PC/PS vesicles) in both the presence and absence of fVa using singlet–singlet energy transfer equations as described previously [14,15]. Noting that all previous FRET studies have utilized this or a similar tripeptidyl-tethered labelling approach to investigate the topography of membrane-bound coagulation enzymes [14–21], we also investigated whether or not the distance between the FPR-tethered FI dye in the active site of fXa and the OR dye at the surface of PC/PS vesicles reflects the actual distance of the active site relative to the membrane surface. Thus we expressed a mutant of fX in which a cysteine residue was substituted for the catalytic residue Ser¹⁹⁵ (chymotrypsinogen numbering [22]) (fX-Cys¹⁹⁵). Following the direct labelling of Cys¹⁹⁵ with the FI dye, the distance between Cys¹⁹⁵ and OR-labelled PC/PS vesicles was measured using FRET in both the presence and absence of fVa.

MATERIALS AND METHODS

Construction, expression and purification of recombinant fX mutants

The expression and purification of fX-desEGF1 in HEK-293 (human embryonic kidney) cells has been described previously [12]. fX-Cys¹⁹⁵ was constructed using PCR mutagenesis methods and expressed in InsectSelect system (Invitrogen) using pIZ/V5-His expression vector transfected to Sf21 insect cells according to the manufacturer's instructions. The construction strategy was such that the sequence of His₆-tag in the vector remained downstream of the stop codon for fX, thus it was not incorporated into the sequence of the mutant fX protein. Similarly to fX-desEGF1, this mutant was purified by a combination of immunoaffinity and ion-exchange chromatography using the HPC4 monoclonal antibody immobilized on Affi-gel 10 and FPLC Mono Q columns respectively as described in [23].

Human plasma proteins fXa and fVa, prothrombin, fX activator from Russell's viper venom (RVV-X) and FI-labelled FPR (FI-FPR) were purchased from Haematologic Technologies. The fluorescent dyes fluorescein-5-maleimide and OR were purchased from Invitrogen. PC and PS were purchased from Avanti Polar Lipids. The chromogenic substrate Spectrozyme FXa (SpFXa) was purchased from American Diagnostica, and S2222, S2765 and S2238 were purchased from Kabi Pharmacia/Chromogenix.

Phospholipid vesicle preparations

PC/PS vesicles containing or lacking different amounts of OR were prepared by an extrusion method as described previously [24]. Briefly, dried phospholipid mixtures were suspended at 1 mg/ml in 0.1 M NaCl and 0.05 M Hepes (pH 7.4), mixed for 10 min and passed 20 times through a 100 nm pore diameter polycarbonate membrane. The OR-containing vesicles received various amounts of the dye in ethyl acetate before freeze-

drying and extrusion. The percentage yield of phospholipids recovery was determined by comparing the amount of choline from small samples before and after the centrifugation step by a colorimetric assay using Wako phospholipid B kit. The percentage recovery for different preparations ranged from 85 to 90 %. Phospholipid vesicles containing 100 % PC were prepared by the same procedures for coating cuvettes. The concentration of OR in the acceptor-containing samples was determined from absorbance at 564 nm using a molar absorption coefficient of $95\,400\text{ M}^{-1}\cdot\text{cm}^{-1}$ as described in [15,16]. The acceptor density of OR (σ , in OR/Å²) was calculated using molecular masses 786.1 and 810.0 Da for PC and PS respectively, assuming that the acceptors were distributed randomly at the phospholipid surface, and assuming that each phospholipid molecule occupies 70 Å² of the surface area as described in [16,25].

Fluorescent labelling

Both wild-type and fXa-desEGF1 (~1 mg) in the Mono Q elution buffer (~0.45 M NaCl and 0.02 M Tris/HCl, pH 7.4) were incubated with a 10-fold molar excess of FI-FPR for 2 h at room temperature (25 °C) in the dark. The extent of active-site labelling was monitored by the loss of the enzymatic activity using SpFXa. Incubation was continued until more than 99.9 % of the activity was inhibited. For the Cys¹⁹⁵ labelling of the fX-Cys¹⁹⁵ zymogen, the same amount of the mutant protein was incubated with fluorescein-5-maleimide (200 µM) in TBS (Tris-buffered saline) containing 25 µM dithiothreitol, to ensure reduction of the free cysteine, for 2 h at room temperature in the dark. This level of reducing agent did not influence the activity of fXa in the prothrombinase complex. The free inhibitor or dye was separated from the labelled proteins by gel filtration on the PD-10 column followed by their extensive dialysis in 0.1 M NaCl and 0.05 M Hepes (pH 7.4) containing 5 mM Ca²⁺ at 4 °C in the dark. A similar procedure was used to label Cys¹⁹⁵ in the fXa mutant (fXa-Cys¹⁹⁵). In this case, fX-Cys¹⁹⁵ was first activated with RVV-X and then separated from the reactants on the Ca²⁺-equilibrated heparin–Sephacrose column as described in [23]. The extent of fluorescence labelling for all proteins was determined as described by Bock [26]. A molar absorption coefficient of $84\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 498 nm was used to calculate the FI concentration and the ratio $\epsilon_{280}/\epsilon_{498}$ of 0.19 was used to correct for the contribution of the dye to 280 nm absorbance of the proteins, as described in [26].

