Interaction of Blood Coagulation Factor Va with Phospholipid Vesicles Examined by Using Lipophilic Photoreagents†

Ute C. Krieg,‡ Benjamin S. Isaacs,‡,§ S. S. Yemul,∥,⊥ Charles T. Esmon,# Hagan Bayley,∥,△ and Arthur E. Johnson*,‡

Department of Chemistry, University of Oklahoma, Norman, Oklahoma 73019, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York 10032, and Section of Hematology and Thrombosis, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Received June 11, 1986; Revised Manuscript Received September 29, 1986

ABSTRACT: Two different lipophilic photoreagents, [^{3}H]adamantane diazirine and 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), have been utilized to examine the interactions of blood coagulation factor Va with calcium, prothrombin, factor Xa, and, in particular, phospholipid vesicles. With each of these structurally dissimilar reagents, the extent of photolabeling of factor Va was greater when the protein was bound to a membrane surface than when it was free in solution. Specifically, the covalent photoreaction with Vl, the smaller subunit of factor Va, was 2-fold higher in the presence of phosphatidylcholine/phosphatidylserine (PC/PS, 3:1) vesicles, to which factor Va binds, than in the presence of 100% PC vesicles, to which the protein does not bind. However, the magnitude of the PC/PS-dependent photolabeling was much less than has been observed previously with integral membrane proteins. It therefore appears that the binding of factor Va to the membrane surface exposes Vl to the lipid core of the bilayer, but that only a small portion of the Vl polypeptide is exposed to, or embedded in, the bilayer core. Addition of either prothrombin or active-site-blocked factor Xa to PC/PS-bound factor Va had little effect on the photolabeling of Vl with TID, but reduced substantially the covalent labeling of Vh, the larger subunit of factor Va. This indicates that prothrombin and factor Xa each cover nonpolar surfaces on Vh when the macromolecules associate on the PC/PS surface. It therefore seems likely that the formation of the prothrombinase complex involves a direct interaction between Vh and factor Xa and between Vh and prothrombin. In the absence of vesicles, the photolabeling of both subunits of factor Va was increased considerably by the removal of calcium from the solvent. This and other data indicate that nonpolar regions are exposed on the polypeptide surfaces when Vl and Vh dissociate and suggest that hydrophobic interactions may contribute to the intersubunit affinity in factor Va.

Membrane-bound protein complexes catalyze certain stages of the blood coagulation cascade, such as the conversion of prothrombin to thrombin by the prothrombinase complex (Jackson & Nemerson, 1980; Nemerson & Furie, 1980). Although many of the interactions which regulate the functional activities of such complexes are known, the molecular mechanisms and topographies of these complexes remain largely undefined.

The prothrombinase complex, the most thoroughly investigated of the complexes, consists of an enzyme (factor Xa), a protein cofactor (factor Va), Ca^{2+} ions, and a phospholipid surface (Jackson & Nemerson, 1980; Nemerson & Furie, 1980). Factor Xa and the substrate prothrombin are elongated molecules that appear to project radially out of the phospholipid surface when bound to vesicles (Lim et al., 1977). Factor Va is also an elongated molecule, with a length near 220 Å and a diameter near 42 Å when modeled as a prolate ellipsoid (Laue et al., 1984). On the basis of light-scattering experiments that suggest that this protein increases the vesicle radius by 46–63 Å upon binding (Pusey et al., 1982; Lampe et al., 1984), it would appear that factor Va either lies "flat" on the vesicle surface or has a large portion of the molecule embedded in the bilayer. On the other hand, recent fluorescence energy-transfer measurements have shown that one domain of factor Va is located at least 90 Å from the surface of the lipid bilayer (Isaacs et al., 1986). Both extremes of orientation (lying flat and projecting radially) were observed in samples of vesicle-bound factor Va prepared for electron microscopy (Lampe et al., 1984).

The nature of the interaction between factor Va and the lipid bilayer is controversial. Two groups have concluded that this association is mediated primarily by electrostatic interactions, based on the ionic strength dependence of the binding of factor Va to phospholipid vesicles (van de Waart et al., 1983) and on the rate of dissociation of factor Va from vesicles (Mayer et al., 1983; Pusey & Nelsestuen, 1984). In addition, the relatively small surface pressure changes observed upon association of factor Va with phospholipid monolayers suggested that the insertion of factor Va into the hydrocarbon region of a membrane, if any, was small (Mayer et al., 1983). On the other hand, Higgins and Mann (1983) found that the binding of factor Va to vesicles was not sensitive to the ionic strength of the medium, and they concluded that the association is

† This work was supported by National Institutes of Health Grants R01 HL 32934 (A.E.J.), R01 GM 29758 (H.B.), R01 HL 29807 (C.T.E.), and R01 HL 30340 (C.T.E.), National Science Foundation Grant PCM 83-02945 (H.B.), American Heart Association Grant 81-1189, with funds contributed in part by the Oklahoma Affiliate (A.E.J.), and an American Heart Association Established Investigatorship with funds contributed in part by the Oklahoma Affiliate (C.T.E.).

