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Factor Va Increases the Affinity of Factor Xa for Prothrombin: A Binding Study Using a Novel Photoactivable Thiol-Specific Fluorescent Probe

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Summary

The multiprotein complex of factor Xa, factor Va, and prothrombin efficiently generates the blood-clotting agent, thrombin. Here, the formation of the factor Xa•prothrombin complex and the effects of factor Va on this complex were examined using a photoactivable thiol-specific fluorescent probe (LWB), which was synthesized and incorporated into the active site of factor Xa. The use of fluorescent LWB illustrated that factor Xa has an increased affinity for prothrombin in the presence of factor Va. Further exposure of these components to UV light resulted in a specific photocrosslinking of LWB-factor Xa to prothrombin, suggesting a physical association between these proteins. These data demonstrate that LWB can successfully function both as a spectroscopic probe and as a photocrosslinking reagent for studying protein-protein interactions.

Introduction

The activation of zymogen prothrombin to its active form, thrombin, is an essential step in blood coagulation [1]. This activation is accomplished at a physiologically relevant rate by the prothrombinase complex, a macromolecular association of the serine protease factor Xa (fXa) with its cognate cofactor, factor Va (fVa), and substrate, prothrombin, on negatively charged phospholipid surfaces (Figure 1) [2–5].

The prothrombinase has been well characterized and is an archetype for other blood clotting complexes, partly because it directly leads to the generation of thrombin, a multifunctional enzyme that can lead to a procoagulant, anticoagulant, and/or antifibrinolytic responses depending on the local environment [6, 7]. Detailed kinetic analyses by several groups show that fVa, the nonenzymatic cofactor in the prothrombinase complex, enhances the ability of fXa to activate prothrombin by over 13,000-fold [3–5]. In this reaction, fVa functions both as a K-type and a V-type effector, decreasing the K_M and increasing the k_{cat}, respectively [8]. Additionally, in the presence of fVa and phospholipid, prothrombin activation occurs exclusively via a meizothrombin intermediate; whereas, in the absence of these two components, the activation proceeds via a prethrombin 2 intermediate [9–12]. Therefore, the substrate specificity of fXa is altered in the presence of fVa and phospholipids.

Fluorescent reporter groups placed strategically in the active site of fXa have provided valuable information about conformational changes that presumably occur in the active sites of fXa upon binding fVa and phospholipids [13–15]. One approach has been to use fluorescent dead-end inhibitors that conjugate specifically to the active site histidine of fXa. Dansyl-linked tripeptide chloromethylketones have been employed quite extensively in such studies (see [16] and the references therein). These studies suggest that fVa stoichiometrically binds to fXa in a calcium-dependent fashion in the presence of negatively charged phospholipids [13, 15, 17]. However, this affinity is as much as 1000-fold weaker in the absence of phospholipid surfaces. fVa may enhance the activity of fXa in the prothrombinase complex by several mechanisms. fVa increases the lipid binding affinity of fXa and also has a direct effect on the active site of fXa, altering the active site conformation [16]. fVa also binds to and alters the conformation of the substrate [18]. Studies using fluorescence resonance energy transfer (FRET) between the donor dyes positioned in the active site of fXa and acceptors on the membrane surface observed substantial differences in the extent of energy transfer between fXa•fVa•phospholipid complexes compared with fXa•phospholipid complexes [15]. The alteration in energy transfer translates to a reorientation of the active site of the enzyme above the membrane surface in the presence of the cofactor protein. Thus, the biophysical studies are in agreement with the kinetic data and additionally provide a structural explanation for the 13,000-fold fVa-dependent increase in fXa activity.

Although the kinetics of prothrombin activation by fXa are well characterized, the binding of factor Xa to prothrombin is less well characterized. This may be partly due to a lack of probes that are sensitive to the interaction between the two proteins, or it may be due to problems encountered in the preparation of an enzyme that does not hydrolyze its substrate upon interaction. Recently, several reports suggest that enzyme-substrate interactions involving vitamin K-dependent serine proteases may be dictated by exosite interactions distal to the active site rather than by the active site [19–21]. These results suggest that active site-specific probes could be used to investigate the prothrombinase complex from a "prothrombin perspective". This approach would enable the direct measurement of the binding interaction between fXa_i and prothrombin and would give insights into the effect of fVa on this interaction.

To detect the formation of the fXa•prothrombin complex, we have taken a two-pronged approach using steady-state fluorescence spectroscopy and a photocrosslinking technique. To do this efficiently, we have synthesized a reagent we call "LWB". LWB features three functional groups: a fluorescent fluorescein reporter; a photoactivable benzophenone group; and a thiol-reactive

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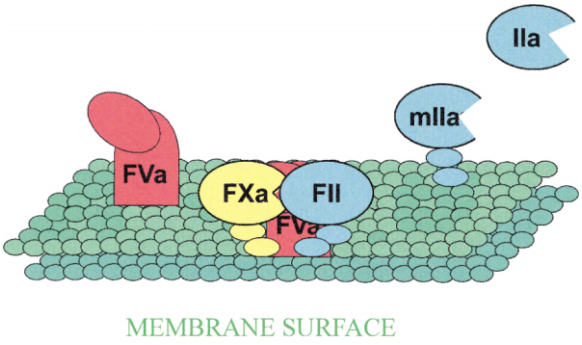

The Prothrombinase Complex

Figure 1. A Schematic Representation of the Prothrombinase Complex

A schematic representation of the prothrombinase complex is shown. Factor Xa (fXa), the serine protease, is shown in yellow. Its cofactor, factor Va (fVa), is depicted in red, and the substrate, prothrombin (fII), is depicted in blue. Also shown are two cleavage products of prothrombin, an intermediate meizothrombin (mIIa) and the product α-thrombin. The membrane surface is shown in green.

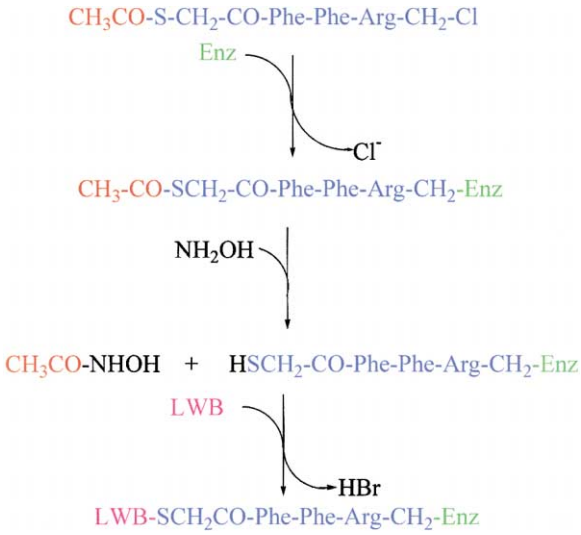

Figure 3. Active Site-Directed Labeling of Factor Xa with LWB

A schematic representation of the active site-directed labeling of fXa with LWB (magenta). Enz (in green) represents factor Xa or any other serine protease. Phe-Phe-Arg represents the phenylalanine, phenylalanine, and arginine residues in the tripeptide chloromethylketone.

bromoacetyl group, aiming to combine the advantages of fluorescence and photocrosslinking techniques.

