Protein S Alters the Active Site Location of Activated Protein C above the Membrane Surface

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The location of the active site of membrane-bound activated protein C (APC) relative to the phospholipid surface was determined both in the presence and absence of its cofactor, protein S, using fluorescence resonance energy transfer (FRET). APC was chemically modified to create the FRET donor species, Fl-FPR-APC, with a fluorescein dye (Fl) covalently attached to the modified to create the FRET donor species, Fl-FPR-APC, nance energy transfer (FRET). APC was chemically modified to create the FRET donor species, Fl-FPR-APC, with a fluorescein dye (Fl) covalently attached to the

...protein S binds to similarly-labeled derivatives of factor Xa, factor IXa, or factor VIIa did not alter the locations of their active sites. This direct measurement demonstrates that the binding of the protein S cofactor to its cognate enzyme elicits a relocation of the active site of protein S relative to the membrane surface and thereby provides a structural explanation for the recently observed protein S-dependent change in the site of factor Va cleavage by APC.

Hemostasis is achieved by maintaining a balance between the molecular interactions that promote blood clot formation and those that inhibit coagulation. Protein C is an important component of one catalytic anticoagulation pathway. When activated, this enzyme (activated protein C or APC) cleaves and inactivates the factor Va (fVa) and factor VIIIa (fVIIa) cofactors that are absolutely required to maintain physiologically-relevant rates of thrombin and hence fibrin formation (for reviews, see Refs. 1–3). Since the fVa and fVIIIa substrates are membrane-bound, their inactivation by APC occurs on a phospholipid surface. APC activity is also modulated by a non-enzymatic protein cofactor, protein S (1–3).

APC inactivation of fVa has been studied much more than that of fVIIIa, in part because a common polymorphism occurs in fV at Arg506, one of the APC-cleavage sites in fVa, and this polymorphism is associated with thrombotic disease (4, 5). This form of fV is generally referred to as fV Leiden, and the resultant phenomenon is referred to as APC resistance. Factor Va inactivation by APC is a complex process involving at least two and probably three proteolytic cleavages (6). Cleavage at Arg506, the polymorphic site leading to APC resistance, results in a partial loss of fVa activity, with the extent of loss depending on the assays employed (6, 7). Cleavage at Arg506 results in complete loss of fVa activity, occurs at a slower rate than cleavage at Arg506 and is enhanced by prior cleavage at Arg506 (6–8).

Protein S binds APC on the membrane surface and increases the affinity of APC for the surface (9). In addition, although fVa is protected from inactivation when bound to factor Xa (10), this protection can be partially overcome by addition of protein S (11, 12). In purified systems, protein S stimulates fVa inac-

1 The abbreviations used are: APC, activated protein C; fVa, factor Va; fXa, factor Xa; protein S, thrombin-inactivated protein S; Fl-FPR-, fluorescein-5(6)-octyl-4-mercaptoprophenylalaniny1-propyl-2-arginyl-; Fl-FPR-APC, APC modified at the active site with Fl-FPR-; Fl-FPR-fXa, fXa modified at the active site with Fl-FPR-; Glu, γ-carboxyglutamic acid; OR, octadecylrhodamine; PC, dioleoylphosphatidylcholine; PS, dioleoylphosphatidylserine; PE, 1,2-dilinoleoylphosphatidylethanolamine; OG, N-succinimidyl 6-glucopyranoside; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; DTT, dithiothreitol.
activation only 2–3-fold (12–16), whereas in plasma, protein S increases the APC anticoagulant activity more than 10-fold (17). Furthermore, protein S deficiency is associated with thrombotic disease (5, 17, 18), which shows that the role of protein S in vivo and in complex systems is more critical and complicated than indicated by assays with purified systems. Recently it was shown that protein S stimulates APC cleavage of fVa at Arg306 and that the rate of this cleavage in the presence of protein S is unaffected by factor Xa (11). Thus, protein S apparently switches the relative rates of the Arg306 and Arg306 cleavages and thereby overcomes protection by factor Xa (11). The mechanism by which this is accomplished has not been identified.

APC and protein S are vitamin K-dependent proteins whose N-terminal Glu residues are post-translationally modified to 4-carboxyglutamic acid (Gla) residues (1–3). As is true of other proteins with Gla domains, both APC and protein S bind to negatively-charged phospholipid surfaces in the presence of Ca²⁺ (9, 19, 20). Based on light scattering data, Nelsenstuen and colleagues (21) concluded that prothrombin and factor X, two elongated vitamin K-dependent proteins, bind to the membrane surface at one end and project radially into the medium. Our subsequent fluorescence energy transfer studies revealed that the active sites of factor Xa (fXa) (22, 23), factor IXa (fIXa) (23), factor VIIa (fVIIa) (24), and meizothrombin (25), a prothrombin derivative, are each located high above the membrane surface, thereby showing that the protease domain was located furthest from the bilayer in each case and confirming that the bound protein was oriented approximately perpendicularly to the plane of the membrane. The same conclusion was reached in another FRET study which showed that the protease domains of prothrombin and meizothrombin are both far above the surface, although in somewhat different locations (26). These spectroscopic results, obtained with membrane-bound proteins under near-physiological conditions, are completely compatible with the recently reported solid-state crystal structures of fIXa (27) and of fVIIa bound to a proteolytic derivative of tissue factor (28). Thus, one goal of the work reported here was to determine to what extent the membrane-bound structure of the anticoagulant APC is similar to those of the procoagulant enzymes.

