Relocating the Active Site of Activated Protein C Eliminates the Need for Its Protein S Cofactor

A FLUORESCENCE RESONANCE ENERGY TRANSFER STUDY*

(Received for publication, November 20, 1998)

Subramanian Yegneswaran‡‡‡, Mikhail D. Smirnov§§, Omid Safa¶, Naomi L. Esmon¶¶, Charles T. Esmon¶¶¶, and Arthur E. Johnson†††

From the ‡‡‡Department of Medical Biochemistry and Genetics, Texas A&M University Health Sciences Center, College Station, Texas 77843-1114, the §§Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, the Departments of ¶¶Pathology and ¶¶¶Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190, the †††Howard Hughes Medical Institute, Oklahoma City, Oklahoma 73104, and the §§§Departments of Chemistry and of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843.

Activated protein C (APC) functions as a potent anticoagulant in vivo by proteolytically inactivating factors Va and VIIIa, two cofactors that are essential for blood clot formation (1–3). APC inactivation of factor Va is a complex process involving at least two proteolytic cleavages. APC cleaves factor Va rapidly at Arg506 and slowly at Arg506, and cleavage at Arg506 totally inactivates factor Va (4–7). Protein S is the cognate cofactor for membrane-bound APC (8), and protein S stimulates the rate of factor Va cleavage at Arg506 by 20-fold (9).

APC is a vitamin K-dependent anticoagulant enzyme that has extensive structural homology to other vitamin K-dependent enzymes (for reviews see Refs. 1–3). The N-terminal module (amino acids 1–36) of human APC contains 9 γ-carboxyglutamic acid (Gla) residues and is termed the Gla domain. The Gla domain is followed by a domain rich in aromatic residues (also known as the aromatic stack), by two domains that are homologous to the epidermal growth factor, and then by a serine protease domain that contains the active site.

As is true for other vitamin K-dependent plasma proteins, APC binds via the Gla domain to membranes containing negatively charged phospholipids in the presence of calcium ions (1–3, 10). Light scattering experiments indicated that two elongated vitamin K-dependent proteins, prothrombin (PT) and factor X, project radially from the surface when bound to the membrane (11), and our fluorescence resonance energy transfer (FRET) experiments showed that the active site of each of the vitamin K-dependent enzymes is located far (>70 Å) above the membrane surface (12–16), thereby indicating that they project approximately perpendicularly from the membrane surface. In the case of membrane-bound APC, its active site is located an average of 94 Å above the surface (assuming k^2 = 2/3; Ref. 12). Our FRET study also revealed that protein S relocates the active site of membrane-bound APC to a unique position above the membrane surface (84 Å, assuming k^2 = 2/3). These FRET results therefore provide a possible structural explanation for the protein S-dependent alteration in the APC cleavage site on factor Va from Arg506 to Arg306.

Because of the sequence similarity of the Gla domains in different vitamin K-dependent proteins, it has been assumed that the Gla domains of all vitamin K-dependent enzymes must be structurally and functionally similar (17). Consistent with this view, exchanging the Gla domain of factor VIIa for that of APC had no effect on APC plasma anticoagulant activity (18). On the other hand, replacing the Gla domain of factor IXa with that of factor VIIa decreased the V_max for factor X activation (19). Furthermore, the membrane binding affinities of vitamin K-dependent plasma proteins differ (e.g. Ref. 10). In addition, unique protein-protein interactions sometimes involve the Gla domain. For example, factor IXa, factor VIIa, and protein C have been shown to bind specifically to collagen IV (20), tissue factor (21), and endothelial protein C receptor (22), respec-
tively, through the Gla domain.

Recently, Smirnov et al. replaced the Gla domain and the aromatic stack of APC with the corresponding domains of prothrombin to form the APC-PTGla chimera (23). Exchange of the Gla domains did not alter the affinity for phosphatidylcholine/phosphatidylserine vesicles significantly but did increase the rate of factor Va inactivation on these vesicles. Furthermore, the activity of the chimera was not increased by protein S. In this study, we test the hypothesis that the location of the active site in the chimera may be similar to that in the APC-protein S complex and thereby explain the increased activity and protein S-independence exhibited by the chimera.

**EXPERIMENTAL PROCEDURES**

*Reagents—*Succinimidyl acetylthioacetate, octadecylrhodamine (OR), and 5-(iodoacetamido)fluorescein were obtained from Molecular Probes, Eugene, OR. Dieoleoylphosphatidylcholine (PC) and dioleoylphosphatidylserine (PS) were purchased from Avanti Polar Lipids, Alabaster, AL. 1-O-Di(3-iodoacetamido)proplyl-1-arginyI (FPR) chloromethylketone was purchased from Calbiochem. Spectrozyme PCa and Spectrozyme TH were purchased from American Diagnostica (New York).