‡ University of Oklahoma.

§ Present address: Biomedical R & D Labs, American Red Cross, Bethesda, MD 20814.

∥ Columbia University.

⊥ Present address: Department of Dermatology, College of Physicians and Surgeons, Columbia University, New York, NY 10032.

Oklahoma Medical Research Foundation.

△ Present address: Howard Hughes Medical Institute, Columbia University, New York, NY 10032.

0006-2960/87/0426-0103$01.50/0 © 1987 American Chemical Society

mediated by hydrophobic interactions between the protein and the lipid core of the bilayer.

The extent to which factor Va is embedded in the membrane can be examined more directly by using lipophilic photoreagents. Since these reagents partition strongly into the nonpolar region of the lipid bilayer, they react covalently and preferentially with those polypeptides within the bilayer at the time of photoactivation [see Bayley (1983) and references cited therein]. In principle, then, the labeling of a protein with a radioactive reagent should increase upon association with a membrane if the polypeptide is buried in the lipid bilayer. Diazirine derivatives have proven particularly useful in such studies because they form carbenes upon photoactivation, and these moieties are both highly reactive and relatively nonspecific, capable of inserting into carbon–hydrogen bonds [see Bayley & Knowles (1980), Brunner & Semenza (1981), Bayley (1983), Hoppe et al. (1984), Kahan & Moscarello (1985), Brunner et al. (1985), and references cited therein].

We have examined the reactivity of factor Va with two different lipophilic photoreagents, focusing primarily upon the labeling that is dependent upon the binding of the protein to a phospholipid surface. In addition, we have investigated the effects of factor Xa, prothrombin, and calcium ions on the reactivity of factor Va with the lipophilic photoreagents.

Experimental Procedures

Proteins. Bovine factor Va and its subunits were prepared as detailed by Esmon (1979). The purified factor Va or subunit (Vl or Vh)[1] fractions were stored in their ion-exchange chromatography elution solvents. Bovine factor Xa was isolated from bovine plasma as described by Guinto (1983), and bovine prothrombin was isolated according to Owen et al. (1974). Factor Xa was stored at 4 °C in 0.1 M NaCl, 5 mM MES/HCl (pH 6.5), 5 mM benzamidine, and prothrombin in 0.1 M NaCl/0.02 M Tris-HCl (pH 7.5) at 4 °C. Prior to use, these proteins were dialyzed into buffer A [0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5), 2 mM $CaCl_2$, and 1 mM benzamidine (Aldrich)] at 4 °C for 12 h against two changes of buffer A (1:1000 v/v). For experiments done in the absence of calcium, factor Va was dialyzed into buffer A containing 5 mM EDTA instead of 2 mM $CaCl_2$.

The protein concentrations were determined by using the following molecular weights and $E^{1\%}_{1cm}$ extinction coefficients at 280 nm, respectively: factor Xa, 45 300 and 12.4 (Fujikawa & Davie, 1976); prothrombin 72 000 and 14.4 (Owen et al., 1974); and from Guinto and Esmon (1982) and Laue et al. (1984), factor Va, 174 000 and 15.0; Vl, 82 500 and 18.0; Vh, 92 300 and 12.7. Protein homogeneity was monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate using a 12% (factor Xa, prothrombin) or a 8.5% (factor Va) resolving gel (Laemmli, 1970). The activities of the factor Va stock solutions were routinely monitored by using one-stage clotting assays (Esmon, 1979).

To prevent enzymatic cleavage of factor Va in the presence of factor Xa (Tracy et al., 1983), *p*-(amidinophenyl)-methanesulfonyl fluoride (*p*-APMSF; Cal-Med, South San Francisco, CA) was used to block the active site of factor Xa (Laura et al., 1980). A 5-fold molar excess of *p*-APMSF in methanol was added to the factor Xa solution and incubated for 10 min at room temperature. This was repeated 3 more times before dialysis of the factor Xa into buffer A. In some experiments, the dialyzed factor Xa solution was made 25 μM in *p*-APMSF 15 min before addition to the sample to be photolyzed. The loss of factor Xa activity was measured by a one-stage clotting assay using factor Xa deficient plasma.

Photoreagents. [^{3}H]Adamantane diazirine (820 mCi/mmol) was synthesized as described elsewhere (Bayley & Knowles, 1980) and stored in the dark at −20 °C in ethanol at 2–5 μCi/μL. 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID) was purchased from Amersham and stored at 4 °C in the dark. The TID stock solutions in ethanol contained 2–5 μCi/μL and specific activities that ranged from 5 to 10 Ci/mmol.