The activity of each Gla-containing procoagulant enzyme is sufficient to support coagulation only when it is bound to a membrane surface and associated with a protein cofactor (29–31). The specific molecular mechanisms by which the cofactors, with no enzymatic activity of their own, stimulate the enzymatic activity and specificity of their cognate enzymes remain largely undefined. However, four general types of cofactor-mediated changes have been observed. First, cofactor association increases the enzyme concentration at the membrane surface by increasing its affinity for the enzyme (9, 32–34). Second, cofactor-dependent alterations in the conformations of enzyme active sites have been detected (22, 23, 33, 35, 36) that may regulate enzyme activity. Third, the cofactors can bind the substrate (37, 38) and elicit conformational changes, as has been observed when fVa binds to a prothrombin activation intermediate (25), that may allow better complementation between the enzyme and substrate and/or alter the sequence of proteolytic cleavages during activation. Fourth, the cofactors can reposition the active site relative to the membrane surface (22, 24, 39). This mechanism may be particularly relevant when a cofactor is known to alter the relative rates of cleavage of two peptide bonds within the substrate, as is the case with the prothrombin (40) and fVα (11) substrates.

This fourth potentially critical mechanism for cofactor regulation of enzyme activity was discovered by examining the effect of cofactor binding on the location of the enzyme active site above the membrane (22). Specifically, the binding of fVα to fXa on the membrane was found to alter the efficiency of fluorescence resonance energy transfer (FRET) between a donor dye in the active site of fXa and acceptor dyes at the membrane surface (22). Thus, the height and/or orientation of the active site probe was changed when fVα associated with fXa on the membrane. This cofactor-dependent movement of the fXa active site groove presumably occurs to position the active site in the intact prothrombinase complex at both the proper height and orientation to optimize the cleavage of peptide bonds in its prothrombin substrate (22). Factor Va also alters the topography of the membrane-bound prothrombin substrate, so a cofactor may align both the enzyme and the substrate on the membrane to maximize the rate of activation (25). Similarly, tissue factor has recently been shown to relocate the active site of fVIIIa upon forming the fVIIIa-tissue factor complex (24), and the binding of thrombin to thrombomodulin also positions the active site of the thrombin-thrombomodulin complex at a specific location above the membrane surface (39). The association of fVIIIa with membrane-bound fIXa did not result in a detectable change in energy transfer (23), but it is not clear whether no cofactor-dependent change in topography occurred or whether the magnitudes of cofactor-dependent rotational and translational movements offset each other so as to yield no net change in energy transfer efficiency. In this paper we show that protein S relocates the active site of APC, thereby providing a structural explanation for the protein S-dependent alteration in APC cleavage of fVa (11) discussed above.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Sucinimydyl acetylatedioacetate, octadecylrhodamine (OR), and 5-iodoacetamido fluorescein were purchased from Molecular Probes, Eugene, OR; dioleoylphosphatidylethanolamine (PC), dioleoylphosphatidylserine (PS), and 1,2-dilinoleoylphosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids, Alabaster, AL. l-3-Phosphatidylethanolamine-1,2-di[1-¹⁴C]oleoyl (1-¹⁴C)PC was purchased from Amer sham. b-Phenylalanyl-l-prolyl-l-arginyl (FPR) chloromethylketone was purchased from Calbiochem, La Jolla, CA. Spectrozyme PCa and Spectrozyme TH were obtained from American Diagnostica (New York). L-α-Octyl-β-n-glucopyranoside (OG) was obtained from Sigma.

**Proteins**—Bovine protein C (10, 41), bovine protein S (13), bovine thrombin (42), human thrombin (43), bovine fVa (44), and bovine anti-thrombin III (45) were prepared according to procedures published earlier. For some experiments, fVa was purchased from Hematologic Technologies, Essex Junction, VT. Proteinase K was purchased from Sigma. Protein concentrations were determined using the following values for molecular weight and ε₅₃₅ for bovine thrombin, 99,000 and 21.0 (43); human thrombin, 99,000 and 21.0 (43); human thrombin, 96,000 and 13.7 (46); protein S, 72,000 and 10.0 (13); bovine thrombin, 37,000 and 21.0 (43); human thrombin, 36,000 and 17.3 (47); fVa, 174,000 (48) and 15.0 (49); and antithrombin III, 56,000 and 6.0 (50).

**Activation of Protein C**—Bovine protein C (1 mg) was activated in buffer A (50 mM HEPES (pH 7.5), 150 mM NaCl) plus 5 mM EDTA by incubation (2.5-ml final volume) with 50 μg of human thrombin at 37 °C, 400 units/ml in a detection mixture and antisera against APC activity. For some experiments, fVa was purchased from Hematologic Technologies, Essex Junction, VT. Proteinase K was purchased from Sigma. Protein concentrations were determined using the following values for molecular weight and ε₅₃₅ for bovine thrombin, 99,000 and 21.0 (43); human thrombin, 99,000 and 21.0 (43); human thrombin, 96,000 and 13.7 (46); protein S, 72,000 and 10.0 (13); bovine thrombin, 37,000 and 21.0 (43); human thrombin, 36,000 and 17.3 (47); fVa, 174,000 (48) and 15.0 (49); and antithrombin III, 56,000 and 6.0 (50).

**Activity Assays**—Chromogenic assays of APC activity were done as described elsewhere (41). Factor Va inactivation was monitored by a three-stage assay similar to that published earlier (51) and described in detail elsewhere (52).