Spectral measurements

The fluorescence measurements were made using an Aminco-Bowman series 2 Spectrophotometer (Spectronic Unicam) as described in [15,16]. Excitation and emission wavelengths of 493 nm and 521 nm and bandwidths of 4–8 nm were used. All fluorescence experiments were performed in 4 mm × 4 mm quartz cuvettes coated with extruded PC vesicles to minimize adsorption of proteins to the cuvettes as described previously [15,16]. The anisotropy of the FI-labelled proteins (25–50 nM) were measured in both the presence and absence of saturating concentrations of PC/PS vesicles (24 µM) and fVa (50 nM) using the same excitation and emission wavelengths as described previously [27]. The anisotropy of OR-labelled PC/PS vesicles containing a limiting concentration of the acceptor ($\sigma = 0.7 \times 10^{-4}$ OR/Å²) was measured as described above, except that the excitation and emission wavelengths were set at 521 nm and 586 nm respectively. Absorbance measurements were made using a Beckman Coulter DU 800 spectrophotometer. The values for the quantum yield (Q), spectral overlap between donor and acceptor dyes (J_{DA}), and the distance between donor and acceptor dyes at the 50 % FRET efficiency (R_0) were calculated as described

previously [15,16]. The values of Q and J_{DA} were measured separately for each FI-labelled protein in both the presence and absence of PC/PS vesicles and fVa.

FRET measurements

FRET measurements were performed as described previously [14,15], except that the donor containing (cuvette D) and both donor- (FI) and acceptor- (OR) containing cuvettes (cuvette DA) each received 50 nM FI-labelled fXa derivatives in 0.1 M NaCl, 0.05 M Hepes (pH 7.4) and 5 mM Ca^{2+} , whereas blanks (cuvette B) and acceptor-containing cuvettes (cuvette A) received 50 nM unlabelled fXa derivatives in the same buffer. For the measurements in the presence of fVa (50 nM), concentrations of the labelled fXa derivatives were reduced to 20 nM. The net initial emission intensities were obtained by subtracting the initial intensities of A from DA ($F_{DA}-F_A$)₀ and B from D (F_D-F_B)₀. Samples D and B were then titrated with PC/PS vesicles lacking the acceptor OR, whereas samples DA and A were titrated with PC/PS vesicles containing the acceptor. Similarly, the intensities of A and B were subtracted from DA and D respectively, and the values were then corrected for dilutions (less than 4% at the end of titration). Following 5 min of incubation, emission intensities were measured. The ratio of the donor quantum yields (Q) in the D and DA samples based on their fluorescence emission intensities (F) is given by eqn (1) as described in [16]:

$$Q_D/Q_{DA} = [(F_D - F_B)/(F_D - F_{B0})]/[(F_{DA} - F_A)/(F_{DA} - F_{A0})] \quad (1)$$

At the end of each experiment, the fluorescently labelled proteins were released from the membrane surface by the addition of EDTA to 10 mM to ensure that energy transfer was reversible. For calculating the distance of the closest approach using eqn (2) below, the Q_D/Q_{DA} value was calculated by dividing the value before the addition of EDTA by the value after the addition of EDTA. This normalization procedure corrects for the contribution of the acceptor inner filter effects and a potential membrane-binding-independent energy transfer as described in [16].

Calculation of the distance of closest approach

Assuming that both donor and acceptor dyes are randomly and uniformly distributed ($\kappa^2 = 2/3$), the distance of closest approach (L) between the plane of the donor dye attached to the active site of the protein and the plane of the acceptor dyes at the surface of PC/PS vesicles can be determined using eqn (2) as described previously [15,16]:

$$Q_D/Q_{DA} = 1 + (\pi\sigma R_0^2/2)(R_0/L)^4 \quad (2)$$

where π is 3.14, σ is the acceptor density at the membrane surface in OR/Å², and R_0 is the distance at which the singlet-singlet energy transfer from the donor dye to the acceptor dye is 50% efficient. The net Q_D/Q_{DA} values were plotted as a function of product between R_0^2 and the acceptor density (σ) for at least 3–13 different energy transfer experiments to obtain the average value of L as described in [15,16].