Preparation of Phospholipid Vesicles. Phospholipid vesicles were prepared from synthetic dioleoylphosphatidylcholine (PC; Sigma) and 98% bovine brain phosphatidylserine (PS; Sigma). Typically, 113 μg of phospholipid (either 100% PC or 75% PC and 25% PS) in chloroform was added to a Pyrex glass tube, and the solvent was evaporated under a stream of nitrogen. To eliminate all of the chloroform, the PL was twice redissolved in less than 1.0 mL of diethyl ether which was then evaporated with N_2. Buffer A (0.1–0.2 mL) was then added, and the mixture was vortexed for 30 s. The resultant dispersion was sonicated to clarity in a bath sonicator (usually 6–10 min total sonication in 2-min intervals).

Photoreactions. Samples (250 μL final volume) were prepared in the cold room in precooled 1 cm × 1 cm quartz fluorescence cuvettes containing small Teflon stirring bars. Samples typically contained 67 μg of factor Va, 36 μg of Vh (the larger subunit of factor Va), or 31 μg of Vl (the smaller subunit of factor Va). The following were included in incubations where indicated: 57 μg of PC or PC/PS vesicles; 21 μg of active-site-blocked factor Xa; 33 μg of prothrombin. This corresponds to a 1.2-fold molar excess of prothrombin or factor Xa over factor Va in the incubations, and to about a 4-fold excess of phospholipid binding sites for factor Va (Higgins & Mann, 1983). The same results were obtained with 500-μL incubations at the same concentrations. In some experiments, glutathione was added to a final concentration of 10–50 mM to serve as a scavenger.

Samples were stirred at 4 °C for 30 min to allow equilibrium to be reached. Until photolysis, all further operations were performed under red light. TID or adamantane diazirine (0.5–2.5 μL) was added with a Hamilton syringe; the total radioactivity per 250-μL sample ranged from 1.3 to 4.3 μCi for TID and from 0.8 to 14 μCi for adamantane diazirine. The final ethanol concentration in the incubations never exceeded 1% in order to avoid disruption of the vesicles. The capped cuvettes were positioned 3 cm from a UVSL-25 ultraviolet lamp (Ultra-Violet Products, San Gabriel, CA), and the lamp, stirrer, and cuvettes were covered with aluminum foil. In some experiments, the cuvettes were placed in a Spex Fluorolog spectrofluorometer with a 450-W xenon lamp for illumination. Following a 60-min incubation in the dark to ensure that the photoreagent partitioning had reached equilibrium, the stirred samples were photolyzed for 30 min using either the long-wavelength range on the UV lamp or 350-nm excitation with a 20-nm band-pass on the spectrofluorometer. When included, glutathione was added to the samples 1 min prior to the initiation of photolysis in order to avoid any loss of factor Va activity due to reduction of disulfide bonds.

Product Analysis. Following photolysis, two 100-μL aliquots from each sample were each mixed with 45 μL of treatment buffer [3% (w/v) sodium dodecyl sulfate, 24% (v/v)

[1] Abbreviations: ADA, [^{3}H]adamantane diazirine; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Vh and Vl, heavy and light chains of factor Va, respectively; *p*-APMSF, *p*-(amidinophenyl)-methanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC, phosphatidylcholine; PS, phosphatidylserine; EDTA, ethylenediaminetetraacetic acid; PL, phospholipid; ANS, 1-anilinonaphthalene-8-sulfonic acid.

glycerol, 0.2 M glycine, 20 mM Trizma base (Sigma), and 0.005% (w/v) bromphenol blue] and 5 μL of neat 2-mercaptoethanol and then incubated at 70 °C for 10 min. Immediately prior to this, most samples that contained prothrombin were first incubated with 27 pmol of active factor Xa at room temperature for 1 min to digest the prothrombin and thereby eliminate problems in distinguishing between Vl- and prothrombin-associated radioactivity on the gels.

Polypeptides were separated by polyacrylamide gel electrophoresis in 0.1% (w/v) sodium dodecyl sulfate as described by Laemmli (1970). The 8.5% polyacrylamide tube gels were 12 cm long with a 0.5-cm stacking gel, and they were electrophoresed at 3 mA/gel. Gels were fixed in 12.5% trichloroacetic acid (20 min, 70 °C), stained (90 min, 70 °C) in 0.1% (w/v) Coomassie Blue, 25% (v/v) 2-propanol, and 10% (v/v) glacial acetic acid, and destained at 70 °C overnight and then at room temperature against three changes of 5% (v/v) glacial acetic acid/7.5% (v/v) methanol. Gels were cut into 2-mm slices, and, in the case of TID samples, were counted directly in a γ counter. Gel slices from [^{3}H]adamantane diazirine samples were incubated overnight at 60 °C in capped scintillation vials with 1.0 mL of 30% H_2O_2 and then counted following the addition of 10 mL of Triton-containing scintillation cocktail. In some cases, gels were not stained and destained prior to slicing.

Functional Assays. Activities were examined by using both the one-stage clotting assay (Esmon, 1979) and a two-stage prothrombin activation assay with the Kabi Diagnostica synthetic chromogenic substrate S-2238 (Helena Laboratories, Beaumont, TX) (Nesheim et al., 1979).