**Active Site-directed Labeling of APC**—Immediately following the activation incubation, APC (1 mg) was incubated at room temperature in 2.5 ml of buffer A plus 5 mM EDTA with a 5–10-fold molar excess of N-[(acetythio)acetyl]-FPR-chloromethylketone that had been synthesized as described by Bock (53). The extent of active site labeling was monitored by the loss of enzymatic activity of APC using Spectrozyme PCa and the labeling reaction was typically allowed to proceed until the APC was 99.9% inactive. The inactivated APC was dialyzed against...
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1 liter of buffer A plus 1 mM EDTA at 4 °C for 15 h with 2 changes to remove unreacted reagent. The resulting preparation was treated with a 25-fold molar excess of 5-(iodoacetamido)flourescein in the presence of 0.1 mM NH₂OH for 2 h at 25 °C, and then chromatographed on a Sephadex G-25 column (0.7-cm, inner diameter, × 20 cm) in buffer A plus 1 mM EDTA. The sample was dialyzed against buffer A plus 1 mM EDTA in a water bath at room temperature to remove free dye by ion-exchange chromatography at room temperature (Mono-Q HR 5/5, Pharmacia) using a linear 100–600 mM NaCl gradient (20 ml total) in 50 mM HEPES (pH 7.5), 1 mM EDTA. The fluorescent APC, designated Fl-FPR, was isolated from a single 450-nm band-pass filter. Fl-FPR-APC was dialyzed into buffer A plus 2 mM CaCl₂ at 4 °C before being aliquoted into convenient sizes, frozen in liquid nitrogen, and stored at −95 °C. In some cases, the Fl-FPR-APC was further purified by gel filtration (Superdex 200, Pharmacia; buffer A plus 5 mM EDTA; room temperature) prior to dialysis and freezing as above. The number of dyes per protein molecule in each Fl-FPR-APC preparation was determined as described by Bock (53). Fl-FPR-αxa (23), Fl-FPR-IXa (23), and Fl-FPR-Vila (24) were prepared as described previously.

Thrombin-cleaved Protein S—Protein S (0.5 mg) was incubated with bovine a-thrombin (2.5 µg) for 2 h at 37 °C in 1 ml of buffer A plus 5 mM EDTA (54). The reaction was stopped by the addition of a 5-fold molar excess of FPR-chloromethylketone over thrombin. The thrombin-cleaved protein S was then dialyzed into buffer A plus 2 mM CaCl₂ at 4 °C.

Phospholipid Vesicles—Small unilamellar vesicles (SUV) of PC/PS (the molar ratio of PC to PS was 4:1) were prepared by sonication and centrifugation as described previously (22). Samples containing OR were prepared in the same way except that the desired amount of OR (in ethyl acetate) was added to the phospholipid prior to lyophilization and sonication (22). PC/PS/PE vesicles were prepared identically, except that the molar ratio of PC to PS to PE was 2:2:1 (51).

Large unilamellar vesicles (LUV) were prepared by dialysis as before (39), except that dioleyl PC and PS were used here. Parallel phospholipid samples (2.5 mg containing trace amounts of [³⁵S]PC and either 0 or 10–35 nmol of OR) were mixed with OG at a molar ratio of 1:20 (phospholipid to OG) and resuspended in 5.2 ml of buffer A. OG was then removed by dialysis against 1 liter of buffer A for 72 h at 4 °C with changes after 24 and 48 h.

The concentration of phospholipid in a purified SUV or LUV sample was determined from the recovery of radioactive [³⁵S]PC as before (22). The concentration of OR in a phospholipid sample was determined from the absorbance at 564 nm using a molar extinction coefficient of 85,400 M⁻¹ cm⁻¹ (55). The surface density of bound dye (e, in OD units) was calculated by using molecular weights of 786 for PC, 810 for PS, and 736 for PE; by assuming that each phospholipid molecule occupies 70 Å² of surface area (56); and by assuming that the acceptors were distributed uniformly and randomly on the surface of the bilayer. Both LUV and SUV vesicles were stored at 4 °C and used within 2 weeks with no detectable change in binding or spectroscopic properties.

Fluorescence Measurements—Fl-FPR-APC binding to vesicles was measured by excitation at 490 nm and emission at 520 nm. Flow cytometry data showed that the background signal was less than 0.1% of the average signal of samples containing Fl-FPR-APC binding to vesicles. The calcium dependence of Fl-FPR-APC binding to vesicles was measured as described in the section on energy transfer. The calcium dependence of Fl-FPR-APC binding to vesicles was measured as described in the section on energy transfer. The calcium dependence of Fl-FPR-APC binding to vesicles was measured as described in the section on energy transfer.
valently tethered via the FPR tripeptide to the active site histidine and serine (61). For the experiments discussed in this paper, the presence of non-fluorescein-labeled APC molecules in a sample does not interfere with the interpretation of the spectroscopic data.

**Spectral Properties of Free and Membrane-bound Fl-FPR-APC**—The corrected wavelength of maximum emission and the average values for the quantum yield ($Q$), steady-state anisotropy ($r$), and fluorescence lifetime ($\tau$) of the fluorescein dye in Fl-FPR-APC were found to be 520 nm, 0.30, 0.20, and 4.0 ns, respectively, in buffer A plus 2 mM CaCl$_2$. Upon addition of either PC/PS or PC/PS/PE vesicles to a sample of Fl-FPR-APC, the spectral properties of the fluorescein did not change significantly (data not shown and, OR data in Fig. 1). Thus, the binding of APC to a membrane surface did not elicit a detectable alteration in the environment of the fluorescein dye in the APC active site.

**Fluorescence Energy Transfer: Active Site to Phospholipid Surface**—The fluorescein in the active site of APC was the donor dye in the singlet-singlet energy transfer experiments, while the rhodamine in OR was the acceptor dye. The rhodamine dye is charged at pH 7.5, so it remains in the aqueous phase while the long octadecyl aliphatic chain of OR partitions into the hydrophobic interior of the bilayer, thereby anchoring the rhodamine moiety at the phospholipid surface (55).