*Proteins—*Human protein C (24), protein C-PTGla chimera (25), thrombin (26), protein S (25), and antithrombin III (29); and antithrombin III, 56,000 and 6.0 (30). Protein C-PTGla and human protein C were then generated essentially as described for bovine APC (12).

**Active Site-directed Labeling of Human APC-PTGla—**The phospholipid and OR in a purified vesicle sample was obtained from Amersham Pharmacia Biotech. D-Phenylalanyl-L-prolyl-L-arginyl (FPR) chloromethylketone was purchased from Calbiochem. Spectrozyme PCa and Spectrozyme TH were purchased from American Diagnostica (New York).

**FRET Measurements—**FRET experiments were performed as before (12), except that the D (donor-containing) and DA (containing donor and acceptor) microcuvets initially received 15 nm FL-FPR-APC-PTGla (the donor), whereas microcuvets B (blank) and A (acceptor-containing) received 15 nm unmodified APC-PTGla. The initial net emission intensity (P, ) was obtained by the subtraction of the signal P from the samples of DA, A, and D. Samples D and B were then titrated with phospholipid vesicles lacking the OR acceptor, whereas samples DA and A were titrated with an equivalent amount of phospholipid vesicles containing OR. The emission intensity of a sample was measured 5 min after each addition of phospholipid, a time that was found to be sufficient to reach thermal equilibrium. The net intensity of D, DA, or A, $I_D$, $I_{DA}$, and $I_A$ respectively, was obtained by subtracting the signal of the background B and then correcting for dilution. The blank signal never exceeded 0.5% of the fluorescent signal of the D or DA samples. To compensate for any signal in the DA sample caused by direct excitation of the acceptor, the net dilution-corrected emission intensity of the A sample was subtracted from that of the DA sample. The intensity of DA was then normalized by comparison with its own initial intensity as shown below, as was that of D. Making the reasonable assumption that the absorbance of the donor dye in the active site is not altered by the presence of the OR at the membrane surface, the ratio of the donor quantum yields in the D and DA samples is given by

$$Q_D/Q_{DA} = (F_D/F_{DA})/(F_{DA} - F_{D})(F_{DA} - F_A)$$

where $F$ is the net dilution-corrected emission intensity of a sample at some point in the titration, and the subscript $o$ is used to denote the intensity before any donor or acceptor was added.

At the end of the phospholipid titration, the membrane-bound FL-FPR-APC-PTGla was released from the membrane surface by the addition of 5 mM EDTA. After donor release from the membrane, the spectral measurements were repeated to determine what fraction of the acceptor-dependent reduction in donor emission intensity was due to FL-FPR-APC-PTGla binding to the membrane. The $Q_D/Q_{DA}$ value used in Equation 2 below was calculated by dividing the $Q_D/Q_{DA}$ value before EDTA addition by the $Q_D/Q_{DA}$ value after EDTA addition. This normalization procedure corrects for the contribution of OR inner filter effects and membrane-binding independent energy transfer to the observed total reduction in donor emission intensity.

For experiments with protein S, 15 nm FL-FPR-APC-PTGla was first titrated with PC/PS (OR) vesicles until the FRET efficiency reached a constant value. Protein S was then titrated into membrane-bound FL-FPR-APC-PTGla up to a final concentration of 300 nm. Identical procedures were used while performing control experiments with human FL-FPR-APC, except that excess DTT instead of EDTA was used to release the fluorescein-labeled heavy chain of FL-FPR-APC from the membrane surface (12).

**RESULTS**

**Extent of Labeling—**Human FL-FPR-APC-PTGla was prepared as described under “Experimental Procedures.” When the fluorescein concentration was determined as described by Bock (53), the number of dyes per protein averaged 0.7 in our preparations of both FL-FPR-APC-PTGla and wild-type human FL-FPR-APC, the same yield that was obtained previously with bovine FL-FPR-APC (12). For the experiments described in this paper, the presence of nonfluorescein-labeled APC-PTGla or APC molecules in the sample does not interfere with our interpretation of the spectroscopic data.

**Spectral Properties of Fluorescein-labeled Proteins—**The corrected wavelength of maximum emission and the average val-
ues of quantum yield and of steady-state anisotropy were 520
nm, 0.30, and 0.20 for human Fl-FPR-APC and Fl-FPR-APC-
PTGla, the same as previously published for bovine Fl-FPR-
APC (12). Thus, there is no significant difference in probe
environment in the active sites of human and bovine APC.
Furthermore, replacing the Gla domain of APC with the Gla
domain of PT did not alter the spectral properties of the fluo-
rescein dye and, hence, did not detectably alter the conforma-
tion of the active site of APC.