RESULTS

Expression, purification and characterization of mutant proteins

The kinetic properties of fXa-desEGF1 have been characterized extensively in a previous study [12]. This mutant appears to

Figure 1 SDS/PAGE analysis of fX-Cys¹⁹⁵ and fXa-Cys¹⁹⁵ mutants expressed in insect cells

Under non-reducing conditions: lane 1, plasma-derived fX; lane 2, plasma-derived fXa; lane 3, fX-Cys¹⁹⁵; lane 4, fXa-Cys¹⁹⁵. MW, molecular-mass standards with the sizes indicated in kDa.

be folded properly as is evident by its normal amidolytic activity and an order of magnitude improved activity towards prothrombin in the absence of fVa [12]. It also exhibits normal affinity for fVa as determined by a prothrombinase assay [12]. The fX-Cys¹⁹⁵ mutant was expressed in Sf21 cells and purified to homogeneity by a combination of immunoaffinity and ion-exchange chromatography using HPC4 monoclonal and Mono Q columns as described in [23]. The mutant fX zymogen was eluted from the ion-exchange column as a single peak at ~0.45 M NaCl. The SDS/PAGE analysis of the purified protein indicated that the fX mutant is homogeneous, migrating with a relative molecular mass that was comparable with that of the plasma-derived counterpart (Figure 1). After activation by RVV-X, a small fraction of the purified protein migrated at approx. 100 kDa under non-reducing conditions (Figure 1). The fX mutant migrated as a single band under reducing conditions, suggesting that Cys¹⁹⁵ in a small fraction of mutant enzyme forms an intermolecular disulfide bond (results not shown). The dimeric form of the molecule is not expected to be labelled, thus it cannot interfere with the spectroscopic measurements. The fXa-Cys¹⁹⁵ mutant did not exhibit any detectable amidolytic activity towards the chromogenic substrates SpFXa, S2222 and S2765, in either the presence or absence of fVa at enzyme concentrations of up to 250 nM (results not shown). The proteolytic activity of the fXa-Cys¹⁹⁵ mutant towards prothrombin was not evaluated.

Labelling and spectral characterization of fX/fXa derivatives

The active sites of both wild-type fXa and fXa-desEGF1 were labelled with FI-FPR. The inhibitor covalently binds to His⁵⁷ and inhibits the catalytic function of fXa and, by doing so, positions the FI dye in the catalytic groove of the protease somewhere in the vicinity of the P3-binding pocket of the protease [16]. The efficiency of labelling was evaluated using the methods described by Bock [26], and the average number of dyes per protein was determined to be 0.8 for both wild-type fXa and fXa-desEGF1. In the case of the fX-Cys¹⁹⁵ and fXa-Cys¹⁹⁵ mutants, the FI dye was directly incorporated into Cys¹⁹⁵ in both the zymogenic and activated forms of the molecule. The average number of dyes per protein was determined to be 0.6 and 0.5 for fX-Cys¹⁹⁵ and fXa-Cys¹⁹⁵ respectively.

The maximum excitation and emission wavelengths for all FI-labelled proteins were determined to be identical values of 493 nm and 521 nm respectively (results not shown). The anisotropy values for the labelled proteins were determined in both the

Table 1 The distance of closest approach between the donor FI dyes in the active sites of fX/fXa derivatives and the acceptor OR dyes incorporated into PC/PS vesicles and anisotropy values for the labelled proteins

The R_0 and L values (in Å) were determined based on the efficiency of energy transfer due to decreases in the emission intensity of the donor FI dyes in the active sites of proteins (20–50 nM) upon their titration with PC/PS vesicles containing the acceptor OR dyes with densities of $(0.6\text{--}5.5) \times 10^{-4}$ OR/Å² in 0.1 M NaCl and 0.05 M Hepes (pH 7.4) containing 5 mM Ca²⁺, assuming a random orientation of transition dipoles for donor and acceptor dyes ($\kappa^2 = 2/3$) as described in the Materials and methods section. ND, not determined since the zymogen fX is not expected to bind fVa. The anisotropy values (r) for fluorescein in the active sites of the proteins were determined at excitation and emission wavelengths of 493 nm and 521 nm respectively, as described in [27].

Donor enzyme	R_0	L (–fVa)	L (+fVa)	r	r (PC/PS)	r (PC/PS + fVa)
FI-FPR-fXa	48 ± 1	72 ± 2	75 ± 1	0.22	0.22	0.27
FI-FPR-fXa-desEGF1	42 ± 1	56 ± 1	63 ± 1	0.21	0.25	0.28
FI-fXa-Cys ¹⁹⁵	41 ± 1	95 ± 6	no FRET	0.17	0.19	0.19
FI-fX-Cys ¹⁹⁵	41 ± 1	97 ± 2	ND	0.17	0.17	0.17

presence and absence of saturating concentrations of PC/PS vesicles and fVa (Table 1). The anisotropies of free and membrane-bound FI-FPR-fXa were identical; however, fVa significantly increased it from 0.22 to 0.27, suggesting that the cofactor restricts the rotation of the FI dye in the active-site groove of the protease (Table 1). A similar change in the anisotropy of the FI-FPR-fXa-desEGF1 was observed upon its interaction with fVa. In this case, however, PC/PS also increased the anisotropy of the mutant protein (Table 1). On the other hand, although PC/PS enhanced the anisotropy of FI-fXa-Cys¹⁹⁵ from 0.17 to 0.19, fVa did not influence the anisotropy of the FI-labelled mutant protease (Table 1). The anisotropy of an OR-containing PC/PS preparation with a density of 0.7×10^{-4} OR/Å² was determined to be 0.15.