When Fl-FPR-APC was titrated with PC/PS vesicles (the donor-only or D sample), no change in fluorescein emission intensity was observed (Fig. 1). However, when Fl-FPR-APC was titrated with PC/PS vesicles containing OR (PC/PS/OR; the donor + acceptor or DA sample), the fluorescein intensity decreased until sufficient rhodopsin had been added to bind all of the Fl-FPR-APC (Fig. 1). To facilitate analysis, the data of Fig. 1 have been normalized and expressed as the ratio of the donor quantum yields in the presence and absence of acceptor (Fig. 2). The OR-dependent decrease in fluorescein intensity evident in Figs. 1 and 2 indicates that the donor dyes are close enough to the acceptor dyes for FRET to occur.

**Reversibility of FRET**—At the low concentrations of Fl-FPR-APC and OR used in our experiments, the average separation between free Fl-FPR-APC and OR molecules would be too large for detectable energy transfer to occur. Thus, if Fl-FPR-APC is released from the membrane surface, no energy transfer should occur and the $Q_{DA}/Q_D$ value should return to 1.0. In previous studies (22–25, 60), excess EDTA was added at the end of tiritations to chelate the calcium ions and thereby release the vitamin K-dependent proteins from the PC/PS surface. However, we found in our initial experiments that EDTA-stimulated dissociation of APC from PC/PS vesicles was too slow and incomplete to allow us to use this approach for examining the reversibility of APC-to-OR FRET. This unexpected property of APC has since been thoroughly characterized and will be described elsewhere.²

We therefore used three different methods to release the donor dye from the membrane surface and test the reversibility of the observed acceptor-dependent decrease in donor emission intensity. In one method, excess DTT was added to the samples to reduce the disulfide bond between the two chains of APC and thereby release the fluorescein-labeled heavy chain from the vesicle surface. In a second approach, excess IVa was added to the samples at the end of the titration to compete with the weaker-binding APC for the limited amount of phospholipid surface and thereby prevent Fl-FPR-APC binding to the PC/PS vesicles. In a third approach, fluorescein donor dyes were released from the membrane surface by proteolytically digesting Fl-FPR-APC with proteinase K. Each approach gave the same results, both qualitatively and quantitatively: the $Q_{DA}/Q_D$ values increased dramatically, but not to the expected value of 1.0 (Fig. 2; only a reversal by DTT is shown here). Instead, final $Q_{DA}/Q_D$ values ranged from 0.94 to 0.99, depending upon the total concentration of OR in the sample, even though gel filtration experiments confirmed that no fluorescein was bound to the vesicles (see below) after any of the treatments.

As we have documented previously (24), the small residual acceptor-dependent decrease in donor intensity (Fig. 2) observed using these three independent approaches is due primarily to an inner filter effect, specifically a reduction in detected fluorescein emission caused by OR absorption of excitation and emitted light. This was confirmed by showing that the observed intensities of samples of disodium fluorescein and DTT-treated Fl-FPR-APC were reduced to the same extent by the addition of equivalent amounts of PC/PS-OR (data not

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when excess Ca\(^{2+}\) concentrations using the methods described above. However, transfer was determined over a wide range of intensity (though Fl-FPR-APC could not bind to the vesicles in these QDA magnitude to the final PC/PS SUV (\(\triangle\)) vesicles with different \(\sigma\) values and the final \(Q_{DA}/Q_{D}\) values are plotted. The best-fit lines, determined by linear regression, have been required to go through (0, 1). The average values of \(L\) determined from these data are 94 and 84 Å in the absence and presence of protein S, respectively, as summarized in Table I.

**Calcium Ion Dependence of FRET—Membrane-binding independent effects on donor emission intensity can also be demonstrated by titrating PC/PS-OR into a sample of Fl-FPR-APC under conditions in which the protein cannot bind to the membrane surface. Since APC binding to a PC/PS surface requires Ca\(^{2+}\) (9, 19, 20), we titrated Fl-FPR-APC with PC/PS (2\(\sigma\)) OR in the absence of calcium ions (buffer A plus 1 mM EDTA). Even though Fl-FPR-APC could not bind to the vesicles in these samples, a small acceptor-dependent decrease in fluorescein intensity \(Q_{DA}/Q_{D}\) was observed (Fig. 2) that was equivalent in magnitude to the final \(Q_{DA}/Q_{D}\) values obtained at the same OR concentrations using the methods described above. However, when excess Ca\(^{2+}\) ions were added to the calcium-free samples, the Fl-FPR-APC bound to the PC/PS surface and the resulting extent of energy transfer was the same as observed in an equivalent sample that had contained Ca\(^{2+}\) from the beginning (Fig. 2). These data therefore demonstrate the calcium dependence of APC binding to the PC/PS vesicles.

These data also show that the small amount of acceptor-dependent reduction in donor emission observed in the absence of Ca\(^{2+}\) (Fig. 2) is not due to energy transfer from membrane-bound Fl-FPR-APC to OR. As a result, we have calculated the distance between the donor and acceptor dyes using only the changes in donor emission that are due to the membrane binding-dependent change in \(Q_{DA}/Q_{D}\).

**Distance of Closest Approach: APC Active Site to Membrane Surface—\(R_{c}\), the distance between donor and acceptor dyes at which FRET efficiency is 50%, was calculated as before (23) assuming that the refractive index was 1.4 and the orientations of the transition dipoles of the dyes were random during the lifetime of the excited state of the donor dye (\(\kappa^2 = 2/3\)). The spectral overlap integral for Fl-FPR-APC and OR was found to be \(3.58 \times 10^{-15} \, \text{M}^{-1} \, \text{cm}^{-1} \, \text{nm}^{-4}\), and this yielded an \(R_c\) of 50.3 Å for this donor-acceptor pair.