When PC/PS vesicles were added to either human Fl-FPR-
APC or Fl-FPR-APC-PTGla, no significant changes in fluo-
rescein spectral properties were detected. In addition, the fluores-
cence lifetime of the fluorescein (3.9 ns) was unaltered when
Fl-FPR-APC-PTGla bound to PC/PS, so the quantum yield of
the fluorescein was unaffected by membrane binding. Thus,
the binding of the protein to a membrane surface did not elicit
a detectable alteration in the environment of the fluorescein
dye in the active site of human APC.

Active Site to Membrane Surface Energy Transfer—In our
FRET experiments, the fluorescein dye in the active site of the
protein is the FRET donor, whereas the rhodamine in OR is the
FRET acceptor. The rhodamine dye is positively charged at pH
7.5 and remains in the aqueous phase, whereas the hydropho-
bic octadecyl aliphatic chain partitions into the lipid bilayer,
thereby anchoring the rhodamine moiety at the aqueous-lipid
interface.

When human Fl-FPR-APC-PTGla was titrated with PC/PS
vesicles, only a very small decrease in fluorescein emission
intensity was detected (see Fig. 1, -OR). However, when Fl-
FPR-APC-PTGla was titrated with PC/PS vesicles containing
OR, the fluorescein intensity decreased until the phospholipid
added was sufficient to bind all of the Fl-FPR-APC-PTGla (Fig.
1, +OR). The association of all of the Fl-FPR-APC-PTGla mol-
eecules with vesicles was confirmed by gel filtration (see below).
This OR-dependent decrease in fluorescein intensity results
largely from FRET from the fluorescein dyes in the active site
of the protein to the rhodamine dyes localized at the phospho-
lipid membrane surface. To facilitate analysis, the data in Fig.
1 were normalized and expressed in Fig. 2 as the ratio of donor
quantum yields in the presence and absence of acceptor using
Equation 1. The OR-dependent decrease in fluorescein intensity
evident in Figs. 1 and 2 shows that the fluorescein dyes are

For comparison, wild-type human Fl-FPR-APC was titrated
in parallel with the same stock of PC/PS vesicles used for the
Fl-FPR-APC-PTGla titrations. When Fl-FPR-APC was titrated
with PC/PS vesicles, no significant change in fluorescein emis-
sion intensity was observed. However, when titrated with PC/
PS vesicles containing OR (PC/PS/OR), the Fl-FPR-APC
emission decreased because of FRET, as shown by the reduc-
tion in $Q_{DA}/Q_D$ (Fig. 2).

As a control, Fl-FPR-APC-PTGla was also titrated with 100%
PC vesicles with or without OR because the PT Gla domain
requires negatively charged phospholipids to bind to a mem-
brane surface (34). As expected, no FRET was observed (Fig. 2).
The small reduction in $Q_{DA}/Q_D$ observed with PC vesicles (Fig.
2) results from an inner filter effect (a reduction in detected
fluorescein emission caused by the absorption of excitation and
emission light by rhodamine), not from FRET, as we have
documented elsewhere (12).

The data in Fig. 2 also show that the $Q_{DA}/Q_D$ values for
wild-type human Fl-FPR-APC titrations were always higher
than those for Fl-FPR-APC-PTGla at each point in the titra-
tion. Because the proteins were titrated with the same PC/
PS/OR) vesicles, the extent of FRET between the fluorescein
dye in the active site of membrane-bound Fl-FPR-APC-PTGla
and OR dyes on the membrane surface was greater than that
between membrane-bound Fl-FPR-APC and OR. The increased
efficiency of energy transfer in the chimeric APC-PTGla rela-
tive to wild-type APC shows that the probe in the active site
of the membrane-bound chimera is not in the same position as
that in membrane-bound APC. Thus, the active sites of mem-
brane-bound wild-type APC and APC-PTGla are positioned at
different locations above the membrane, with the active site of
the chimera closer to the surface and/or rotated so that the
relative orientation of the donor and acceptor transition dipoles
is more parallel.