Results

Synthesis of LWB and Active Site-Directed Labeling of Factor Xa with LWB

LWB (Figure 2) was synthesized by manual SPPS procedure for Boc-chemistry [22]. Prior to cleavage from the resin, the Fmoc group on the ε-amino group of the Lys residue was removed and the Lys residue was bromoacetylated. The cleaved reagent was then purified by HPLC and was characterized by electrospray/ionization mass spectrometry (ESI-MS). LWB eluted as a single sharp peak at ~44% acetonitrile. The molecular weight of LWB was determined by ESI-MS to be 877.4 ± 0.5 amu, in agreement with the calculated average isotopic mass of 877.7 amu. The compound was lyophilized and was stored in the dark at −20°C for future use.

To prepare human LWB-FFR-fXa$_i$, we have utilized an approach similar to one proposed by Bock (Figure 3) [23]. fXa was inactivated by the modified inhibitor N^{α}-([acetylmercapto] acetyl)-(D-phenylalanyl)-phenylalanyl-arginyl-chloromethylketone. After purification of the inhibited enzyme from the excess unreacted reagents using conventional biochemical techniques, the inhibited reagent was treated with an excess of LWB in the presence of a nucleophile such as NH_2OH. The resulting free thiol generated in situ on the inhibited

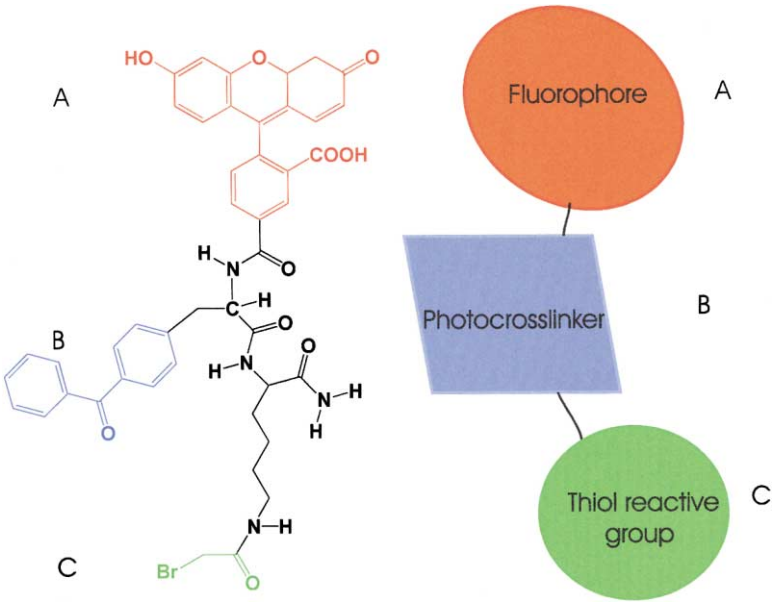

Figure 2. Chemical Structure and a Schematic Representation of LWB

The structure of LWB is shown as a chemical structure and a schematic, respectively.
(A) The fluorophore, fluorescein (shown in orange).
(B) The photocrosslinker, benzophenone, is shown in blue.
(C) The thiol reactive moiety, a bromoacetyl group, is shown in green.

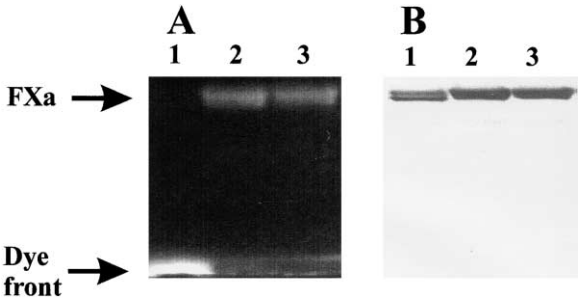

Figure 4. SDS-PAGE Analysis of Active Site-Specific Labeling Procedure

(A) AMA-FFR-fXa$_i$ (11 μM) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA was incubated with LWB in the presence of 0.1 M NH_2OH for 1 hr at 25°C. The reaction mixture was then purified according to procedures detailed in the Experimental Procedures. Purified product (20 μl) was analyzed using nonreduced SDS-PAGE on a 4%–12% gradient gel. The fluorescence of LWB-FFR-fXa$_i$ was visualized using an UV illuminator prestaining with Coomassie blue (lanes 2 and 3). Lane 1 contains samples from a mock labeling reaction of AMA-FFR-fXa$_i$ with LWB in the absence of NH_2OH.
(B) Figure 4A stained using Coomassie blue G250.

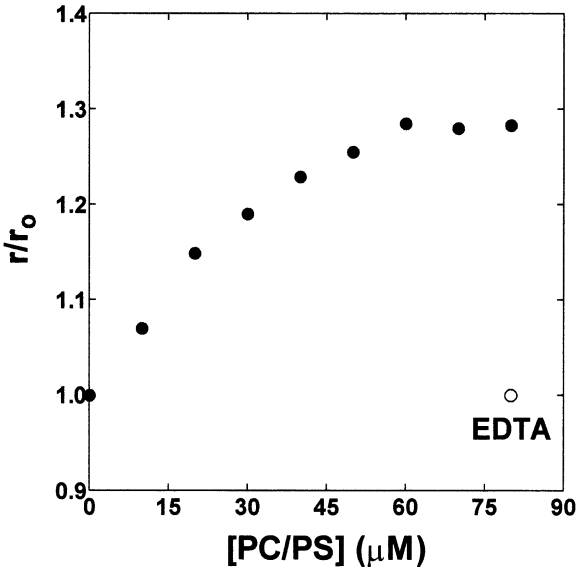

Figure 5. PC/PS Dependence of LWB-FFR-fXa$_i$ Fluorescence

LWB-FFR-fXa$_i$ (initially 200 nM in cuvette) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ was titrated with PC/PS vesicles, and the steady-state anisotropy of the fluorescein moiety in LWB-FFR-fXa$_i$ was monitored at 490 nm excitation and 520 nm emission (closed circles). At the end of the titration, 5 mM EDTA (final) was added to the cuvette (open circle).

enzyme reacts site specifically with the bromoacetyl group on LWB to generate LWB-FFR-fXa$_i$. LWB-FFR-fXa$_i$ was purified from the excess reagents and was stored in the dark at −80°C for future use.