Since the magnitude of the observed FRET depends upon the density of OR at the membrane surface (\(\sigma\)), the extent of energy transfer was determined over a wide range of \(\sigma\) values. The results from 17 independent experiments are shown in Fig. 3 (open symbols), and the data show the expected (see Equation 2) linear relationship between \(Q_{DA}/Q_{D}\) and \(\sigma R_c^2\). In these experiments, the distance of closest approach between the plane of donor dyes in the active sites of membrane-bound APC and the plane of acceptor dyes at the membrane surface, ranged from 87 to 98 Å in these experiments, with an average value and standard deviation of 94 \(\pm\) 4 Å. The uncertainty noted here reflects the random experimental error in our experiments. There is no apparent dependence of \(L\) on the method used to release Fl-FPR-APC from the membrane because the average \(L\) values obtained for titrations reversed by the addition of DTT, proteinase K, or IVa were 94, 93, and 94 Å, respectively.

The accuracy of this determination of \(L\) depends in part on the homogeneity of the sample in two important respects: do all of the Fl-FPR-APC molecules bind to the PC/PS vesicles, and is OR distributed uniformly and randomly in the bilayer? To address the first issue, we incubated Fl-FPR-APC with a large excess of PC/PS vesicles in the presence of Ca\(^{2+}\) and then analyzed the sample using gel filtration. We have shown previously that by using a substantial excess of PC/PS, the Fl-FPR-APC molecules that dissociate from a PC/PS vesicle during the chromatography are much more likely to reassociate with another vesicle than to enter the pore of a gel filtration bead (24). As a result, Fl-FPR-APC molecules that bind to PC/PS vesicles will elute near the void volume under these conditions, while any Fl-FPR-APC molecules unable to bind to the PC/PS surface will elute in the included volume. Since virtually all of the fluorescent material co-eluted from the column in the void volume with the radioactive vesicles (Fig. 4A), we conclude that all (>98%) of the Fl-FPR-APC molecules in our preparations bind to the PC/PS vesicle surface and therefore participate in FRET. In contrast, no fluorescence was detected co-eluting with the radioactive vesicle peak when the incubation lacked Ca\(^{2+}\) (Fig. 4B) or after DTT had been added to membrane-bound Fl-FPR-APC (Fig. 4C). Our Fl-FPR-APC preparations were also homogeneous chromatographically, electropheretically, and spectroscopically: the single discrete fluorescein lifetime observed in samples containing Fl-FPR-APC indicates that all of the dyes were in the same environment. Thus, the Fl-FPR-APC is fully active in terms of binding to PC/PS vesicles.

The linear dependence of \(Q_{DA}/Q_{D}\) on \(\sigma\) in our experiments (Fig. 3) strongly indicates that the distribution of OR at the membrane surface was both uniform and random in our experiments because any other distribution of OR, such as a local increase in \(\sigma\) caused by OR binding to APC or protein S, would lead to nonlinearity. Furthermore, no changes in OR absorbance or fluorescence were observed when saturating APC and/or protein S was added to PC/PS-OR vesicles, which suggests that OR does not bind to either APC or protein S. Functional assays also showed no sensitivity to the presence of OR. The rate of inactivation of IVa by APC in the presence of protein S was the same using OR-free PC/PS vesicles or PC/PS vesicles containing a high concentration of OR (\(\sigma = 5.5 \times 10^{-4}\) OB/Å\(^2\)). We therefore conclude that the binding of APC and protein S to the vesicles did not detectably alter the random and uniform distribution of OR.

Thus, the samples appear homogeneous. However, some uncertainty in \(L\) remains because \(\kappa^2\) cannot be determined experimentally. The above \(L\) values and those in Table I were calculated assuming that \(\kappa^2 = 2/3\), but this value is correct only when the donor and acceptor dyes can sample all orientations during the excited state lifetime of the donor. In fact, the actual rotational freedom is somewhat restricted for both the donor and acceptor dyes, as shown by the anisotropies of 0.20 for Fl-FPR-APC and 0.22 for membrane-bound OR (22). Using these anisotropy values to calculate the maximum range of \(\kappa^2\)
values for this donor-acceptor pair (62), the actual \( R_0 \) will be somewhere between \( 2^{19} \) and \( 2^{26} \) of the \( R_0 \) value. However, because the acceptor dyes are oriented randomly in the plane of the membrane (63, 64) and also because the separation between the donor and acceptor dyes is significantly greater than \( R_0 \) (65), a more reasonable estimate of the uncertainty in \( R_0 \) due to \( \kappa^2 \) is \( \pm 10\% \) (55, 63). Thus, the FRET data clearly demonstrate that the active site of membrane-bound APC is located far above the phospholipid surface.

**Active Site Location Is Unaffected by PE and Vesicle Curvature**—The 17 experiments shown in Fig. 3 include some important variations. First, although FI-FPR-APC was titrated in most cases with PC/PS(±OR) SUV, in two instances the FI-FPR-APC was titrated with PC/PS/PE(±OR) vesicles because PE has been shown to increase substantially the affinity of APC for a phospholipid surface (51). When FI-FPR-APC was titrated with PC/PS/PE vesicles containing OR, the fluorescein emission intensity decreased and reached a plateau after the addition of only 11 \( \mu \)M PC/PS/PE (data not shown). This contrasts with the 30 \( \mu \)M PC/PS that was typically required to obtain complete binding of FI-FPR-APC in a comparable experiment (cf. Fig. 1). Our energy transfer results therefore confirm the significantly higher affinity of APC for PC/PS/PE than for PC/PS. On the other hand, the tighter binding does not alter the overall topography of the membrane-bound APC because there was no significant difference in the average \( L \) values calculated from experiments using PC/PS or PC/PS/PE (Table I). We conclude that the orientation of the elongated APC is the same when it bound to vesicles containing either PC/PS or PC/PS/PE, even though the latter vesicles bind much more tightly to APC and may have a different surface topography because of the PE.