Reversibility of Energy Transfer—At the low concentrations
of fluorescent-labeled protein and OR used in our experiments,
the average separation between free protein and OR molecules
is too large for detectable FRET to occur. Thus, if PC/PS-bound
Fl-FPR-APC-PTGla or Fl-FPR-APC is released from the mem-

![Fig. 1. OR dependence of fluorescence of membrane-bound Fl-FPR-APC-PTGla. Samples containing 15 nM Fl-FPR-APC-PTGla were titrated with PC/PS vesicles that contained (○) or lacked OR (□). $F_0$ is the initial fluorescence intensity of a sample before the addition of any vesicles; $F$ is the emission intensity of the sample at any point in the titration. The acceptor density ($\sigma$) in this titration was $4.73 \times 10^{-4}$ OR/Å$^2$.](Image 62x550 to 284x729)

![Fig. 2. Gla domain and lipid dependence of Fl-FPR-APC-PTGla FRET to OR. The data of Fig. 1 are replotted here as $Q_{DA}/Q_D$ (●). At the end of the titration, the membrane-bound Fl-FPR-APC-PTGla was released from the membrane by the addition of EDTA to a final concentration of 5 mM (□). In a parallel experiment, Fl-FPR-APC was titrated with PC/PS vesicles in the presence or absence of OR dyes (▲). The membrane-bound Fl-FPR-APC was released from the membrane by the addition of DT to a final concentration of 200 mM (▲). In another experiment, Fl-FPR-APC-PTGla (initially 15 nM) was titrated with 100% PC vesicles (■). The $\sigma$ was $2.90 \times 10^{-4}$ OR/Å$^2$ in the PC experiment and $4.73 \times 10^{-4}$ OR/Å$^2$ in the others.](Image 311x559 to 551x729)
brane surface at the end of the experiment, no FRET should occur, and the $Q_{DA}/Q_D$ value should return to 1.0. Because vitamin K-dependent proteins require calcium ions to bind to negatively charged phospholipid surfaces, an excess of EDTA is commonly used to chelate the calcium ions and dissociate the protein-membrane complex (e.g. Refs. 13–16).

However, as documented in the case of bovine Fl-FPR-APC (12), we observed that the EDTA-stimulated dissociation of the human Fl-FPR-APC-PC/PS complex was too slow and incomplete to allow us to use this approach for examining the reversibility of Fl-FPR-APC-to-OR FRET (data not shown). We therefore used an excess of DTT to reduce the disulfide bond between the two chains of Fl-FPR-APC and thereby release the fluorescein-labeled heavy chain from the vesicle surface. Upon addition of excess DTT, the value of $Q_{DA}/Q_D$ increased to a value close to 1.0 (0.94–0.99 depending on the acceptor density). We have shown earlier that this small residual OR-dependent decrease in donor intensity (Fig. 2, open circle) that cannot be reversed by DTT (or EDTA for the chimera; see below) is caused by an inner filter effect (12). Thus, only the changes in $Q_{DA}/Q_D$ resulting from membrane binding were used to calculate the distance of closest approach between the fluorescein and rhodamine dyes (i.e. the DTT- or EDTA-reversible $Q_{DA}/Q_D$).

Interestingly, in contrast to wild-type APC, when excess EDTA was added to PC/PS-bound Fl-FPR-APC-PTGla, the chelation of the calcium ions resulted in an immediate increase in fluorescein emission intensity in the DA sample cuvette such that the value of $Q_{DA}/Q_D$ increased to a value close to 1.0 (Fig. 2, open circle). This spectral change results from the dissociation of Fl-FPR-APC-PTGla from the membrane surface. The Fl-FPR-APC-PTGla release was confirmed by gel filtration chromatography (see below). Thus, the replacement of the Gla domain of wild-type human APC with that of the Gla domain of PT yields a chimeric protein with the membrane-binding properties that correspond, as expected, to PT, not APC.

**Phospholipid Dependence of Fl-FPR-APC-PTGla to OR Energy Transfer**—Because prothrombin does not bind to membrane surfaces that lack acidic phospholipids (10), one would not expect to see FRET if Fl-FPR-APC-PTGla was titrated with PC vesicles containing OR. As shown in Fig. 2 (open squares), only a very small decrease in fluorescein intensity was observed when Fl-FPR-APC-PTGla was titrated with PC(OR). This OR-dependent decrease in donor intensity was due to an inner filter effect rather than to membrane-binding-dependent FRET, as evidenced by the fact that the magnitude of this decrease was nearly equivalent to that observed when Fl-FPR-APC-PTGla and Fl-FPR-APC were dissociated from the membranes with excess EDTA and DTT, respectively (Fig. 2).