Characterization of LWB-FFR-fXa$_i$

The dye-to-protein ratio in LWB-FFR-fXa$_i$ averaged 0.75, assuming a molar extinction coefficient of 84,000 M^{-1} cm^{-1} for the fluorescein moiety in LWB [23]. Figure 4A (lanes 2 and 3) shows a nonreduced SDS-PAGE analysis of the purified LWB-FFR-fXa$_i$ on a 4%–12% gradient gel. The fluorescence of LWB-FFR-fXa$_i$ was visualized using a UV illuminator. Staining with Coomassie blue was used to visualize protein bands (Figure 4B). The nonreduced LWB-FFR-fXa$_i$ migrated as a fluorescent band corresponding to 45.7 kDa, the molecular weight of unlabeled fXa. The detection of intense fluorescence with the protein band indicates that fluorescein was covalently attached to fXa. A mock labeling was performed either in the absence of the nucleophile, NH_2OH (lane 1, Figure 4A), or in the absence of the Bock inhibitor (data not shown). Lane 1 in Figure 4A shows the absence of a fluorescent band comigrating with the stained protein band (lane 1, Figure 4B) when the AMA-FFR-fXa$_i$ was reacted with LWB in the absence of the hydroxylamine. Similarly, an absence of conjugation between fXa and LWB was observed without the Bock inhibitor (data not shown). Thus, LWB conjugated to the active site of fXa only in the presence of the hydroxylamine-treated Bock reagent.

Using the same labeling procedure described in the Experimental Procedures, LWB was successfully used to label two other serine proteases, thrombin and factor VIIa (data not shown). The labeling of these three serine proteases demonstrated that the labeling procedure was nonspecific for trypsin-like serine proteases.

Spectral Properties

In the fluorescence studies, corrected wavelength maxima for excitation and emission of LWB-FFR-fXa$_i$ in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ were 500 and 525 nm, respectively. The steady-state anisotropy of the free LWB-FFR-fXa$_i$ in the same buffer averaged 0.205 ± 0.001. The quenching constant (K_{sv}) for NaI quenching at room temperature was determined to be 8.79 mM for LWB-FFR-fXa$_i$ and 8.71 mM for free fluorescein, indicating that the fluorescein probe in the labeled fXa was highly accessible for collisional quenching.

LWB-FFR-fXa$_i$ Binding to Phospholipid Vesicles

fXa binds negatively charged phospholipid vesicles in a calcium-dependent fashion via the Gla domain [13, 15, 24–26]. Upon the addition of phosphatidylcholine/phosphatidylserine (PC/PS) vesicles, the anisotropy of free LWB-FFR-fXa$_i$ increased from 0.205 ± 0.001 to 0.237 ± 0.002. Figure 5 illustrates the PC/PS dependence of LWB-FFR-fXa$_i$ anisotropy. At the end of the PC/PS titration, EDTA added to the samples dissociated the membrane-bound LWB-FFR-fXa$_i$. The emission intensity of the fluorescein moiety in LWB-FFR-fXa$_i$ was also sensitive to the presence of PC/PS vesicles. When LWB-FFR-fXa$_i$ was titrated with PC/PS, the emission intensity decreased by ~33% and reached a minimum at ~40 μM PC/PS. Upon the addition of EDTA to reverse membrane binding, the emission intensity increased and reached a value similar to that of free LWB-FFR-fXa$_i$. Thus, both the anisotropy and the emission intensity of LWB-FFR-fXa$_i$ were sensitive to binding to PC/PS.

Cofactor Dependence of LWB-FFR-fXa$_i$ Fluorescence

fVa forms a complex with PC/PS-bound fXa [16]. To investigate the cofactor dependence of LWB-FFR-fXa$_i$ fluorescence, samples of PC/PS-bound LWB-FFR-fXa$_i$

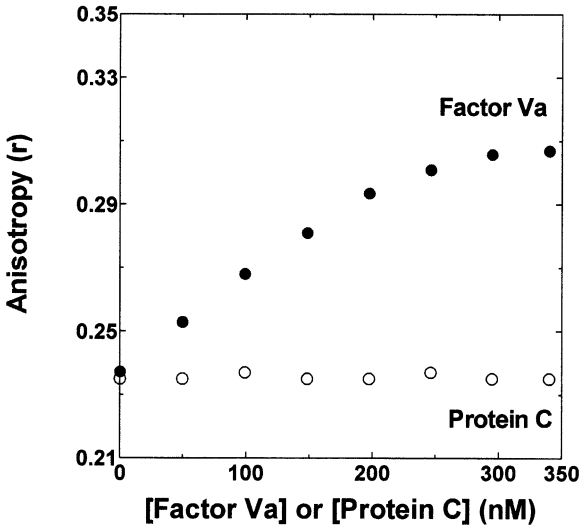

Figure 6. Factor Va Dependence of LWB-FFR-fXa$_i$ Fluorescence

LWB-FFR-fXa$_i$ (initially 200 nM in cuvette) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ was titrated with PC/PS vesicles. The anisotropy value increased from a value of 0.205 (for free LWB-FFR-fXa$_i$) to a value of 0.237 upon the addition of 80 μM PC/PS. At this point, factor Va was titrated into the cuvette (closed circles). In a parallel titration, the LWB-FFR-fXa$_i$•PC/PS complex was titrated with protein C (open circles).

were prepared with sufficient vesicles to bind both fXa and an excess of fVa. When the LWB-FFR-fXa$_i$•PC/PS complex was titrated with factor Va, the fluorescein anisotropy increased by ~30% before reaching a plateau value (Figure 6). Similarly, a 50% increase in emission intensity of the probe was observed upon the addition of fVa (data not shown). An excess of EDTA was added at the end of the factor Va titration to test if the spectral changes were calcium ion dependent because the fXa•fVa•PC/PS complex dissociates in the absence of calcium ions [14]. The addition of EDTA changed the anisotropy and emission intensity of the LWB-FFR-fXa$_i$•PC/PS•fVa complex to values equal to those of free LWB-FFR-fXa$_i$, indicating that calcium ions were required for the formation of this ternary complex. Protein C is the zymogen form of the anticoagulant serine protease-activated protein C (reviewed in [27]). To test the specificity of fVa-induced spectral changes in LWB-FFR-fXa$_i$, protein C, which binds negatively charged phospholipid vesicles but not fXa [27], was added to a preformed LWB-FFR-fXa$_i$•PC/PS complex. No changes in the anisotropy (Figure 6) or emission intensity (data not shown) of LWB-FFR-fXa$_i$ were observed, implying that the spectral changes in the LWB-FFR-fXa$_i$•PC/PS complex caused by fVa are due to specific interactions between fXa and fVa.