Membrane-dependent energy transfer

The changes in the emission intensity of the donor FI dye in the active-site grooves of both FI-FPR-fXa and FI-fXa-desEGF1, upon titration with PC/PS vesicles either lacking or containing the acceptor dye OR, are presented in Figure 2. The FI emission intensity was decreased for both proteases with both types of PC/PS vesicle, reaching saturation at $\sim 10 \mu\text{M}$ phospholipid. The decrease in the emission intensity of FI in the presence of OR was significantly greater than that in the absence of the acceptor dye for both proteases. Thus the OR-dependence of the decrease in the emission intensities are due to singlet–singlet energy transfer between FI in the active sites of the proteases and OR at the surface of PC/PS vesicles. Interestingly, the maximum OR-dependent decrease in the emission intensity of the donor dye in the active site of fXa-desEGF1 was considerably larger than that of the donor dye in the active-site groove of fXa, suggesting that singlet–singlet energy transfer is more efficient in the mutant protease. Energy transfer was dependent on binding fXa to PC/PS vesicles, since no energy transfer was observed in the absence of Ca²⁺ in EDTA. The EDTA reversible ratio of the donor quantum yields in the presence and absence of the acceptor ($Q_{\text{DA}}/Q_{\text{D}}$) was calculated according to eqn (1). The results presented in Figure 2(C) suggest that, relative to FI-FPR-fXa, there is $\sim 5\text{--}10\%$ larger OR-dependent decrease in the emission intensity of FI in the active-site groove of FI-FPR-fXa-desEGF1 (at the same acceptor density of 3.1×10^{-4} OR/Å²). The OR-independent change in the emission intensity of the donor dye probably represents a conformational change in the active-site groove of fXa which has been observed previously in similar studies [14]. Using the singlet–singlet energy transfer eqn (2) described in

Figure 2 Titration of FI-fXa and FI-fXa-desEGF1 with PC/PS vesicles

(A) Changes in the emission intensity of FI in the active site of FI-FPR-fXa (50 nM) were monitored upon titration with PC/PS vesicles lacking (○) or containing OR (●) as described in the Materials and methods section. (B) As in (A), except that FI-fXa-desEGF1 was used for the titration. (C) Results shown in (A) and (B) are re-plotted for both FI-fXa (○) and FI-fXa-desEGF1 (●) according to eqn (1) as described in the Materials and methods section. Acceptor densities were 3.1×10^{-4} OR/Å² for both FRET measurements.

the Materials and methods section, the average distance of the closest approach (L) between FI in the active-site grooves of fXa and fXa-desEGF1 and OR on PC/PS vesicles was calculated at several acceptor densities of $(0.6\text{--}5.5) \times 10^{-4}$ OR/Å², assuming a random orientation of transition dipoles for donor and acceptor dyes ($\kappa^2 = 2/3$) as described previously [15,16]. The average L values for FI-FPR-fXa were determined to be 75 ± 1 Å (mean $\pm$ S.D.; $n = 6$) and 72 ± 2 Å (mean $\pm$ S.D.; $n = 7$) in the presence and absence of fVa respectively (Table 1). The same values for fXa-desEGF1 were 63 ± 1 Å (mean $\pm$ S.D.; $n = 3$) and 56 ± 1 Å (mean $\pm$ S.D.; $n = 4$) in the presence and absence of fVa respectively. These results suggest that, relative to wild-type fXa, the FI dye in the active-site groove of the fXa-desEGF1 mutant is ~ 16 Å closer to the membrane surface, assuming a κ^2 value of 2/3 for both proteins. Since the magnitude of the energy transfer depends upon the density of the acceptor OR at the membrane surface [16], it was ensured that the plot of $Q_{\text{D}}/Q_{\text{DA}}$ exhibited a linear dependence on the product between R_0^2 and the acceptor density (σR_0^2) as demonstrated in Figure 3.

To determine whether the average L value for the FI-FPR-fXa reflects the distance of the active site of fXa relative to the membrane surface, the same FRET measurements were carried out for the FI-labelled fXa-Cys¹⁹⁵ mutant. The titration of FI-fXa-Cys¹⁹⁵ with PC/PS vesicles lacking OR did not result in a significant change in the emission intensity of the donor FI dye attached to the mutant catalytic residue (Figure 4A). However, the intensity of the donor dye was decreased $\sim 10\text{--}12\%$

Figure 3 Linear dependence of FRET upon acceptor density

The R_0 values for FI-FPR-fXa were determined by its titration with OR-containing PC/PS vesicles at acceptor densities of $(0.6\text{--}5.0) \times 10^{-4}$ OR/Å² as described in the Materials and methods section.