**Association of Fl-FPR-APC-PTGla with Membranes Detected by Gel Filtration**—The magnitude of FRET can be determined accurately only if the fraction of membrane-bound Fl-FPR-APC-PTGla molecules is known. To address this issue, Fl-FPR-APC-PTGla was incubated with a large excess of PC/PS vesicles in the presence of $Ca^{2+}$, and the distribution of free and membrane-bound chimera was then determined using gel filtration. Fl-FPR-APC-PTGla bound to PC/PS vesicles will elute in the excluded volume, whereas unbound Fl-FPR-APC-PTGla will elute in the included volume. More than 98% of the Fl-FPR-APC-PTGla fluorescence co-eluted with the radioactive vesicles (Fig. 3A), thereby demonstrating that essentially all of the Fl-FPR-APC-PTGla molecules can bind to the PC/PS vesicles and participate in FRET. In contrast, no fluorescence was detected co-eluting with the radioactive vesicle peak when the incubation lacked $Ca^{2+}$ (Fig. 3B).

**Distance of Closest Approach: Active Site to Membrane Surface**—$R_o$, the distance at which the efficiency of FRET is 50% efficient, was determined as before (12), assuming that the refractive index of the medium between the donor and acceptor is 1.4 and that the transition dipoles of the donor and acceptor dyes are oriented randomly during the excited state lifetime of the donor (i.e. $\kappa^2 = 2/3$). The spectral overlap integral ($J_{DA}$) for fluorescein in Fl-FPR-APC-PTGla and OR totaled $3.62 \times 10^{-15} \text{ m}^{-1} \text{ cm}^{-1} \text{ nm}^4$, and this yielded an $R_o$ of 50.3 Å, the same $R_o$ value obtained for both human and bovine Fl-FPR-APC-to-OR FRET (12).

Because 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 range of $\sigma$ values. The results from eight independent experiments at five different $\sigma$ values with human Fl-FPR-APC-PTGla and from five independent experiments with human Fl-FPR-APC are tabulated in Table 1. $L$, the distance of closest approach between the plane of donor dyes in the active sites of membrane-bound enzymes and the plane of OR acceptor dyes at the membrane surface, averaged 88.7 Å for human Fl-FPR-APC-PTGla, whereas the average $L$ value of the human Fl-FPR-APC was 94.3 Å. The uncertainty noted in Table 1 reflects the random experimental error in our experiments. The absolute values of $L$ reported in Table 1 are also uncertain because the relative orientation of the donor and acceptor transition dipoles cannot
be determined experimentally, and we have assumed $\chi^2 = 2/3$. As discussed previously, the uncertainty in $R_o$ in our experiments because of this assumption is approximately $\pm 10\%$ (Ref. 12 and references therein). However, for the purposes of this study, the absolute value of $L$ is not important, whereas the relative efficiencies of FRET for the chimera and wild-type proteins are very important. There was no dependence of $L$ on the method used to release Fl-FPR-APC-PTGla from the membrane, because the same average $L$ value was obtained for titrations reversed by the addition of excess EDTA or DTT.

The average $L$ value obtained for human Fl-FPR-APC-PTGla, assuming a $\chi^2$ value of 2/3, was 5.6 Å shorter than either human or bovine Fl-FPR-APC. Because the spectral properties of the fluorescein dye in Fl-FPR-APC-PTGla are the same as those in both human and bovine Fl-FPR-APC, the difference in FRET efficiency between the chimera and the wild-type proteins must arise from a difference in the heights of the active sites of the membrane-bound proteins above the membrane surface and/or from a difference in fluorescein orientation ($\chi^2$). A difference in $\chi^2$ could arise either from different orientations of the protease domains of the chimeric and wild-type APC relative to the membrane surface or from different rotational freedoms of the donor dyes in the active sites of the two proteins. Because fluorescein anisotropy was the same for the wild-type APC species and the chimera, we conclude that the active-site probe has the same rotational freedom in each enzyme.

When the FRET results obtained with human Fl-FPR-APC-PTGla are compared with those obtained with human Fl-FPR-APC, the S.D. values for the average $L$ values appear to overlap (Table 1). However a rigorous statistical analysis of these data using the Tukey HSD method to compare the means (35) reveals that the average $L$ values for these membrane-bound proteins are different at the 98% confidence level ($p < 0.02$). (The critical criterion is not whether the limit on individual means overlap but rather whether the limit on the differences between the means includes zero. Stated another way, the S.E. of the differences is more important than the differences in the S.E. values.) Therefore the difference between the locations of the active sites of APC and APC-PTGla above the membrane is statistically significant.