Substrate Dependence of LWB-FFR-fXa$_i$ Fluorescence

The effect of the physiological substrate of fXa, prothrombin (reviewed in [1, 2]), on LWB-FFR-fXa$_i$ fluorescence was investigated. When PC/PS-bound LWB-FFR-fXa$_i$ was prepared with sufficient vesicles to bind both labeled fXa and an excess of prothrombin and was titrated with prothrombin, a dose-dependent increase in fluorescein anisotropy was observed (Figure 7A). This prothrombin-dependent change in anisotropy reached a maximum value of 0.266 at ~1400 nM prothrombin. Similarly, the emission intensity of the LWB-FFR-fXa$_i$•PC/PS complex also showed a prothrombin-dependent increase of ~32% before reaching a plateau (data not shown). These hyperbolic titration curves suggest the formation of a LWB-FFR-fXa$_i$•PC/PS•prothrombin ternary complex.

Figure 7B shows the effects of fVa on fXa-prothrombin interactions. First, PC/PS vesicles were titrated into LWB-FFR-fXa$_i$ until the PC/PS-dependent change in LWB-FFR-fXa$_i$ anisotropy reached a plateau. An excess of PC/PS was added so that sufficient vesicles were present to bind an excess of fVa and prothrombin. Second, fVa was added to the LWB-FFR-fXa$_i$•PC/PS solution until the fVa-dependent change in LWB-FFR-fXa$_i$ anisotropy reached a plateau. Finally, prothrombin was titrated into the LWB-FFR-fXa$_i$•PC/PS•fVa ternary complex. A further prothrombin-induced increase in LWB-FFR-fXa$_i$ anisotropy that reached a plateau at ~150 nM prothrombin was observed. At the end of the prothrombin titration, the addition of EDTA reversed the fluorescein anisotropy to a value similar to that of LWB-FFR-fXa$_i$ free in solution, suggesting that all changes caused by phospholipid vesicles, fVa, and prothrombin were calcium dependent. These data suggest that the inactive LWB-FFR-fXa$_i$ forms a quaternary complex with prothrombin in the presence of fVa, negatively charged phospholipids, and calcium ions.

LWB as a Photoactivable Crosslinker

The interaction of fXa with prothrombin was also monitored using photocrosslinking. LWB-FFR-fXa$_i$ was incubated for 5 min at room temperature with prothrombin and excess PC/PS in the presence of calcium ions before being irradiated with UV light for 2 min. After exposure to UV light, the reaction mixture was analyzed using SDS-PAGE and immunoblotting with anti-fX antibodies (Figure 8A). Lane 1 (Figure 8A), which contained the complete reaction mixture, shows the appearance of a crosslinked product (indicated by an arrow) migrating with an apparent molecular weight of ~114.5 kDa. This molecular weight corresponds to the theoretical mass of a fXa-prothrombin-crosslinked protein band. Approximately 28% of the total fXa seem to crosslink to prothrombin according to estimates using Un-Scan-It (5.1) software (Silk Scientific). The controls in lanes 2 and 3, containing samples lacking prothrombin or LWB-FFR-fXa$_i$, respectively, showed that both prothrombin and LWB-FFR-fXa$_i$ were necessary to generate the 114.5-kDa band.

To examine the specificity of photocrosslinking, protein C was incubated with the LWB-FFR-fXa$_i$•PC/PS complex. After exposure to UV light, the reaction mixture was analyzed on immunoblots using a monoclonal anti-human protein C (C-3) antibody (Figure 8B). No high-molecular weight adduct of protein C with fXa was observed (lane 3 compared with lanes 2 and 4), suggesting that, although protein C was present on PC/PS vesicles with LWB-FFR-fXa$_i$, it did not crosslink to fXa. Therefore, these experiments suggest that the photocrosslinks that

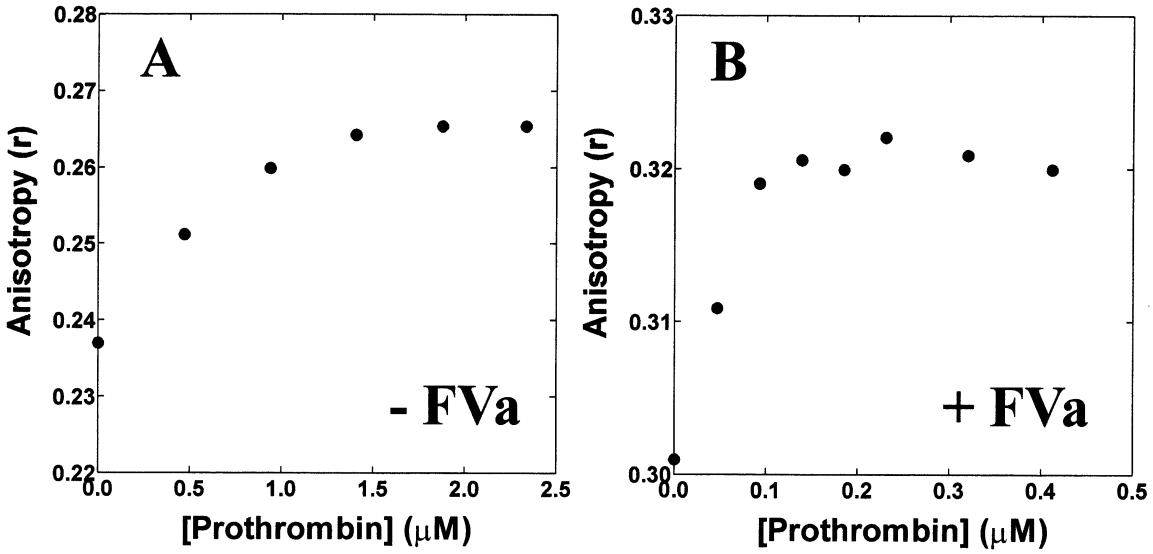

Figure 7. Prothrombin Dependence of LWB-FFR-fXa$_i$ Fluorescence

(A) LWB-FFR-fXa$_i$ (initially 200 nM in cuvette) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ was titrated with PC/PS vesicles. The anisotropy value increased from a value of 0.205 (for free LWB-FFR-fXa$_i$) to a value of 0.237 upon the addition of 80 μM PC/PS. At this point, prothrombin was titrated into the cuvette (closed circles).

(B) LWB-FFR-fXa$_i$ (initially 200 nM in cuvette) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ was titrated with 80 μM PC/PS vesicles. Then, the LWB-FFR-fXa$_i$•PC/PS complex was titrated with fVa as described above. Finally, the LWB-FFR-fXa$_i$•PC/PS•fVa complex was titrated with prothrombin (closed circles).

were observed in Figure 8A were due to specific protein-protein interactions.

Discussion

One aim of this study was to monitor the binding of the enzyme fXa to its substrate prothrombin and to directly measure the effect of fVa on this enzyme•substrate complex. To this end, we have designed and synthesized LWB, a trifunctional compound (Figure 2) that can monitor fXa•prothrombin complex formation using two different techniques, namely, fluorescence spectroscopy and photocrosslinking.