Results

Photolabeling in the Absence of Phospholipid. Soluble proteins typically have nonpolar interiors and may have small nonpolar regions interspersed among the hydrophilic moieties on their surfaces. Hence, factor Va would be expected to react covalently to some extent with lipophilic photoreagents even in the absence of phospholipid vesicles. To assess the membrane-independent photolabeling, factor Va was photolyzed with either [^{3}H]adamantane diazirine or TID in a solvent containing 2 mM Ca^{2+}. The samples were then analyzed by gel electrophoresis under denaturing conditions. Two major Coomassie Blue stained bands were observed in each gel, corresponding to the light (Vl; M_r 82 500) and heavy (Vh; M_r 92 300) subunits of the protein (Esmon, 1979; Laue et al., 1984). Each of these bands was radioactive, demonstrating that TID and adamantane diazirine each labeled both subunits of factor Va upon photolysis. The coincidence of the radioactivity with the stained bands is shown in Figure 1B for the case of TID.

On the basis of the considerable structural difference between TID and adamantane diazirine, it was anticipated that the photolabeling of factor Va would differ for these two reagents. As shown in Table I, this turned out to be the case. Adamantane reacted slightly more with Vl than with Vh, while the reverse was true for TID. The fraction of radioactivity incorporated into factor Va in a sample was about 3-fold less for adamantane diazirine than for TID. The extent of covalent reaction with each subunit was reproducible for duplicate incubations, and the results of parallel incubations could be compared directly as long as the TID used in the experiments came from the same lot. However, we found that the extent of protein photolabeling varied from batch to batch of the TID obtained from Amersham, suggesting that there was a batch variation in the amount of intact diazirine. In order to compare directly the data obtained from all of our experiments, we

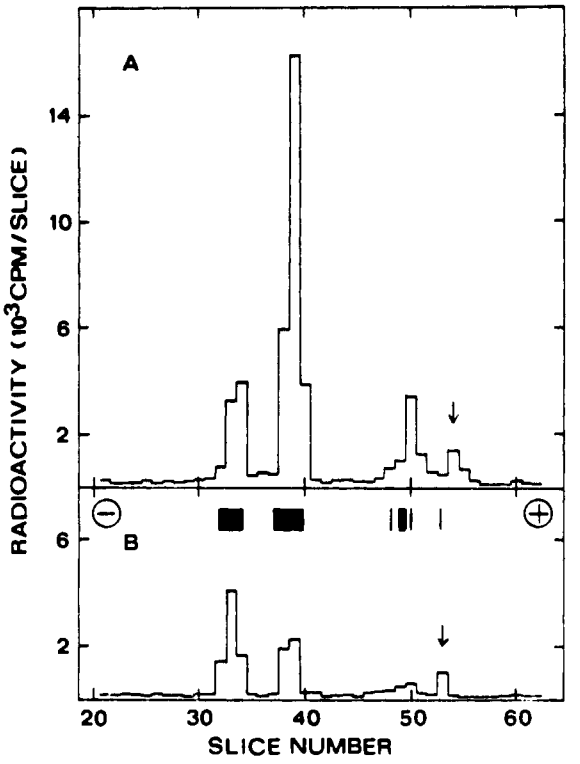

FIGURE 1: Calcium dependence of the photolabeling of factor Va with TID in the absence of phospholipid. Samples loaded on gels contained 27 μg of factor Va and 0.8 μCi of TID and were treated as described under Experimental Procedures. The Coomassie-stained bands were located in the gels at the positions indicated by the black rectangles in panel B; Vh was located at slices 32–34, Vl at slices 38–40, and "peak 3" at slices 48–51 in these gels. The arrow indicates the position of the marker dye. These gels were destained before slicing. (A) Buffer A containing 5 mM EDTA and no $CaCl_2$. (B) Buffer A containing 2 mM $CaCl_2$.

Table I: Photolabeling of Factor Va in the Presence and Absence of Ca^{2+} Ions[a]

photoreagent	calcium[b]	n[c]	Vl labeling/Vh labeling: average	Vl labeling/Vh labeling: range
[^{3}H]adamantane diazirine	+	4	1.2	1.1–1.3
[^{3}H]adamantane diazirine	–	3	1.6	1.4–1.7
[^{125}I]TID	+	10	0.8	0.6–0.9
[^{125}I]TID	–	4	4.0	3.0–5.6

[a] Experiments were performed as detailed under Experimental Procedures. [b] Buffer A is represented by "+", while buffer A containing 5 mM EDTA and lacking $CaCl_2$ is represented by "–". [c] The number of separate experiments is given by n. This table includes data obtained both from stained/destained gels (see Experimental Procedures) and from gels which were counted immediately after electrophoresis and were not stained.

analyzed the photolabeling results (and have expressed them in the tables) in terms of the ratio of covalent reactions with the subunits (cpm Vl/cpm Vh). This ratio was both reproducible and independent of the batch of TID used.