**Protein S Dependence of FRET**—The active site of membrane-bound bovine APC moves upon binding to bovine protein S (12). Because the effect of protein S on APC has been shown to be species-specific (36), we wanted to determine whether the protein S-dependent alteration in the topography of membrane-bound APC observed with the bovine proteins also occurs in the human system. Thus, we have here examined the effect of human protein S on the location of the active sites of membrane-bound human Fl-FPR-APC and human Fl-FPR-APC-PTGla. Samples of human Fl-FPR-APC were first titrated with sufficient PC/PS vesicles (-OR) to bind all of the Fl-FPR-APC and an excess of protein S cofactor. When human protein S was titrated into these samples, little change in fluorescein emission intensity was observed in the D sample lacking OR (data not shown). However, when human protein S was titrated into the human Fl-FPR-APC-PC/PS sample containing PC/PS/OR (the DA cuvette), the donor intensity decreased until all of the Fl-FPR-APC was bound to protein S. This protein S-dependent change in human Fl-FPR-APC emission is expressed in Fig. 4 as the relative $Q_{DA}/Q_D$, specifically the ratio $(Q_{DA}/Q_D)_{protein S}/(Q_{DA}/Q_D)_{protein S}$. This spectral change was saturatable with respect to protein S concentration, suggesting that it reflects APC:protein S complex formation. The protein S-dependent change in FRET efficiency seen in Fig. 4 can occur either because of translational (closer to the membrane) and/or rotational (more parallel alignment of donor-acceptor transition dipoles) movement of the active site of membrane-bound Fl-FPR-APC relative to the membrane surface. Assuming that this movement is solely translational, the average height of the fluorescein in this membrane-bound APC:protein S complex would be 84 Å above the membrane surface. Thus, the human protein S relocates the active site of human APC to the same extent observed with the bovine proteins.

Although human and bovine protein S appear to elicit the same topographical change upon binding to their cognate membrane-bound enzymes, these changes are species-specific. When human protein S was titrated into a sample of bovine Fl-FPR-APC, no change in FRET efficiency was observed (Fig. 4). Thus, the absence of a cofactor-dependent structural change correlates with the inability of human protein S to stimulate
bogonic APC function (36, 37).

Strikingly, when the human Fl-FPR-APC-PTGla/PC/PS(±OR) complex was titrated with human protein S, no protein S-dependent increase in the efficiency of energy transfer was observed, even when high concentrations of protein S were added to the complex (Fig. 4). Because human protein S does not elicit any fluorescein spectral changes upon association with human Fl-FPR-APC and because protein S does not stimulate APC-PTGla activity (23), we cannot ascertain whether or not protein S is binding to Fl-FPR-APC-PTGla in these experiments. However, it is clear that protein S does not elicit the same change in the topographies of APC-PTGla/PC/PS and wild-type APC-PC/PS. Given the absence of any protein S-dependent stimulation of APC-PTGla activity (23), it is interesting that protein S also does not have any influence on the location of the active site of membrane-bound Fl-FPR-APC-PTGla.

**Inactivation of Factor Va Leiden**—Because the active sites of the chimera and of APC in the presence of protein S have similar locations, it raised the possibility that the chimera and the APC-protein S complex might be functionally similar. If the protein S-dependent enhancement of factor Va cleavage at Arg\(^{306}\) by APC (9) is caused by the movement of the APC active site, then the chimera might cleave factor Va at Arg\(^{306}\) at a rate similar to that of APC-protein S. In factor Va Leiden, the most common source of APC resistance, Arg\(^{306}\) is mutated to Gln, leaving only Arg\(^{306}\) available for APC inactivation of factor Va (9, 38). We therefore compared the dose-response curves of factor Va Leiden inactivation by APC in the presence and absence of protein S to that of the chimera in the absence of protein S (Fig. 5). Consistent with the above proposal, the dose-response curve for the inactivation of factor Va by the chimera is very similar to that of APC plus protein S, and both curves are shifted to the left relative to APC in the absence of protein S. Consistent with earlier findings of the Rosing group (9), protein S had a much greater effect on factor Va Leiden inactivation than we or others had observed on normal factor Va (23).

**FIG. 5. Inactivation of factor Va Leiden by APC and APC-PTGla.** Factor Va Leiden (0.2 nM) was inactivated by APC (○, ●) or the chimera (□) at the concentrations indicated for 30 min at room temperature. When present (●), the protein S concentration was 70 nM. The reaction mixtures contained 10 \(\mu\)g/ml PC/PS vesicles. Remaining factor Va activity was determined by prothrombinase assays containing 1 nM factor Xa, 1.4 M prothrombin, 10 \(\mu\)g/ml PC/PS.