Second, the same \( L \) value was obtained for both large and small unilamellar vesicles (Table I). Thus, the radius of curvature of the membrane surface and the method of vesicle preparation did not detectably alter either the angle at which APC projects from the membrane surface or the distance between the active site and the surface. This result is most easily explained if the APC projects approximately perpendicularly from the bilayer.

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**TABLE I**

**Summary of FI-FPR-APC to OR fluorescence energy transfer results**

| Protein S | Phospholipid | Vesicle | \( n \) | \( L \) Å |
|-----------|--------------|---------|-------|--------|
| +         | PC/PS        | SUV     | 13    | 94 ± 3 |
| -         | PC/PS        | LUV     | 2     | 95     |
| -         | PC/PS/PE     | SUV     | 2     | 91     |
| +         | PC/PS        | SUV     | 7     | 84 ± 3 |
| +         | PC/PS        | LUV     | 2     | 81     |
| +         | PC/PS/PE     | SUV     | 2     | 83     |

\( ^a \) \( L \) was calculated using Equation 2 and an \( R_0 \) of 50.3 Å (\( \kappa^2 = 2/3 \)). Average values for \( n \) independent determinations are shown. Standard deviations are reported for PC/PS SUV determinations; individual \( L \) values were within 2 Å of the average value for the other experiments.

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**FIG. 4. FI-FPR-APC binding to PC/PS vesicles.** FI-FPR-APC (50 nM) was incubated with 560 \( \mu \)M PC/PS SUV vesicles in buffer A containing either 2 mM CaCl\(_2\) (A), 5 mM EDTA (B), or 2 mM CaCl\(_2\) and 200 mM DTT (C) and then chromatographed over a Superdex 200 gel filtration column. Each fraction was examined for FI-FPR-APC content by fluorescence intensity (●) and for phospholipid content ([14C]PC) by scintillation counting (○).
**Protein S Relocates the Active Site of Membrane-bound APC**

**Cofactor Dependence of Fl-FPR-APC Emission**—When an excess of protein S cofactor was added to a sample of Fl-FPR-APC bound to PC/PS vesicles, no changes in fluorescence emission intensity, lifetime, or anisotropy were observed. The binding of the cofactor to APC therefore does not appear to alter the conformation of the active site near S in a manner that influences the environment of the probe.

**Protein S Dependence of Energy Transfer**—To investigate the effect of the cofactor on the Fl-FPR-APC to OR energy transfer, samples of membrane-bound Fl-FPR-APC were prepared with sufficient PC/PS vesicles (≥OR as necessary) to bind both the APC and an excess of the protein S cofactor. When Fl-FPR-APC/PC/PS was then titrated with protein S, no change in fluorescence emission was observed (Fig. 5A, −OR). However, when protein S was titrated into the Fl-FPR-APC/PC/PS-OR sample, the donor intensity decreased until reaching a constant value when, presumably, every Fl-FPR-APC molecule was bound to protein S (Fig. 5A, +OR). The protein S-dependent decrease in $Q_{DA}/Q_D$ (Fig. 5B) reveals that the fluorescein-to-rhodamine FRET is more efficient for the membrane-bound Fl-FPR-APC-protein S complex than for membrane-bound Fl-FPR-APC. This is clearly evident when one compares the 11 measurements of $L$ made in the presence of protein S and the 17 measurements made in the absence of protein S (Fig. 3). The difference in the average $Q_{DA}/Q_D$ values obtained in the presence and absence of protein S depicted in Fig. 3 is significant at the 99% confidence level (unpaired $t$ test, $p < 0.01$).

This cofactor-dependent change in FRET occurred only with functional protein S. When protein S was inactivated by thrombin cleavage (66) and then titrated into a sample containing Fl-FPR-APC/PC/PS-OR, no change in $Q_{DA}/Q_D$ was observed (Fig. 5B). This observation strongly suggests that the functional activity of protein S and its ability to relocate the APC active site are linked.

The increased energy transfer results from a protein S-dependent movement of the active site probe relative to the membrane surface. This movement could be translational (closer to the membrane and hence a change in $L$), rotational (to a more parallel alignment of the donor and acceptor transition dipoles and hence a change in $R_0$), or a combination of the two. Since protein S elicited no change in fluorescein lifetime, the quantum yield of the fluorescein donor was unaffected by protein S binding to Fl-FPR-APC. Similarly, the spectral overlap was the same in the presence and absence of protein S. Thus, any change in $R_0$ would have to originate from a change in $\kappa^2$. A change in $\kappa^2$ could occur either because the entire active site groove and protease domain rotated relative to the bilayer surface or because changes in the local environment altered the rotational freedom of the donor dye. Since the fluorescein anisotropy was unchanged by the addition of protein S, the local active site environment of the fluorescein was unaltered by protein S binding to Fl-FPR-APC. Thus, the cofactor may alter $\kappa^2$ (and hence $R_0$) by reorienting the active site groove upon binding to Fl-FPR-APC. If, however, we assume that $\kappa^2$ is unchanged and equal to two-thirds, then the $R_0$ value for this donor-acceptor pair is the same in the presence and absence of protein S. Using 50.3 Å for $R_0$, the average value and standard deviation of $L$, the distance of closest approach between the membrane surface and the active site in the APC-protein S complex, is $84 ± 4$ Å (Table I). Thus, if one assumes that the protein S effect is entirely translational (i.e., $\kappa^2$ is unaltered by protein S binding), then the formation of the enzyme cofactor complex causes the active site groove to move 10 Å closer to the membrane surface. Protein S binding to APC therefore substantially alters the height and/or the orientation of the active site above the membrane.