Radioactivity was also found associated with one of the minor stained bands in the gels that was designated "peak 3" (Figure 1). The peak 3 polypeptide (M_r 50 000) was derived from Vl, since a stained peak 3 band was found in gels of purified Vl, but not in gels of purified Vh. The amount of peak 3 material varied from preparation to preparation, but this variation did not correlate with differences in the activities of factor Va preparations. Since the factor Va traveled as a single band on native gels, we conclude that this particular cleavage of Vl does not release the peak 3 polypeptide from the protein and does not affect factor Va activity. In our data analysis,

we routinely determined the cpm Vl/cpm Vh labeling ratio both with and without the peak 3 associated radioactivity included in the Vl labeling. Although inclusion of the peak 3 radioactivity altered the absolute cpm Vl/cpm Vh values, it did not alter any conclusions based on a comparison of cpm Vl/cpm Vh ratios obtained in two different experiments. To simplify the table in this paper, the peak 3 associated radioactivity was not included in the total radioactivity associated with Vl, and we have shown the cpm Vl/cpm Vh values calculated from the radioactivities found only in the Vl and Vh peaks.

Of the total photoreagent in the photolysis incubations, typically 0.3% of the adamantane diazirine and from 0.5% to 1.0% of the TID were covalently attached to protein.

Calcium Dependence of Photolabeling in the Absence of Vesicles. Calmodulin was recently shown to exhibit a calcium-dependent conformational change that was detected by increased TID photoreaction with the protein, presumably because the hydrophobic sites that reacted with TID were increased in number, size, or affinity for TID (Krebs et al., 1984). Since factor Va requires Ca^{2+} for activity and for subunit association (Esmon, 1979; Guinto & Esmon, 1982; Laue et al., 1984), it was of interest to examine the effect of calcium ions on the photolabeling of Vl and Vh. Factor Va was therefore dialyzed for 24 h at 4 °C in the presence of 5 mM EDTA in order to remove the Ca^{2+} and effect subunit dissociation. The EDTA-treated protein sample did not show significant clotting activity, which confirmed that the calcium had been lost, but did regain full activity upon readdition of Ca^{2+}.

When calcium-free protein was photolyzed with TID, both Vl and Vh were labeled (Figure 1A), and the extent of photolabeling of Vl was typically 6-fold greater in the absence than in the presence of Ca^{2+} (compare panels A and B of Figure 1). The labeling of Vh with TID was also increased in the absence of calcium, usually 50–100% over the value in the presence of calcium. When calcium-free protein was photolyzed with adamantane diazirine, the extent of Vl labeling was an average of 47% higher than it was in parallel samples containing calcium, and this increase was 3-fold higher than the EDTA-dependent increase in Vh labeling. As a result, the cpm Vl/cpm Vh ratio also increased (Table I). Thus, despite the structural dissimilarity of the lipophilic photoreagents, each showed that the number or affinity of hydrophobic sites on the polypeptides was higher in the absence of Ca^{2+} ions that in their presence. Furthermore, in each case the photolabeling of Vl was more sensitive to the presence of calcium ions than that of Vh.

Photolabeling of Factor Va in the Presence of Phospholipid Vesicles. Several studies have shown that the binding of factor Va to the lipid bilayer requires the presence of acidic phospholipids (Bloom et al., 1979; van de Waart, 1983; Mayer et al., 1983). We therefore utilized this property of factor Va to investigate whether or not the binding of the protein to a phospholipid surface leads to an increased reaction with a lipophilic probe. This was done by comparing the photolabeling of factor Va in samples which contained equal concentrations of either PC/PS vesicles, to which the protein binds, or PC vesicles, to which the protein does not bind. Any difference in the labeling of factor Va represents a membrane binding dependent photoreaction.

In order to evaluate the influence of membrane binding on the photolabeling of factor Va, it is essential to have most of the protein bound to the vesicle surface. Incubations were therefore prepared with a 4-fold excess of phospholipid as