**Discourse**

Our previous FRET studies have revealed that a feature shared by most coagulation enzyme-cofactor complexes is an alteration in the topography of the membrane-bound enzyme upon binding to the cofactor. For example, tissue factor was found to alter the location of the active site of membrane-bound factor VIIa relative to the membrane surface (15), and this position was dictated by the cofactor, not by the membrane binding Gla domain of factor VIIa (39). Also, factor Va binding to factor Xa on a membrane surface causes translocation and/or rotation of the active site of the enzyme relative to the membrane surface (13). In addition, the cofactor may alter the conformation of the zymogen and/or the location of the scissile bond in the substrate above the membrane surface, as is the case with factor Va and the prothrombin activation intermediate, meizothrombin (16). The alignment of enzyme active sites with the scissile bond in the substrates cannot account for all cofactor effects, because most cofactors can stimulate zymogen activation by their cognate enzyme in the absence of membrane surfaces (40). Protein S differs from other cofactors in that it cannot enhance factor Va inactivation by APC in the absence of phospholipid (41). This observation makes the protein S system ideally suited to examine the importance of aligning the active site of the enzyme with the scissile bond of the substrate, particularly because we recently observed that protein S moves the active site of APC as much as 10 Å closer to the membrane surface (12).

An approach to testing the functional significance of the latter protein S-dependent change in membrane topography is to develop an APC homologue in which the location of the active site above the membrane surface in the absence of protein S is similar to that of the APC-protein S complex. The FRET measurements reported here indicate that the topography of the active site of the chimera and the APC-protein S complex are similar. That this change in topography has functional consequences is borne out by the observation that factor Va Leiden is inactivated on PC/PS vesicles at comparable rates by the APC-protein S complex and by the chimera in the absence of protein S. This is true despite the fact that the chimera and APC bind PS/PC vesicles with comparable affinity (23). This suggests that protein S functions, at least in part, by aligning the APC active site with the Arg\(^{306}\) cleavage site in factor Va.

The fact that the PT Gla domain can be substituted for the APC Gla domain without reducing the rate of factor Va inactivation argues strongly that the Gla domain is not directly involved in substrate recognition by APC. It is extremely unlikely that the prothrombin Gla domain improves the activity of the chimera in the absence of protein S over wild-type APC because of direct interactions between the prothrombin Gla domain and factor Va. For example, in the absence of membranes, the heavy chain of factor Va binds intact prothrombin and prethrombin 1, a derivative of prothrombin that lacks the Gla domain, with the same affinity (42). The observation that APC-PTGla inactivates membrane-bound factor Va Leiden at the same rate as the APC-protein S complex demonstrates that replacing the Gla domain of APC with that of prothrombin is functionally equivalent to binding protein S to APC.

The nature of the FRET-detected difference in active-site locations between membrane-bound APC and APC-PTGla cannot be determined unambiguously. Assuming that the observed increase in FRET efficiency for Fl-FPR-APC-PTGla relative to Fl-FPR-APC was due solely to translational movement, the average height of the active site above the membrane was about 6 Å less with APC-PTGla than with APC (Table I). If the exchange of the Gla domains also caused the active site to rotate relative to the planar bilayer surface, then the actual
change in height caused by the domain swap could be more or less than 6 Å. Although the FRET measurements cannot tell us the exact magnitude of the structural change, they do show that exchanging the Gla domains caused the active site of the enzyme to move significantly relative to the membrane surface, which, as discussed above, probably accounts for the selective enhancement of cleavage at Arg in factor Va.

Although the position of the active site above the membrane surface has been postulated to be functionally important (12, 13, 15, 16, 43), the data reported here constitute the first direct demonstration that modulation of active site location can alter enzyme activity and that the cofactor can be rendered irrelevant by the appropriate repositioning of the active site. Thus, we conclude that protein-dependent modulation of topography (specifically, structure relative to the membrane surface) is an effective means to regulate the activity of membrane-bound enzymes involved in hemostasis and likely in other membrane-dependent processes.

Acknowledgments—We thank Dr. Armando D’Angelo (H S Rafaele, Milan, IT) for supplying the factor V Leiden plasma, Dr. Alan Nicewander (Defense Manpower Data Center, Monterey, CA) for doing the statisti-