Figure 4 Titration of FI-fXa-Cys¹⁹⁵ and FI-fX-Cys¹⁹⁵ with PC/PS vesicles

(A) Changes in the emission intensity of FI covalently attached to residue 195 of the fXa-Cys¹⁹⁵ mutant (50 nM) were monitored upon titration with PC/PS vesicles lacking (○) or containing (●) OR as described in the Materials and methods section. (B) As in (A), except that FI-fX-Cys¹⁹⁵ zymogen was used for the titration. Acceptor densities were 3.1×10^{-4} OR/Å² for both FRET measurements.

at the same acceptor density (3.1×10^{-4} OR/Å²) when the mutant protease was titrated with PC/PS vesicles containing OR, suggesting that FRET occurs between the two donor-acceptor dye pairs (Figure 4A), yielding an average L value of 95 ± 6 Å (mean $\pm$ S.D.; $n = 13$) for Cys¹⁹⁵ relative to the membrane surface (Table 1). These results suggested that the actual distance of the

active site of fXa relative to the membrane surface, assuming a κ^2 value of 2/3 for both proteins, may be ~ 23 Å higher than the distance obtained between FI in the active site of the FPR-inhibited fXa and OR at the vesicle surface. In the presence of fVa, no value for L could be estimated, since the efficiency of FRET was decreased to $\sim 1\%$. No increase in FRET was observed even if the acceptor density was increased to 5.5×10^{-4} OR/Å². Such a minor reduction in the emission intensity could not be utilized for the distance measurement, since previous studies have indicated that this level of decrease may not be due to FRET, but rather due to an inner filter effect caused by the absorption of excitation and emission light by rhodamine [15]. Nevertheless, the results support the previous observation that fVa alters the topography of the active site of fXa bound to a negatively charged membrane surface [14]. A similar $\sim 10\text{--}12\%$ decrease in the emission intensity of FI-labelled zymogen fX-Cys¹⁹⁵ was observed, yielding an L value of 97 ± 2 Å (mean $\pm$ S.D.; $n = 5$) for Cys¹⁹⁵ of the zymogen fX relative to the membrane surface assuming $\kappa^2 = 2/3$ (Figure 4B, Table 1). The comparable L values obtained for both fX-Cys¹⁹⁵ and fXa-Cys¹⁹⁵ mutants suggest that the activation peptide of fX loops out of the molecule and does not contribute to the height of the active site from the membrane surface. In agreement with these results, the plots of Q_{DA}/Q_D exhibited essentially identical OR-dependent decreases in the emission intensity of FI in the active sites of both fX-Cys¹⁹⁵ and fXa-Cys¹⁹⁵ mutants (Figure 4C). As with FI-FPR-fXa (Figure 3), the plots of Q_D/Q_{DA} exhibited a linear dependence on the product between R_0^2 and the acceptor density (σR_0^2) for all FRET measurements described above (results not shown).

DISCUSSION

In the present study, on the basis of the efficiency of fluorescence energy transfer, we have measured the distance of closest approach between the donor FI dyes in the active sites of both fXa and fXa-desEGF1 and OR dyes on the PC/PS vesicles and found that, relative to fXa, the distance of the active site of fXa-desEGF1 is ~ 16 Å closer to the membrane surface, suggesting that EGF1 plays a spacer function in maintaining the active site of fXa at an appropriate height above the membrane surface. Such a function for the EGF1 domain of fXa, and probably other vitamin K-dependent coagulation proteases, may be crucial for maintaining both the protease active site and the substrate scissile bond at a similar distance above the membrane surface for efficient bond cleavage. Consistent with this hypothesis, previous data obtained with activated Protein C and Protein S have shown that altering the height of the active site of activated Protein C eliminates the need for Protein S [15]. These results are also consistent with molecular models showing extended conformations for vitamin K-dependent coagulation proteases when assembled with their respective cofactors and substrates on membrane surfaces [28–30].

In agreement with previous results [14], the efficiency of energy transfer was decreased with both fXa derivatives, thus yielding a 3–7 Å greater distance between the active sites of the FI-FPR-inhibited proteases and the membrane surface, suggesting that fVa alters the topography of the membrane-bound enzyme in the prothrombinase complex. Previous functional prothrombin activation data with fXa-desEGF1 provides further support for this hypothesis as is evident by the observation that the fXa-desEGF1 mutant activated prothrombin with improved catalytic efficiency by an order of magnitude in the absence of fVa, but the fXa-desEGF1 mutant activated prothrombin with 3-fold lower activity in the presence of the cofactor [12]. On the basis of similar

FRET results with meizothrombin, it has been hypothesized that the cofactor function of fVa may also exert a similar effect on the topography of the fXa-recognition site on the membrane-bound substrate [17]. To test this hypothesis, we also labelled an R320C mutant of prothrombin with FI and attempted to measure its distance from OR dye at the vesicle surface. Unfortunately, however, for unknown reasons, we did not observe any change in the emission intensity of FI-Cys³²⁰ in the membrane-bound mutant zymogen in either the presence or absence of fVa in order to measure the distance of Cys³²⁰ relative to membrane surface (results not shown). We also obtained an identical anisotropy value of 0.15 for the FI-labelled prothrombin-Cys³²⁰ both in the free form and in the presence and absence of PC/PS vesicles and fVa, thus no evidence for a cofactor-induced spectral change in the environment of the dye at P1 site of the mutant protein was detected in order to speculate whether or not fVa alters the orientation and/or the height of the fXa-recognition site on the membrane-bound prothrombin.