The synthesis of LWB was designed to be general for the production of trifunctional molecules. Because of the modular nature of the trifunctional probe, the fluorescein can be easily replaced with any other functional group that can be conjugated to the N terminus of the probe. If desired, a spacer group could also be inserted between A and B (Figure 2). Next, a functional amino acid, in this case, a benzoyl phenylalanine, was incorporated. Alternatively, a variety of commercially available fluorophores or biotinylated amino acids could be substituted. A differentially protected C-terminal Lys residue (Boc-Lys [Fmoc]-OH) enables the reactive conjugating group to be added in the last step of the synthesis.

In this study, LWB was designed to contain a thiol-reactive bromoacetyl group and two equally spaced functional groups, a photoactivable benzophenone group and a fluorescent fluorescein moiety. The fluorescein and benzophenone groups (benzoylphenylalanine) were chosen for two reasons. First, they can be incorporated by SPPS, and, second, they provide relatively high quantum yields in fluorescence and photocrosslinking, respectively [28, 29]. In addition, the availability of anti-fluorescein antibodies enables this group to double as an affinity tag. The bromoacetyl group enables site-specific conjugation to thiols such as Cys side chains or, in this case, a thiol introduced on an irreversible inhibitor. Several multifunctional reagents have been synthesized, and a few are commercially available [30–35]. However, the advantage of LWB lies in the simplicity of its synthesis, the site specificity of its labeling, and its utility as a detector of effector-induced conformational changes in proteins. The compound was synthesized by standard SPPS using the in situ neutralization/HBTU activation procedure for Boc-chemistry.

After synthesis, LWB was incorporated into the active site of fXa using an approach similar to that of Bock [23]. Purified fXa was labeled with LWB in the presence of the Bock inhibitor and hydroxylamine. The protein-to-dye ratio indicates not more than one label per protein. Since essentially no labeling of fXa is observed in the absence of either the Bock reagent or hydroxylamine, we conclude that LWB attaches specifically to the active site histidine of fXa via a thioacetyl Phe-Phe-Arg tether.

The fluorescein moiety in LWB-labeled factor Xa (LWB-FFR-fXa$_i$) had excitation and emission maxima that were ~5 nm red shifted compared with fluorescein-Phe-Phe-Arg-fXa$_i$ (Fl-FFR-fXa$_i$) [36]. However, the emission maximum of LWB-FFR-fXa$_i$ was very similar to that observed previously using fluorescein-Glu-Gly-Arg-fXa$_i$ (Fl-EGR-fXa$_i$) [36]. The anisotropies of LWB-FFR-fXa$_i$ and Fl-FFR-fXa$_i$ are comparable. These data suggest that the addition of the Lys and (benzoyl)-Phe amino acids to the tripeptide tether does not dramatically change the spectral properties of the fluorescein dye in Fl-FFR-fXa$_i$.

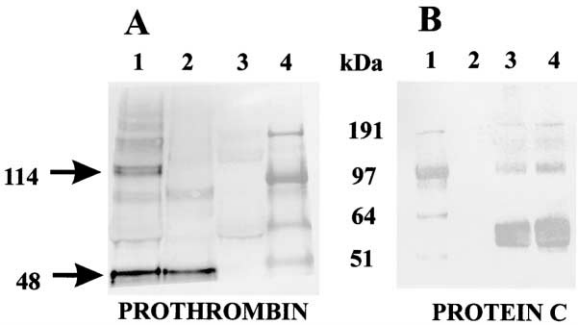

Figure 8. LWB as a Photoactivable Probe

(A) LWB-FFR-fXa$_i$ (50 nM) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ was incubated with 2 μM prothrombin and 100 μM PC/PS vesicles for 5 min at room temperature. The sample was then irradiated with 254 nm UV light as described in the Experimental Procedures. The reaction mix was analyzed on a 4%–12% SDS-PAGE electrophoresis gel. The protein bands were then transferred to a membrane and were analyzed by Western blots. The primary rabbit polyclonal antibody was directed against human fXa. The secondary biotin-coupled antibody used here was anti-rabbit IgG. The bands were finally visualized by using streptavidin-coupled alkaline phosphatase as described in the Experimental Procedures. Lanes 1, 2, 3, and 4 contain the reaction mixture, the reaction performed in the absence of prothrombin, the reaction performed in the absence of LWB-FFR-fXa$_i$, and prestained molecular weight standards, respectively.

(B) LWB-FFR-fXa$_i$ (1 μM) in 50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$ was incubated with 2 μM protein C and 100 μM PC/PS vesicles (reaction mix) for 5 min at room temperature. The sample was then irradiated with 254 nm UV light as described in the Experimental Procedures. The sample was then diluted 1000-fold in the reaction buffer, and the reaction mix was analyzed on a 4%–12% SDS-PAGE electrophoresis gel. The protein bands were then transferred to a membrane and were analyzed by Western blots. The primary monoclonal antibody was directed against human protein C (C-3). The secondary biotin-coupled antibody used here was anti-mouse IgG. The bands were finally visualized by using streptavidin-coupled alkaline phosphatase as described in the Experimental Procedures. Lanes 1, 2, 3, and 4 contain the prestained molecular weights, the reaction mix without protein C, the reaction mix, and the reaction performed in the absence of LWB-FFR-fXa$_i$, respectively.

Next, the fluorescein moiety in LWB-FFR-fXa$_i$ was tested for its ability to sense conformational changes induced in fXa due to lipid and protein interactions. Both the anisotropy and the emission intensity of LWB-FFR-fXa$_i$ were sensitive to phospholipid binding, in contrast to previous studies in which the fluorescence of Fl-FFR-fXa$_i$ is insensitive to membrane binding [36]. The fluorescein dye in Fl-FFR-fXa$_i$ resides near the S4 pocket of fXa. Perhaps moving the spectroscopic probe from the S4 pocket, possibly to the S6 pocket, makes the fluorescein more sensitive to the fXa-PC/PS vesicle interaction. Alternatively, this increased sensitivity to lipid binding could be due to the alteration in the orientation of the fluorescein dye in the active site region of fXa. The mercaptoacetyl group on the Bock reagent binds to the ϵ-amino group end of the Lys on LWB, hence changing the orientation of the fluorescein moiety in the fXa active site compared with Fl-FPR-fXa$_i$. The lipid binding titration curves obtained with LWB-FFR-fXa$_i$ were similar to those obtained with a low-quantum yield dansyl-labeled fXa (DEGR-fXa$_i$), indicating that the incorporation of LWB into the active site of fXa did not cause major changes in the enzyme-lipid interaction [16].