The protein S effect on APC topography is not sensitive to the presence or absence of PE at the membrane surface, since the same $L$ values were obtained ± protein S for PC/PS and PC/PS/PE vesicles (Fig. 3; Table I). Similarly, the same protein S-dependent change in $L$ was observed for both SUV and LUV (Fig. 3; Table I).

**Is the Protein S Effect Specific for APC?**—To determine whether protein S only elicits a change in the membrane-bound topostructure of its cognate enzyme, the effect of protein S on the active site-to-OR FRET efficiency was examined using three other fluorescent-labeled vitamin K-dependent enzymes. When an excess of protein S was titrated into a sample containing membrane-bound Fl-FPR-IXa, Fl-FPR-tXa, or Fl-FPR-tXIIa, no alteration in the efficiency of energy transfer was observed in any of the three cases (Table II). Thus, even though protein S has been shown to bind to membrane-bound IXa (67, 68) and we confirmed the protein S-dependent spectral changes upon binding Fl-FPR-tXa (data not shown), that association does not cause a detectable movement in the protease domain of IXa.
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**TABLE II**

| Fluorescent protein | Without protein S | With protein S | ΔL′ |
|---------------------|-------------------|----------------|-----|
|                     | A                 | A              | A   |
| FI-FPR-APC          | 50.3              | 17             | 11  |
| FI-FPR-fXa          | 57.7              | 5              | 5   |
| FI-FPR-fIXa         | 56.8              | 2              | 2   |
| FI-FPR-fVIIa        | 53.6              | 2              | 2   |

*ΔL′ values were determined from the ratio of the observed lifetimes for the FRET donor and acceptor, respectively, in the absence and presence of protein S. The values are given as the average of at least three independent experiments. The standard deviations for each experiment were typically less than 10%.

**DISCUSSION**

The present study reveals that the active site of membrane-bound APC is approximately 94 Å from the membrane surface. As discussed above, this direct measurement by FRET under near-physiological conditions has an uncertainty of less than ±10%. This distance is entirely compatible with the recent crystal structures of APC lacking the Gla domain (Gla-domainless APC) (61) and of the Gla domain in prothrombin fragment 1 (69). In Gla-domainless FPR-APC, the distance from the D-Phe to the base of the first epidermal growth factor domain is approximately 65 Å. If one assumes that the Gla domains of prothrombin fragment 1 and protein C are structurally similar, as predicted from the high sequence identity in the two domains and the similarity in their function, then the Gla domain would add between 20 and 30 Å to the height of the active site, depending on the extent to which the hydrophobic loop of the Gla domain inserts into the membrane (70). Assuming no insertion, the active site-to-membrane surface distance would be about 95 Å, while this distance would be about 85 Å if the Gla domain is maximally inserted. This estimate further assumes that the epidermal growth factor domains of APC sit on top of the Gla domain. Thus, the values for L determined experimentally by FRET and estimated from crystallographic data agree quite closely. It is therefore clear that the elongated APC protein binds to the membrane at one end and extends radially from the surface, a topographical motif that minimizes the phospholipid surface area occupied by the protein. Similar distances and conclusions have been obtained in previous studies with VIIa (24), IXa (23), FXa (22, 23), meizothrombin (25, 26), and prothrombin (26). Thus, many of the proteolytic cleavages that effect and regulate hemostasis occur far above the membrane surface.

The association of protein S with APC on the membrane surface increases the efficiency of FRET, thereby showing that the active site of APC moves upon binding to its cofactor. This structural change is specific, since inactive, thrombin-cleaved protein S, did not elicit the change, nor did protein S alter the locations of the VIIa, IXa, or IXa active sites. If one assumes that the FRET-detected movement of the active site probe is solely translational (see “Results”), then the formation of the protein S-APC complex causes the APC active site to move 10 Å closer to the membrane. A possible functional reason for this relatively large structural change is suggested by the strong correlation between two protein S-dependent events: the FRET-detected movement of the APC active site and the shift in APC cleavage of FVa from Arg506 to Arg506, the site that completely inactivates FVa and is not protected by IXa (11). Since a protein S-dependent increase in APC membrane binding affinity would not be expected to mediate a change in cleavage site, and since the effect of protein S on Arg506 cleavage is IXa-independent, this change in cleavage site must result from a direct interaction between protein S and APC that alters its active site topography and/or conformation. Since the FRET data show that the binding of protein S to APC repositions its active site at a unique location above the membrane, it seems likely that this topographical change directs the APC active site to a different cleavage site on the FVa.

Finally, it seems more than a coincidence that protein S, tissue factor (24), thrombomodulin (39), and FVa (22) each relocate the active sites of their cognate enzymes above the membrane, and that FVa alters the orientation of its membrane-bound substrate (25). We therefore suggest that protein-dependent topographical changes provide a general mechanism for regulating enzyme activity when substrate, enzyme, and cofactor must all bind to a common plane of reference.