All FRET measurements for estimating the height of the active site of coagulation proteases relative to membrane surface are derived using proteases inhibited with tripeptidyl-chloromethane inhibitors which contain a donor FI or dansyl dye tethered at their N-terminal residues [14–21]. This strategy labels the active site of the protease by the inhibitor covalently attaching itself to His⁵⁷, thus positioning the dye somewhere in the vicinity of the S3 subsite of the protease. This labelling approach has provided clear evidence in support of coagulation proteases interacting with phospholipid membranes in an elongated manner, with their protease domains being located far above the membrane surface. However, since the fluorescent label is estimated to be located some 15 Å away from Ser¹⁹⁵ in the active-site groove [16], it is not known whether the distance between the FI dye tethered to the inhibitor and the OR dye incorporated to the vesicle surface reflects the actual distance of the active-site residue from the membrane surface. In an attempt to provide an answer to this question and also determine whether fVa can alter the height and/or the topography of the catalytic residue 195 of fXa in the prothrombinase complex, we attached FI dye directly to this residue by mutating it to cysteine. FRET measurements with FI-fXa-Cys¹⁹⁵ and FI-FPR-fXa using the same OR-containing PC/PS vesicles revealed that the measured *L* value in the fXa-Cys¹⁹⁵ mutant is ~23 Å longer than the same value in fXa labelled with FI-FPR. The efficiency of energy transfer was drastically reduced in the presence of fVa, suggesting that the cofactor alters the height and/or orientation of the membrane-bound fXa in the prothrombinase complex, although no distance could be measured because of lack of FRET. A longer *L* value for the fXa-Cys¹⁹⁵ mutant apparently suggests that the FI dyes, attached either to the tripeptidyl inhibitor in the active-site groove of FI-FPR-fXa or directly to Cys¹⁹⁵, are positioned at different heights relative to the plane of membrane. It should be noted that this hypothesis assumes a similar randomly oriented transition dipoles for the donor and acceptor dyes during the lifetime of the donor excited state in the catalytic grooves of both proteins ($\kappa^2 = 2/3$). However, the orientation and/or the rotational freedoms of the FI dyes in the active sites of FI-FPR-fXa and fXa-Cys¹⁹⁵ may not be identical, and thus there is uncertainty associated with the κ^2 value of the donor dyes between the two proteins. Although it is not possible to determine experimentally the orientation of the FI dyes in the active sites of the two proteins, nevertheless, the maximum uncertainty in distance measurements due to orientational effects was calculated from the measured steady-state anisotropies of FI in either FI-FPR-fXa ($r = 0.22$) or FI-fXa-Cys¹⁹⁵ ($r = 0.17$) and the membrane-bound OR dyes in PC/PS vesicles ($r = 0.15$), which ranged from ± 21 to $\pm 33\%$ for the labelled enzymes

respectively [31]. The actual uncertainty in R_0 values is expected to be lower (± 10 – 15%) owing to the random orientation of the OR dye in the plane of the membrane as described previously [16]. Thus it cannot be asserted with certainty that the difference in the measured *L* values between the two proteins is not due to the uncertainty in κ^2 values between the two proteins.

An interesting observation with anisotropy measurements was noting a lower value of *r* for the FI dye covalently attached to Cys¹⁹⁵, suggesting that its rotation is less constricted than the dye in the active site of FI-FPR-fXa. Furthermore, unlike FI-FPR-fXa, where fVa significantly enhanced the anisotropy of the FI dye from 0.22 to 0.27, the cofactor had no effect on the anisotropy of the dye attached to Cys¹⁹⁵ of the membrane-bound mutant protease ($r = 0.19$ in both the presence and absence of fVa on PC/PS vesicles). These results are in agreement with previous reports and suggest that fVa may not detectably alter the conformation of the active-site residue of fXa [32]. Unlike the ~15% decrease in the emission intensity of FI in the FI-FPR-fXa bound to PC/PS vesicles lacking the acceptor OR dye and a ~25–30% decrease with the vesicles containing the acceptor dye, there was no change in the intensity of FI attached to Cys¹⁹⁵ on PC/PS vesicles lacking OR and only ~10–12% decrease in intensity was observed on the OR-containing PC/PS vesicles. These results suggest that all decreases in the emission intensity with both the fX-Cys¹⁹⁵ and fXa-Cys¹⁹⁵ mutant proteins can be attributed to FRET occurring between the donor FI dye attached to Cys¹⁹⁵ and the acceptor OR dye at the vesicle surface. Thus the membrane-induced spectral changes in FI-FPR-fXa observed in the present study as well as in similar previous FRET studies with fXa [14], may not reflect a conformational change in the active-site residue of fXa, but rather reflect a change in the environment of FI in the vicinity of the extended P3-binding pocket of the protease. This interpretation of results is consistent with previous FRET studies concluding that fVa primarily affects the conformation of the active site of fXa approx. 15 Å from the active-site Ser¹⁹⁵ [14,16].