When PC/PS-bound LWB-FFR-fXa$_i$ was titrated with fVa, increases in the anisotropy and emission intensity of the probe were observed. No increase in anisotropy or intensity was observed when protein C, another membrane binding protein, was added to PC/PS-bound LWB-FFR-fXa$_i$. These data suggest that the LWB probe specifically detects the fXa-fVa interaction on the phospholipid surface. A 1:1.5 stoichiometry for fXa:fVa was observed, similar to reports published previously [13, 15, 36, 37]. Since the lifetime of the fluorescein dye is only ~4 nsec, time enough to sense only local changes, the fVa-dependent increase in LWB-FFR-fXa$_i$ anisotropy suggests an alteration in the local environment of the dye upon fVa binding. The emission intensity of LWB-FFR-fXa$_i$ was also sensitive to fVa binding. Because Fl-FFR-fXa$_i$ fluorescence intensity does not change significantly upon binding fVa [36], LWB-FFR-fXa$_i$ is a unique probe for monitoring fXa-fVa interactions using fluorescence.

Although one must assume the formation of a fXa•prothrombin complex, i.e., an enzyme•substrate complex, none of the active site fluorescent probes used so far are sensitive to fXa-prothrombin interactions. In solid-phase binding assays, fXa appears to bind prothrombin via the Kringle 2 domain of prothrombin [38]. One possible explanation for the inability of active site-specific fluorescent probes to sense the fXa-prothrombin interaction could be that the occupancy of the fXa active site by these probes blocks the formation of an enzyme•substrate complex due to steric hindrance. However, increasing evidence suggests that enzyme-substrate interactions involving serine proteases involved in blood coagulation are driven by exosite interactions that are quite removed from the active site and that the affinity of the enzyme for its macromolecular substrate is not affected by the occupancy of the active site by small substrates, inhibitors, and activation peptides [19–21]. When LWB-FFR-fXa$_i$•PC/PS was titrated with prothrombin, an initial increase in both the anisotropy and intensity of fluorescein emission that reached a plateau at ~1.5 μM prothrombin ($K_{d\,app}$ = 816 nM) was observed. This binding curve suggests the formation of an LWB-FFR-fXa$_i$•PC/PS•prothrombin ternary complex. The concentration of prothrombin in plasma ranges from 1.1 to 1.7 μM, suggesting that this fXa-prothrombin interaction, though weak, is physiologically relevant [39, 40]. Thus, the development of LWB provides us with a unique spectroscopic tool to study fXa-prothrombin interactions. Additionally, our data provide further evidence that a small inhibitor such as LWB-FFR-chloromethylketone, in the active site of fXa, does not preclude the formation of a LWB-FFR-fXa$_i$•PC/PS•prothrombin complex.

When LWB-FFR-fXa$_i$ was irradiated with UV light in the presence of prothrombin, PC/PS vesicles, and calcium ions, high-molecular weight fXa species corresponding to a fXa-prothrombin adduct were generated. This suggests that prothrombin and fXa were covalently linked in a 1:1 complex after photoactivation of LWB. From densitometry estimates, ~28% of the fXa could be crosslinked to prothrombin. This relatively high photo-

crosslinking yield may be due to the fact that α, β-unsaturated ketones react from their UV-excited triplet state and the unreacted excited species relax to the ground state and may be excited repeatedly until they react [28]. As a negative control, when LWB-FFR-fXa$_i$ was irradiated with UV light in the presence of protein C, PC/PS vesicles, and calcium ions, no high-molecular weight bands appeared, suggesting the absence of crosslinks between protein C and fXa. This would suggest that fXa and protein C are not close enough to each other on vesicles to crosslink, presumably because they do not interact with each other. Therefore, the photocrosslinking data confirm the spectroscopic observation that prothrombin and fXa form a specific complex on negatively charged vesicles even when the fXa active site is blocked.

The dual functionality of LWB enabled both the anisotropy and crosslinking studies to be performed on the same sample. For example, a steady-state fluorescence experiment at 490 nm yielded fluorescence changes but no crosslinks; however, when the same sample cuvette was exposed to 254 nm light after complex formation, as detected by the anisotropy changes, high-molecular weight adducts of prothrombin and fXa could be observed on SDS-PAGE (data not shown). These experiments demonstrate that both fluorescence and crosslinking experiments can be performed on the same sample using LWB.

fVa increases k_{cat} and decreases K_M for prothrombin activation by fXa [8, 12]. To determine the effect of fVa on the fXa-prothrombin interaction, preformed LWB-FFR-fXa$_i$•PC/PS•fVa ternary complexes were titrated with prothrombin. The prothrombin-dependent increase in fluorescein anisotropy and intensity reached a plateau value at less than 150 nM of prothrombin. Accounting for the dilution of sample in the cuvette during titration, a 1:1 stoichiometry for fXa:prothrombin was observed in the presence of fVa ($K_{d\ app}$ = 15 nM). This suggests that the fVa increases 50-fold the affinity of fXa for prothrombin and that this enhanced affinity does not require binding of prothrombin to the fXa active site. Thus, our spectroscopic studies are in agreement with the kinetic data that indicate that the K_M for the activation of prothrombin by fXa is markedly reduced by fVa. Several groups have reported a weak interaction between fVa and prothrombin [41–43]. However, all of these data are in the absence of phospholipids. Therefore, it is hard to tell if the fXa-prothrombin and the fVa-prothrombin interactions are synergically enhanced in the prothrombinase complex in the presence of phospholipds. The anisotropy changes observed upon titrating prothrombin into the LWB-FFR-fXa$_i$•PC/PS•fVa ternary complex represent the cumulative sum of changes observed during the titration of LWB-FFR-fXa$_i$•PC/PS with fVa and LWB-FFR-fXa$_i$•PC/PS with prothrombin. This suggests the formation of an LWB-FFR-fXa$_i$•PC/PS•fVa•prothrombin complex. Therefore, in addition to enhancing the membrane binding affinity of fXa, increasing the catalytic efficiency of fXa, and reorienting the active site of fXa, fVa also increases the inherent affinity of fXa for prothrombin.

Because LWB was designed to perform dual functions, such as sensing local environmental and conformational changes as well as crosslinkink to other proteins, an extended spacer group was not added to extend the crosslinking radius of the benzophenone. The structure of human active site-inhibited des(1-45)-fXa shows that the active site cleft of fXa is very similar to that of thrombin [44]. When D-Phe-Pro-Arg-thrombin was crystallized, the tripeptide inhibitor was observed to nestle tightly into the active site depression with a third of the inhibitor (D-Phe group) partially exposed outside the active site groove [45]. LWB is coupled to the active site of fXa via the D-Phe-Phe-Arg inhibitor. Molecular models of LWB were constructed to estimate the crosslinking radius of the molecule. The photoreactive carbonyl in the benzophenone of LWB is approximately 15 Å away from the bromoacetyl group on the Lys. Therefore, from these studies, we estimate the crosslinking radius of the reactive benzophenone in LWB-FFR-fXa$_i$ to be 15–20 Å.