References

1. Eson, C. T. (1997) J. Biol. Chem. 272, 2134–2138
2. Egan, J. O., Kalafatis, M., and Mann, K. G. (1997) Protein Sci. 6, 2016–2027
3. McCallum, C. D., Su, B., Neueneschwander, P. F., Morrissey, J. H., and Johnson, A. E. (1997) J. Biol. Chem. 272, 30160–30166
4. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
5. Walker, F. J. (1981) J. Biol. Chem. 256, 11128–11131
6. Luckow, A. E., Lyons, D. A., Ridgeway, T. M., Eson, C. T., and Laue, T. M. (1989) Biochemistry 28, 2348–2354
7. Lu, R., Eson, N. N., Eson, C. T., and Johnson, A. E. (1989) J. Biol. Chem. 264, 12956–12962
8. Yegneswaran, S., Wood, G. M., Eson, C. T., and Johnson, A. E. (1997) J. Biol. Chem. 272, 25013–25021
9. Husten, E. J., Eson, C. T., and Johnson, A. E. (1987) J. Biol. Chem. 262, 12953–12961
10. Mutucumarana, V. P., Duffy, E. J., Lollar, P., and Johnson, A. E. (1992) J. Biol. Chem. 267, 17012–17021
11. McCallum, C. D., Harak, R. C., Neueneschwander, P. F., Morrissey, J. H., and Johnson, A. E. (1996) J. Biol. Chem. 271, 28168–28175
12. Armstrong, S. A., Husten, E. J., Eson, C. T., and Johnson, A. E. (1996) J. Biol. Chem. 261, 6410–6418
13. Soriano-Garcia, M., Padmanabhan, K., deVos, A. M., and Tulinsky, A. (1992) Biochemistry 31, 2554–2566
14. Geng, J.-P., and Castellino, F. J. (1997) Thromb. Haemostasis 77, 926–933
15. Afzal, S., Rawala-Sheikh, R., Cheung, W. F., Jameson, B. A., Stafford, D. W., and Walsh, P. N. (1994) Biochemistry 33, 12048–12055
16. Cheung, W., van der Born, J., Kühn, K., Kjellén, L., Hudson, B. G., and Stafford, D. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11068–11073
17. Bannister, D. W., D’Arcy, A., Chen, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y., and Kirchhofer, D. (1996) Nature 380, 41–46
18. Regan, L. M., Mollica, J. S., Rezaie, A. R., and Eson, C. T. (1997) J. Biol. Chem. 272, 26279–26284
19. Sminov, M. D., Safa, O., Regan, L., Mather, T., Stearns-Kurosawa, D. J., Kurosawa, S., Rezaie, A. R., Eson, N. L., and Eson, C. T. (1998) J. Biol. Chem. 273, 9031–9040
20. Eson, C. T., Eson, N. L., Le Benniec, B. F., and Johnson, A. E. (1993) Methods Enzymol. 222, 359–385
21. Taylor, F., Chang, A., Ferrer, G. L., Mather, T., Catlett, R., Bick, K., and Eson, C. T. (1991) Blood 78, 357–363
22. Owen, W. G., Eson, C. T., and Jackson, C. M. (1974) J. Biol. Chem. 249, 594–605
23. Esmon, C. T. (1977) in Chemistry and Biology of Thrombin (Lundblad, R. L., Fenton, J. W., and Mann, K. G., eds) pp.403–411, Ann Arbor Science, Ann Arbor, MI
24. DiScipio, R. G., and Dave, E. W. (1979) Biochemistry 18, 899–904
25. Thompson, A. R., Enfield, D. L., Ericsson, L. H., Legaz, M., and Fenton, J. W., II (1977) Arch. Biochem. Biophys. 176, 356–367
26. Kurachi, K., Schmer, G., Hermodson, M. A., Teller, D. C., and Dave, E. W. (1976) Biochemistry 15, 368–373
27. Dewey, T. G., and Hammes, G. G. (1980) Biophys. J. 33, 1023–1036
28. Holowka, D., and Baird, B. (1983) Biochemistry 22, 3466–3474
29. Bock, E. P. (1988) Biochemistry 27, 6633–6639
30. Nelsestuen, G. L., and Broderius, M. (1977) Biochemistry 16, 4172–4177
31. Hochberg, Y., and Tamhane, A. C. (1987) Multiple Comparison Procedures, pp. 80–84, John Wiley, New York
32. Walker, F. J. (1981) Thromb. Res. 22, 321–327
33. Dahlback, B. (1986) J. Biol. Chem. 261, 12022–12027
34. Egan, J. O., Kalafatis, M., and Mann, K. G. (1997) Protein Sci. 6, 2016–2027
35. McCallum, C. D., Su, B., Neueneschwander, P. F., Morrissey, J. H., and Johnson, A. E. (1997) J. Biol. Chem. 272, 30160–30166
36. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Annu. Rev. Biochem. 57, 915–956
37. Walker, F. J. (1981) J. Biol. Chem. 256, 11128–11131
38. Luckow, A. E., Lyons, D. A., Ridgeway, T. M., Eson, C. T., and Laue, T. M. (1989) Biochemistry 28, 2348–2354