The observation that the measured *L* value for FI-fXa-Cys¹⁹⁵ mutant was similar to the same value for the FI-fX-Cys¹⁹⁵ mutant suggests that the activation peptide sequence of fX (52 residues) does not have an elongated conformation, but rather it loops out horizontally without contributing to the height of the molecule on the membrane surface. Noting that the active site of the Factor VIIa-tissue factor complex is also located ~75 Å above the membrane surface, we predict that the fX scissile bond is also located at similar height above the membrane surface. In this context, the EGF1 domain of the fX is also expected to play a spacer role to maintain the scissile bond above the membrane surface. In support of this hypothesis, our previous results indicated that fX-desEGF1 does not function as a substrate for Factor VIIa in complex with soluble tissue factor, but rather inhibits the catalytic activity of the protease toward wild-type fX in the extrinsic fXase complex by a competitive manner [12]. In contrast with a complete loss of activity for the fX-desEGF1 zymogen, a modest 3-fold loss of catalytic activity for the fXa-desEGF1 mutant was observed in the prothrombinase complex [12]. Thus the spacer function of EGF1 is indispensable for the zymogenic activity of fX. The basis for such dramatic differences in the activity of zymogen and enzyme forms of the fX mutant is likely to be due to the observation that, unlike the lack of an interactive site between either the Gla or EGF1 domain of fXa [11], both the Gla and EGF1 domains of the zymogen interact with tissue factor on the membrane surface [33,34]. We hypothesize that such an interaction can stabilize the substrate in an unproductive activation complex in which the activation peptide of the membrane- and tissue-factor-bound fX-desEGF1 is spatially aligned at a much lower height than the active site of

Factor VIIa in the extrinsic fXase complex. On the other hand, the Gla domain of fXa in the fXa-desEGF1 mutant protease does not interact with the cofactor fVa [11], thus the mutant protease has much more flexibility to access the activation peptide of the substrate in the prothrombinase complex. Taken together, these results suggest that EGF1 plays an essential spacer function in fX activation by the extrinsic fXase complex.