Significance

Biological processes, such as blood coagulation, occur in a complex and dynamic environment that is not amenable to structural imaging techniques such as X-ray crystallography and NMR. As a result, techniques such as fluorescence spectroscopy and chemical crosslinking are useful for obtaining a spatial and temporal understanding of multiprotein complex formation in these systems, but the results of these experiments can be hard to interpret. During blood coagulation, a complex is formed between prothrombin, factor Xa (fXa), and factor Va (fVa), which results in cleavage of the proenzyme to its active form, thrombin. Here, the formation of the fXa•fVa•prothrombin complex is examined using LWB, a newly developed reagent, which was designed to conjugate to thiols in a site-specific manner, photocrosslink specific enzyme•substrate complexes, and allow for the detection of conformational changes in the active site of enzymes by fluorescence spectroscopy. In this study, LWB is specifically attached to the active site of fXa and is successfully used to monitor the interaction of fXa with different components of the prothrombinase complex. Further, the use of LWB shows that the affinity of fXa for prothrombin increases by ~50-fold in the presence of fVa and negatively charged phospholipids. Together, the experiments described in this work and the reagent characterized in this study (LWB) provide researchers with insight into the formation of the prothrombinase complex and a tool with which to pursue further studies.

Experimental Procedures

Materials

Boc-Lys (Fmoc)-OH was purchased from Midwest Biotech. Boc-*p*-benzoyl-Phe-OH was obtained from Bachem Biosciences, and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Nova Biochem. *N*, *N*-diisopropylethylamine (DIEA) was purchased from Applied Biosystems. Trifluoroacetic acid (Halocarbon), hydrogen fluoride (HF) gas (Matheson Gas), and *p*-methylbenzhydrylamine HCl (MBHA) resin (Peninsula Laboratories) used in peptide synthesis were purchased as indicated. *N*, *N*′-diisopropylcarbodiimide (DIC), bromoacetic

acid, and piperidine were obtained from Sigma-Aldrich Research Company. 5-carboxyfluorescein and succinimidylacetylthioacetate were obtained from Molecular Probes. D-phenylalanylphenylalanyl-arginyl chloromethylketone was purchased from Calbiochem. Dioleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) were obtained from Avanti Polar Lipids. L-3-phosphatidylcholine-1,2-di[1-^{14}C]oleoyl ([^{14}C]PC) was purchased from Amersham Pharmacia Biotech. *N*-benzoylisoleucylglutamylglycylarginyl-*p*-nitroaniline (S2222) was obtained from DiaPharma.

Proteins

Human fXa was activated from human fX using Russell's viper venom (RVV-X, Enzyme Research Laboratories) and was purified from the reactants according to previously described procedures [46]. Human α-thrombin, factor VIIa, and fXa (for some experiments) were obtained from Enzyme Research Laboratories. fVa was purchased from Haematologic Technologies.

Polyclonal rabbit anti-human fX was obtained from Celsus Laboratories. Enzyme-linked immunosorbent assay kits for fXa were obtained from Affinity Biologicals. Monoclonal anti-human protein C (C-3) was prepared according to procedures published earlier [47]. Biotin-labeled secondary antibody, alkaline phosphatase-conjugated streptavidin, 5-bromo-4-chloro-3′-indolyphosphate-*p*-toluidine chloride (BCIP), and nitro-blue-tetrazolium chloride (NBT) substrate were purchased from Pierce.

Synthesis of LWB

LWB (*N*$^{\alpha}$-fluorescein-p benzoyl phenylalanyl-lysyl [*N*$^{\epsilon}$ bromoacetyl] amide, Figure 2) was synthesized by manual solid-phase peptide synthesis, typically on a 0.2-mmol scale using the in situ neutralization/HBTU activation procedure for Boc-chemistry [22]. After the neutralization of the MBHA resin with DIEA (2 $\times$ 10% in DMF for 2 min), the Boc-Lys (Fmoc)-OH (0.44 mmol) was activated with 0.4 mmol (800 μl) HBTU and 200 μl DIEA and was coupled to the resin for 30 min. A quantitative ninhydrin test was performed to determine the extent of coupling [48]. After >99% of the lysine was coupled to the resin, the excess uncoupled lysine was then washed away with three flow washes of DMF.

Next, for the coupling of Boc-*p*-benzoyl-Phe-OH, the *N*$^{\alpha}$-Boc protecting group on Boc-Lys (Fmoc)-OH was removed from the fully protected peptide-resin by two 1-min washes with TFA, and the resin was washed with DMF. The phenylalanyl derivative was activated with HBTU as described above and was coupled to Lys (Fmoc)-MBHA resin (30 min, >99%). The peptide resin was washed with DMF and was neutralized with 10% DIEA. The 5-carboxyfluorescein dye (0.75 mmol) was activated with 0.25 mmol DIC in CH_2Cl_2 (15 min on ice) and was coupled to the peptide resin. The reaction was allowed to proceed until >99% of the fluorescein was coupled to the resin.

The Fmoc group on the Lys residue was removed by treatment with 20% piperidine in DMF for 5–10 min. Then, 2-bromoacetic acid (2 mmol) in 500 μl CH_2Cl_2 was activated with 1 mmol DIC (15 min on ice). The resulting symmetric anhydride was added to the resin and was coupled for 15 min. This procedure bromoacetylates the ϵ-amino group of the Lys on the peptide.

The fluorescein-labeled peptide was then simultaneously cleaved from the resin and was deprotected by treatment with anhydrous HF for 1 hr at 0°C with 4% *p*-cresol as a scavenger. After cleavage, the peptide was precipitated and washed with ice-cold diethylether, dissolved in 50% acetic acid, filtered, and lyophilized. Finally, LWB was redissolved in 30% buffer B containing 2, 2, 2-trifluoroethanol and was purified from *p*-cresol and other reaction side products by semipreparative reverse phase HPLC on a Rainin HPLC system using a Vydac C-18 column (10 μm, 1.0 $\times$ 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute the bound peptide. Dual wavelength detection at 214 and 480 nm was employed to monitor the elution of the fluorescent peptide from the column. The product was confirmed by ESI-MS analysis. The theoretical mass of the compound was calculated using ChemDraw Pro 6.0 version software (Cambridge Soft).