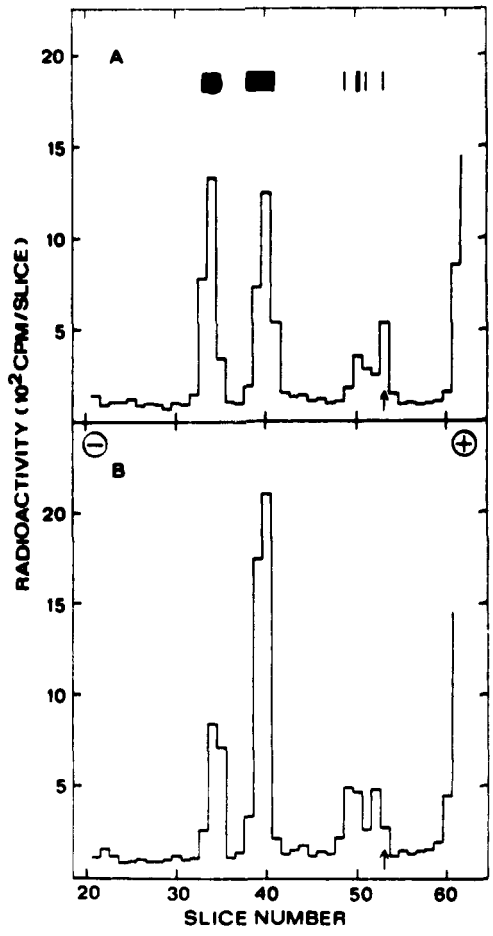

FIGURE 2: Photolabeling of factor Va with adamantane diazirine in the presence of phospholipid. Samples loaded on gels contained 27 μg of factor Va in the presence of either 100% PC vesicles and 4.4 μCi of adamantane diazirine (A) or PC/PS (3:1) vesicles and 4.9 μCi of adamantane diazirine (B). Details are given under Experimental Procedures. The locations of the Coomassie-stained bands and the marker dye are shown as noted in Figure 1. These gels were destained before slicing.

described under Experimental Procedures. Relative 90° light-scattering measurements, done as described by Higgins and Mann (1983) at concentrations one-tenth those used in the photolysis incubations, confirmed that greater than 95% of the factor Va was bound to vesicles in a PC/PS sample (data not shown). No binding of factor Va to PC vesicles was observed (data not shown).

The adamantane diazirine photoreaction with factor Va in parallel samples containing either PC/PS or PC vesicles is shown in Figure 2. In the experiment shown, the photolabeling of Vl and the peak 3 polypeptide was much higher in the PC/PS sample than in the PC sample, while the covalent reaction with Vh was lower with PC/PS vesicles than with PC vesicles. The ratio of photolabeling of the light and heavy chains (cpm Vl/cpm Vh) was therefore greater in the PC/PS sample than in the PC sample. This was observed in every experiment, and Table II shows the range in the ratios of subunit photolabeling observed in our experiments. The Vl reaction with adamantane diazirine was, on average, 2-fold greater in PC/PS incubations than in parallel PC incubations. Thus, the binding of factor Va to the phospholipid surface increased the covalent reaction between Vl and adamantane diazirine.

Similar results were obtained when TID was used as the lipophilic photoreagent rather than adamantane diazirine. In four sets of parallel reactions, the covalent reaction of Vl with

Table II: Photolabeling of Factor Va in the Presence of Phospholipid[a]

photoreagent	phospholipid	n^b	Vl labeling/Vh labeling	
			average	range
[^{3}H]adamantane diazirine	PC	6	1.0	0.8–1.1
[^{3}H]adamantane diazirine	PC/PS	6	1.9	1.7–2.7
[^{125}I]TID	PC	6	1.2	1.1–1.5
[^{125}I]TID	PC/PS	9	3.9	2.6–5.0

[a] Experiments were performed as detailed under Experimental Procedures using samples in buffer A containing either 100% PC or PC/PS (3:1) vesicles. [b] Defined in Table I.

Table III: Effect of Prothrombin and Factor Xa on the Photolabeling of Factor Va with TID[a]

proteins in sample	n^b	Vl labeling/Vh labeling	
		average	range
factor Va	9	3.9	2.6–5.0
factor Va, prothrombin	3	7.0	6.5–7.8
factor Va, factor Xa	4	5.2	3.3–7.7

[a] Experiments were performed as detailed under Experimental Procedures using samples which contained PC/PS (3:1) vesicles in buffer A. [b] Defined in Table I.

TID averaged about 2-fold higher in the PC/PS samples than in the PC samples, as was true above with adamantane diazirine. The photolabeling of Vh was again less (in the case of TID, by 43%) in the PC/PS samples than in the PC samples. The mean ratios of labeling Vl and Vh with TID in the two situations are shown in Table II, along with the range of radiolabeling observed in individual experiments.

The photolabeling of phospholipids by adamantane diazirine and TID was extensive in all of the vesicle-containing samples. This labeled material interfered somewhat with initial efforts to determine the extent of labeling of polypeptide material because the gels of the samples had high "background" counts throughout, as well as a peak of labeled phospholipid that slightly overlapped the radioactive peak associated with the peak 3 polypeptide. Both of these problems were eliminated when we stained and destained the gels prior to slicing them to examine their radioactivity content. However, the results were similar whether or not the gels were stained and destained.

The extents of labeling of factor Va with adamantane diazirine or with TID ranged from 0.10% to 0.25% of the added photoreagent in the samples which contained vesicles.

Effect of Lipophilic Photoreagents upon Prothrombinase Activity. Since the presence of the lipophilic photoreagents in the phospholipid vesicles could conceivably disrupt the normal binding of factor Va, factor Xa, or prothrombin to the phospholipid surface, we examined the effect of the probes on the functional activity of the prothrombinase complex. Neither TID nor adamantane diazirine altered the rate of thrombin production detected by using the two-stage S2238-based assay described by Nesheim et al. (1979), modified only so that phospholipid was limiting in the incubations (data not shown). Also, as expected, there was a stimulation of prothrombinase activity by PC/PS vesicles, but not by PC vesicles. Hence, the photoreagents did not perturb the phospholipid surface or its interaction with the prothrombinase complex.