**REFERENCES**

1. Esmon, C. T. (1989) J. Biol. Chem. 264, 4743–4746
2. Walker, F. J., and Fay, P. J. (1992) FASEB J. 6, 2561–2567
3. Esmon, C. T. (1990) in Hemostasis, Basic Principles and Practices (Hoffman, R. B., Esmon, C. T., Foucar, E. E., Scott, R. P., and Schumacher, R. H., eds) Vol. 2, pp. 1597–1605, Churchill Livingstone, New York
4. Bertina, R. M., Koelman, B. P. C., Koster, T., Rosendaal, F. R., Dirven, R. J., van Rood, H., van den Velden, P. A., and Retisma, P. H. (1994) Nature 369, 64–67
5. Dahlöf, B. (1994) J. Clin. Invest. 94, 923–927
6. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) J. Biol. Chem. 269, 31889–31890
7. Nicolaes, G. A. F., Tans, G., Thomassen, M. C. L. G. D., Hemker, H. C., Fabinger, I., Varadi, K., Schwarz, H. W., and Rosing, J. (1995) J. Biol. Chem. 270, 21158–21166
8. Kalafatis, M., Bertina, R. M., Rand, M. D., and Mann, K. G. (1995) J. Biol. Chem. 270, 4053–4057
9. Walker, F. J. (1991) J. Biol. Chem. 266, 11288–11311
10. Walker, F. J., Sexton, P. W., and Esmon, C. T. (1979) Biochem. Biophys. Acta 571, 333–342
11. Rosing, J., Hoekema, L., Nicolaes, G. A. F., Thomassen, M. C. L. G. D., Hemker, H. C., Varadi, K., Schwarz, H. W., and Tans, G. (1995) J. Biol. Chem. 270, 27852–27858
12. Polymyos, S., Tucker, M. M., and Tracy, P. B. (1988) J. Biol. Chem. 263, 14884–14890
13. Walker, F. J. (1980) J. Biol. Chem. 255, 5521–5524
14. Dahlöf, B. (1986) J. Biol. Chem. 261, 12222–12227
15. Tans, G., Rosing, J., Thomassen, M. C. L. G. D., Heeb, M. J., Zwaal, R. F. A., and Griffin, J. H. (1991) Blood 77, 2641–2648
16. Bakker, H. M., Tans, G., Janssen-Claessen, T., Thomassen, M. C. L. G. D., Hemker, H. C., Griffin, J. H., and Rosing, J. (1992) Eur. J. Biochem. 208, 171–178
17. Comp, P. C., Nixon, R. R., Cooper, M. R., and Esmon, C. T. (1984) J. Clin. Invest. 74, 2082–2088
18. Walker, F. J. (1992) Proc. Soc. Exp. Biol. Med. 200, 285–295
19. Nelsestuen, G. L., Kisel, W., and Di Scipio, R. G. (1978) Biochemistry 17, 2134–2138
20. Krishnaswamy, S., Williams, E. B., and Mann, K. G. (1986) J. Biol. Chem. 261, 9684–9689
21. Lim, T. K., Bloomfield, V. A., and Nelsestuen, G. L. (1977) Biochemistry 16, 4177–4181
22. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) J. Biol. Chem. 262, 12953–12961
23. Mutueumara, Y. P., Duffy, E. J., Lollar, P., and Johnson, A. E. (1992) J. Biol. Chem. 267, 17015–17021
24. McCallum, C. D., Hapak, R. C., Neuen schwander, P. F., Morrissey, J. H., and Johnson, A. E. (1996) J. Biol. Chem. 271, 28168–28175
25. Armstrong, S. A., Husten, E. J., Esmon, C. T., and Johnson, A. E. (1990) J. Biol. Chem. 265, 6210–6218
26. Chen, Q., and Lentz, B. R. (1997) Biochemistry 36, 4701–4711
27. Brandstetter, H., Bauer, M., Huber, R., Lollar, P., and Bode, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9796–9800
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28. Banner, D. W., D’Arcy, A., Chene, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y., and Kirchhofer, D. (1996) Nature 380, 41–46
29. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) J. Biol. Chem. 254, 10952–10962
30. van Dieijen, G., Tans, G., Rosing, J., and Hemker, H. C. (1981) J. Biol. Chem. 256, 3433–3442
31. Bom, V. J. J., and Bertina, R. M. (1990) Biochem. J. 265, 327–336
32. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Hemker, H. C. (1980) J. Biol. Chem. 255, 274–283
33. Nesheim, M. E., Kettner, C., Shaw, E., and Mann, K. G. (1981) J. Biol. Chem. 256, 6537–6540
34. van Rijn, J. L. M. L., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Rosing, J. (1984) Biochemistry 23, 4557–4564
35. Duffy, E. J., Parker, E. T., Mutucumarana, V. P., Johnson, A. E., and Lollar, P. (1992) J. Biol. Chem. 267, 17006–17011
36. Ye, J., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1991) J. Biol. Chem. 266, 23016–23021
37. Luckow, E. A., Lyons, D. A., Ridgeway, T. M., Esmon, C. T., and Laue, T. M. (1989) Biochemistry 28, 2348–2354
38. Vehar, G. A., and Davie, E. W. (1980) Biochemistry 19, 401–410
39. Lu, R., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1989) J. Biol. Chem. 264, 12956–12962
40. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) J. Biol. Chem. 262, 3291–3299
41. Esmon, C. T., Esmon, N. L., Le Bonniec, B., and Johnson, A. E. (1993) Methods Enzymol. 222, 359–385
42. Mileitch, J. P., Jackson, C. M., and Majerus, P. W. (1978) J. Biol. Chem. 253, 6908–6916
43. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) J. Biol. Chem. 249, 594–603
44. Esmon, C. T. (1979) J. Biol. Chem. 254, 964–973
45. Esmon, C. T. (1977) in Chemistry and Biology of Thrombin (Lundblad, R. L., Fenton, J. W., II, and Mann, K. G., eds) pp. 403–411, Ann Arbor Science, Ann Arbor, MI
46. Kiesel, W., Ericsson, L. H., and Davie, E. W. (1976) Biochemistry 15, 4893–4900
47. Thompson, A. R., Enfield, D. L., Ericsson, L. H., Legaz, M., and Fenton, J. W., II (1977) Arch. Biochem. Biophys. 178, 356–367
48. Laue, T. M., Johnson, A. E., Esmon, C. T., and Yphantis, D. A. (1984) Biochemistry 23, 1339–1348
49. Guinto, E. R., and Davie, E. W. (1980) Biochemistry 19, 401–410

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