Synthesis of N$^{\alpha}$-([acetylmercapto]acetyl)-(D-Phenylalanyl)-Phenylalanyl-Arginyl-Chloromethylketone

N$^{\alpha}$-([acetylmercapto]acetyl)-(D-phenylalanyl)-phenylalanyl-arginyl-chloromethylketone (AMA-FFR-CK, Bock reagent) was synthesized and purified [23]. (D-phenylalanyl)-phenylalanyl-arginyl-chloromethylketone (8.7 mM) in 50 mM Na_2HPO_4 (pH 7.0) was incubated with succinimidylacetylthioacetate (47 mM) in methanol for 30 min. The reaction mix was then diluted 5-fold with 25 mM NaH_2PO_4 (pH 3.0), and the pH was adjusted to 3.0 with *o*-phosphoric acid. The product was purified from the reactants on a Rainin HPLC system using a semipreparative reverse phase Vydac C-18 column (10 μm, 1.0 $\times$ 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute the AMA-FFR-CK from the column. The synthesis of the product was confirmed by ESI-MS. The concentration of AMA-FFR-CK was determined by a colorimetric assay using 5, 5′-dithio bis-(2-nitrobenzoic acid) (DTNB) as described by Bock [23].

Active Site-Directed Labeling of Factor Xa with LWB

Following the activation and purification of factor Xa from zymogen factor X and RVV-X, factor Xa (5 mg) was incubated at room temperature with a 5-fold molar excess of AMA-FFR-CK in 5 ml of 50 mM HEPES (pH 7.4), 150 mM NaCl, plus 2 mM $CaCl_2$ (buffer A) [23]. The extent of active site labeling was monitored by the loss of enzymatic activity of factor Xa using a chromogenic substrate S2222 until factor Xa was >99.9% inactive. The inactivated factor Xa (AMA-FFR-fXa$_i$) was dialyzed against 1 liter of buffer A at 4°C with two changes to remove unreacted reagent. The resulting preparation was treated with a 5-fold molar excess of LWB in DMF (final concentration of 5% in solution) in the presence of 0.1 M hydroxylamine for 1 hr at 25°C in the dark. Finally, LWB-mercaptoacetyl-Phe-Phe-Arg-factor Xa (LWB-FFR-fXa$_i$) was purified from the excess of unreacted reagents by initially passing the reaction mix over a Microcon centrifugal device (30-kDa cutoff; Millipore), followed by extensive dialysis against buffer A (2000 ml $\times$ 3 changes) at 4°C in the dark. LWB-FFR-fXa$_i$ was aliquoted, quick frozen, and stored at −80°C.

Preparation of Phospholipid Vesicles

Small unilamellar vesicles of phosphatidylcholine/phosphatidylserine (PC/PS, 4:1 mole ratio) were prepared using sonication and centrifugation procedures as described [15]. Lipid recovery post-centrifugation was quantified using a trace amount of [^{14}C]phosphatidylcholine in the sonication mixture [15].

Spectral Measurements

Steady-state fluorescence intensity and anisotropy measurements were made using a SLM 8100 photon-counting spectrofluorometer or a SLM AB2 Luminescence spectrometer (SLM Aminco) equipped with a 450-W xenon arc lamp, two holographic gratings in the excitation light path, and an IBM computer for data analysis. Fluorescein fluorescence was detected at its emission and excitation maxima wavelengths. A circulating water bath was used to maintain the sample compartment at 25°C. All fluorescence experiments were performed in 5 mm $\times$ 5 mm quartz cuvettes using a 345-nm cutoff filter (Schott Glass) in the excitation beam path. Samples were mixed using a small teflon-coated 2 mm $\times$ 2 mm magnetic spinbar as described before [49]. Absorption of protein to the cuvette walls was minimized by coating the cuvettes with 100% PC vesicles as described [50].

For a typical fluorescence experiment, two samples were prepared in parallel. The sample cuvette (S) received 200 nM LWB-FFR-fXa$_i$ in 50 mM HEPES (pH 7.4), 150 mM NaCl, and 2 mM $CaCl_2$, whereas the blank cuvette (B) received 200 nM FFR-fXa$_i$ in the same buffer. The initial net fluorescence intensity of the sample, designated F_o, was obtained by the subtraction of a dye-free blank from the S cuvette signal. For the phospholipid titrations, small unilamellar vesicles of PC/PS were added to each cuvette. After the signal observed in the absence of the dye (sample B) was subtracted from that in the presence of the dye (Sample S), the net volume-corrected signal was designated F at that point in the titration. Protein titrations were performed in the presence of saturating phospholipids analogously.

Steady-state anisotropy was measured using Glan-Thompson prism polarizers on both the excitation and emission beams. The

emission intensity measured when the sample was excited by vertically (V) plane-polarized light and the emission detected through a horizontal (H) polarizer is termed I_{VH}. I_{HH}, I_{HV}, and I_{VV} are defined analogously. The component intensities of a dye-free blank were subtracted from the component sample intensities to give the net emission intensities. Anisotropy (r) was then calculated from the net intensities using:

$$r = (I_{VV} - GI_{HV})/(I_{VV} + 2GI_{VH}), \quad (1)$$

where the grating factor, G, equals I_{HV}/I_{HH}.

Determination of Apparent Dissociation Constants

The apparent K_ds for the LWB-FFR-fXa$_i$•PC/PS complex binding to prothrombin in the presence or absence of fVa were obtained at saturating phospholipid concentrations as described before [17]. GraphPad Prism version 3.0 was used to calculate the apparent K_d values.

Photocrosslinking

Photocrosslinking experiments were performed using a CL-1000 Ultraviolet Crosslinker (UltraVioletProducts). Storing the samples in the dark until photocrosslinking experiments were performed prevented adventitious crosslinking. Samples were placed in microfuge tubes approximately 15 cm from the light source on ice during the photocrosslinking procedure and were then irradiated with 254 nm ultraviolet light (5 × 8 W discharge bulbs) for 0–5 min. Immediately following the photocrosslinking experiments, samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4%–12% gradient; Novex).

Immunoblots

Immunoblots were performed as described [51]. The protein bands from the polyacrylamide gel electrophoresis analysis were transferred overnight using 200 mA current onto an Immobilon-PVDF (Millipore) membrane with a transfer apparatus (Hoefer Scientific Instruments). The membrane was then incubated with 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$, and 1% casein for 1 hr. Then, the membrane was incubated with the primary antibody against the protein for 1 hr, followed by a 30-min incubation with the biotin-conjugated secondary antibody directed against the primary antibody. Finally, alkaline phosphatase-conjugated streptavidin was added to the membrane for a time period of 30 min. This step was followed by the addition of alkaline phosphatase substrate, namely, a preprepared mixture of BCIP and NBT (from Pierce). Color development was terminated by washing the substrates off the membrane with water.

Enzyme-Linked Immunosorbent Assays (ELISA)

The concentration of factor Xa antigen in LWB-FFR-fXa$_i$ was determined by ELISA kits for factor Xa using manufacturer's conditions. Standard curves for factor Xa antigen were obtained using known concentrations of FFR-inhibited fXa determined by A_{280}.

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