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REFERENCES

- Mann, K. G., Jenny, R. J. and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem.* **57**, 915–956
- Rosing, J., Tans, G., Govers-Riemsag, J. W. P., Zwaal, R. F. A. and Hemker, H. C. (1980) The role of phospholipids and factor Va in the prothrombinase complex. *J. Biol. Chem.* **255**, 274–283
- Furie, B. and Furie, B. C. (1988) The molecular basis of blood coagulation. *Cell* **53**, 505–518
- Davie, E. W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance and regulation. *Biochemistry* **30**, 10363–10370
- Jackson, C. M. and Nemerson, Y. (1980) Blood coagulation. *Annu. Rev. Biochem.* **49**, 765–811
- Nesheim, M. E., Kettner, C., Shaw, E. and Mann, K. G. (1981) Cofactor dependence of factor Xa incorporation into the prothrombinase complex. *J. Biol. Chem.* **256**, 6537–6540
- Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P. and Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood* **76**, 1–16
- Stenflo, J. (1991) Structure–function relationships of epidermal growth factor modules in vitamin K-dependent clotting factors. *Blood* **78**, 1637–1651
- Persson, E., Björk, I. and Stenflo, J. (1991) Protein structural requirements for Ca²⁺ binding to the light chain of factor X: studies using isolated intact fragments containing the γ -carboxyglutamic acid region and/or the epidermal growth factor-like domains. *J. Biol. Chem.* **266**, 2444–2452
- Persson, E., Hogg, P. J. and Stenflo, J. (1993) Effects of Ca²⁺ binding on the protease module of factor Xa and its interaction with factor Va: evidence for two Gla-independent Ca²⁺-binding sites in factor Xa. *J. Biol. Chem.* **268**, 22531–22539
- Thiec, F., Cherel, G. and Christophe, O. D. (2003) Role of the Gla and first EGF-like domains of factor X in the prothrombinase and tissue factor–factor VIIa complex. *J. Biol. Chem.* **278**, 10393–10399
- Kittur, F. S., Manithody, C. and Rezaie, A. R. (2004) Role of the N-terminal epidermal growth factor-like domain of factor X/Xa. *J. Biol. Chem.* **279**, 24189–24196
- Lim, T. K., Bloomfield, V. A. and Nelsestuen, G. L. (1977) Structure of the prothrombin– and blood clotting factor X–membrane complexes. *Biochemistry* **16**, 4177–4181
- Husten, E. J., Esmon, C. T. and Johnson, A. E. (1987) The active site of blood coagulation factor Xa: its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex. *J. Biol. Chem.* **262**, 12953–12962
- Yegneswaran, S., Smirnov, M. D., Safa, O., Esmon, N. L., Esmon, C. T. and Johnson, A. E. (1999) Relocating the active site of activated protein C eliminates the need for its protein S cofactor. *J. Biol. Chem.* **274**, 5462–5468
- Mutucumarana, V. P., Duffy, E. J., Lollar, P. and Johnson, A. E. (1992) The active site of factor IXa is located far above the membrane surface and its conformation is altered upon association with factor VIIIa: a fluorescence study. *J. Biol. Chem.* **267**, 17012–17021
- Armstrong, S. A., Husten, E. J., Esmon, C. T. and Johnson, A. E. (1990) The active site of membrane-bound meizothrombin: A fluorescence determination of its distance from the phospholipid surface and its conformational sensitivity to calcium and factor Va. *J. Biol. Chem.* **265**, 6210–6218
- McCallum, C. D., Su, B., Neuenschwander, P. F., Morrissey, J. H. and Johnson, A. E. (1997) Tissue factor positions and maintains the factor VIIa active site far above the membrane surface even in the absence of the factor VIIa Gla domain. *J. Biol. Chem.* **272**, 30160–30166
- Lu, R. L., Esmon, N. L., Esmon, C. T. and Johnson, A. E. (1989) The active site of the thrombin–thrombomodulin complex: a fluorescence energy transfer measurement of its distance above the membrane surface. *J. Biol. Chem.* **264**, 12956–12962
- McCallum, C. D., Hapak, R. C., Neuenschwander, P. F., Morrissey, J. H. and Johnson, A. E. (1996) 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. *J. Biol. Chem.* **271**, 28168–28175
- Yegneswaran, S., Woods, G. M., Esmon, C. T. and Johnson, A. E. (1997) Protein S alters the active site location of activated protein C above the membrane surface: a fluorescence resonance energy transfer study of topography. *J. Biol. Chem.* **272**, 25013–25021
- Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. and Hofsteenge, J. (1989) The refined 1.9 Å crystal structure of human α -thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* **8**, 3467–3475
- Chen, L., Manithody, C., Yang, L. and Rezaie, A. R. (2004) Zymogenic and enzymatic properties of the 70–80 loop mutants of factor X/Xa. *Protein Sci.* **13**, 431–442
- Smirnov, M. D. and Esmon, C. T. (1994) Phosphatidylethanolamine incorporation into vesicles selectively enhances factor Va inactivation by activated protein C. *J. Biol. Chem.* **269**, 816–819
- Huang, C. and Mason, J. T. (1978) Geometric packing constraints in egg phosphatidylcholine vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 308–310
- Bock, P. E. (1988) Active site selective labeling of serine proteases with spectroscopic probes using thioester peptide chloromethyl ketones: demonstration of thrombin labeling using N^ε-(acetylthio)acetyl-D-Phe-Pro-Arg-CH₂Cl. *Biochemistry* **27**, 6633–6639
- Rezaie, A. R. and Kittur, F. S. (2004) The critical role of the 185–189-loop in the factor Xa interaction with Na⁺ and factor Va in the prothrombinase complex. *J. Biol. Chem.* **279**, 48262–48269
- Kirchofer, D., Eigenbrot, C., Lipari, M. T., Moran, P., Peek, M. and Kelley, R. F. (2001) The tissue factor region that interacts with factor Xa in the activation of factor VII. *Biochemistry* **40**, 675–682
- Borledge, B. V., Petrovan, R. J., Ruf, W. and Olson, A. J. (2003) The tissue factor/factor VIIa/factor Xa complex: a model built by docking and site directed mutagenesis. *Proteins* **53**, 640–648
- Autin, L., Steen, M., Dahlbäck, B. and Villoutreix, B. O. (2006) Proposed structural models of the prothrombinase (Fxa–FVa) complex. *Proteins* **63**, 440–450
- Dale, R. E., Eisinger, J. and Blumberg, W. E. (1979) The orientational freedom of molecular probes: the orientation factor in intramolecular energy transfer. *Biophys. J.* **26**, 161–194
- Walker, R. K. and Krishnaswamy, S. (1993) The influence of factor Va on the active site of factor Xa. *J. Biol. Chem.* **268**, 13920–13929
- Kirchofer, D., Lipari, M. T., Moran, P., Eigenbrot, C. and Kelley, R. F. (2000) The tissue factor region that interacts with substrates factor IX and factor X. *Biochemistry* **39**, 7380–7387
- Zhong, D., Bajaj, M. S., Schmidt, A. E. and Bajaj, S. P. (2002) The N-terminal epidermal growth factor-like domain in factor IX and factor X represents an important recognition motif for binding to tissue factor. *J. Biol. Chem.* **277**, 3622–3631

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