The activity of factor Va in the one-stage clotting assay was routinely determined before and after the photolysis of each sample, and it was found that factor Va activity was unaltered either by the incubation conditions or by the photolysis.

Effect of Glutathione on Photoreactions. Although both adamantane diazirine and TID partition strongly into the lipid bilayer or nonpolar domains on the protein, it is possible that some photoreaction with the protein occurs nonspecifically following diffusion of activated photoreagents through the aqueous phase. It was therefore appropriate to consider to what extent the photoreactions, especially those in the presence of PC/PS vesicles, were sensitive to the presence of scavengers in the aqueous phase.

The presence of 50 mM glutathione, sufficient to quench effectively the covalent reaction of lipophilic photoreagents with molecules in the aqueous phase [see Bayley and Knowles (1980) and references cited therein], had no significant effect on the photolabeling of factor Va; whether in the presence or absence of vesicles, either PC or PC/PS, the covalent reaction was nearly the same for parallel samples with and without glutathione. Thus, nonspecific, diffusion-mediated covalent labeling does not account for the photolabeling patterns observed in our experiments.

Effect of Prothrombin and Factor Xa on Factor Va Photolabeling. Since factor Va associates with both factor Xa (Tucker et al., 1983; Guinto & Esmon, 1984) and prothrombin (Guinto & Esmon, 1984), the assembly of the prothrombinase complex on the phospholipid surface and the presence of its substrate prothrombin may alter the reactivity of factor Va with a lipophilic photoreagent. To examine this possibility, the covalent reaction of TID with factor Va bound to PC/PS vesicles was measured in the presence of prothrombin or factor Xa.

The effect of prothrombin on the TID photoreaction with factor Va bound to PC/PS vesicles was determined by using parallel samples, one with and one without prothrombin. As shown in Table III, the cpm Vl/cpm Vh ratio was 2-fold higher in the presence of prothrombin than in its absence. This change resulted primarily from an effect of prothrombin on the labeling of Vh, since the fraction of TID covalently attached to Vh averaged 58% lower in each sample containing prothrombin than in its parallel prothrombin-free control. In contrast, the photolabeling of Vl in parallel samples was lowered only 14% by prothrombin.

Similarly, the presence of active-site-blocked factor Xa in an incubation increased the cpm Vl/cpm Vh ratio (Table III), primarily because of its effect on the photoreaction with Vh. The photolabeling of Vh by TID averaged 53% less in the samples containing factor Xa than in control samples without factor Xa, while the labeling of Vl was reduced by an average of 16%.

Thus, both prothrombin and factor Xa cause a large reduction in the photolabeling of Vh, but have only a small effect on the TID reaction with Vl.

Discussion

Lipophilic photoreagents have been used as probes of the structure of factor Va and of its interaction with the other components of the prothrombinase complex, particularly the phospholipid surface. Our results demonstrate that these reagents constitute sensitive reporter groups for the presence or absence of calcium, phospholipid, prothrombin, and factor Xa in factor Va containing samples, and that the structural information provided by them is a useful complement to information obtained by using a variety of other techniques.

Factor Va binds a single Ca^{2+} ion (Guinto & Esmon, 1982) and is inactive in the absence of calcium (Esmon, 1979). The exact nature of the structural change(s) which cause(s) the loss in functional activity is unclear, but could result from subunit separation (Esmon, 1979; Laue et al., 1984), polypeptide conformational changes, or both. The hydrodynamic

frictional coefficients of the subunits, and presumably their overall shapes, are very similar in the presence and absence of calcium (Laue et al., 1984), as is the protein secondary structure detected by circular dichroism (Laue et al., 1982). In addition, the calcium-dependent intrinsic fluorescence changes are small, suggesting that the tertiary structures of Vh and Vl are similar in the presence and absence of Ca^{2+} (Laue et al., 1982). Nevertheless, the removal of calcium causes a large increase in the extent of covalent reaction of the factor Va polypeptides with either adamantane diazirine or TID in the absence of phospholipid (Table I and accompanying text). Because this labeling is insensitive to glutathione and is therefore not collisional in origin, the difference in the extents of photoreaction must result from a change in the number and/or the affinity of nonpolar sites on the polypeptide surface to which the lipophilic photoreagents bound. This interpretation is consistent with results we have obtained using 1-anilinonaphthalene-8-sulfonic acid (ANS), a dye whose fluorescence intensity increases greatly upon association with a nonpolar site on a protein (Weber & Laurence, 1954; Stryer, 1965). The ANS emission intensity of a calcium-free solution of Vl, Vh, and ANS decreased substantially upon titration with Ca^{2+}, presumably because of the loss of ANS binding sites when nonpolar regions on Vl and Vh associated in the presence of calcium (U. C. Krieg, R. Lu, and A. E. Johnson, unpublished data). Thus, the most direct interpretation of the photolabeling experiments in the absence of phospholipid vesicles is that hydrophobic domains, normally inaccessible when the subunits are associated, become exposed after the subunits dissociate. The probable juxtaposition of nonpolar surfaces on Vl and Vh suggests that hydrophobic interactions contribute to the high affinity between the factor Va subunits in the presence of Ca^{2+}.

The increased photolabeling of Vl in the presence of PC/PS vesicles (Table II and text) demonstrates that a region of Vl is exposed to the lipophilic reagents upon binding to a phospholipid surface and suggests that a portion of Vl inserts into the lipid bilayer. The specificity of Vl labeling is consistent with previous work which has shown that factor Va binding to vesicles is mediated by the Vl subunit (Higgins & Mann, 1983; Tracy & Mann, 1983; van de Waart et al., 1983; Pusey & Nelsestuen, 1984). The extent of Vl insertion must be small because the magnitude of the Vl photolabeling was considerably lower than has been observed in other studies which examined intrinsic membrane proteins. For example, the extents of photochemical labeling of (Na–K)-ATPase with adamantane diazirine (Farley et al., 1980) and of lipophilin with TID (Kahan & Moscarello, 1985) were on the order of 20-fold higher than the corresponding extent of photochemical reaction with factor Va, even though the concentrations of TID and admantane diazirine in the lipid bilayer were about the same in the corresponding experiments. Since the individual peptides exposed to the lipophilic reagents in each of these cases may not be equally reactive with the reagents, one must be cautious about drawing quantitative conclusions from these results. However, because the photolabeling of the intrinsic membrane proteins was more than an order of magnitude greater than the labeling of factor Va, it appears that only a small amount of Vl polypeptide was exposed to the photoreagents upon binding to the phospholipid surface.

The exposure of Vl to hydrophobic probes upon its binding to a phospholipid surface is consistent with the earlier proposal of Higgins and Mann (1983) that hydrophobic interactions contribute to the high affinity between factor Va and a phospholipid surface. At the same time, factor Va binding to vesicles requires phosphatidylserine and has been reported to be ionic strength dependent (van de Waart et al., 1983; Mayer et al., 1983; Pusey & Nelsestuen, 1984), which suggests that electrostatic interactions are also involved. The involvement of both hydrophobic and electrostatic components may be a common feature in the binding of the blood coagulation proteins to membranes. Recent experiments with prothrombin fragment 1 have indicated that the association of prothrombin with a negatively charged phospholipid membrane involves nonelectrostatic, Ca^{2+}-independent interactions in addition to the widely recognized electrostatic interactions (Lentz et al., 1985).

Two alternative explanations for our results cannot be excluded. First, the small extent of PC/PS-dependent labeling may not result from the thermodynamically favored insertion of a region of Vl into the bilayer, but rather from dynamic fluctuations at the surface which transiently expose the surface-bound Vl to the nonpolar core of the bilayer. Second, it is conceivable that binding to the phospholipid surface causes a conformational change in Vl which increased the size or affinity of its aqueous-exposed nonpolar surface area, and that this resulted in the increased reaction with the lipophilic reagents shown in Table II. However, there was no change in intrinsic protein fluorescence when factor Va was titrated with PC/PS vesicles (B. S. Isaacs and A. E. Johnson, unpublished data), which suggests that there is no major change in factor Va conformation upon binding to a phospholipid surface. Also, since an increase in the nonpolarity of the aqueous-exposed surface area of Vl would presumably occur in order to facilitate and strengthen the association of prothrombin and/or factor Xa with the membrane-bound factor Va, one would expect that this surface area would be covered, and the PC/PS-dependent photolabeling of Vl eliminated, when factor Va associated with prothrombin or factor Xa. Yet the addition of prothrombin or active-site-blocked factor Xa had little effect upon the extent of photolabeling of Vl by TID. These results therefore suggest that the PC/PS-dependent reaction of TID with Vl was not due to a conformational change in Vl.

It is important to emphasize that the increased labeling of Vl was observed with both adamantane diazirine and TID, two nonpolar reagents with very different structures. The increase in photolabeling is therefore unlikely to have resulted from a specific interaction of Vl with a particular reagent. It is also important to note that the photolabeling was insensitive to the presence of the scavenger glutathione, and therefore did not result from collisional labeling by activated photoreagents in the aqueous phase.

Our data indicate that Vh is not embedded in the membrane, because the photolabeling of Vh with both TID and adamantane diazirine was lower with PC/PS vesicles than with PC vesicles. The number or the affinities of the nonpolar sites on the Vh surface were apparently reduced when factor Va associated with PC/PS vesicles, and this suggests that Vh undergoes a membrane binding dependent conformational change. The absence of any PC/PS-dependent increase in Vh photolabeling, the low affinity of Vh for PC/PS vesicles (Higgins & Mann, 1983; Tracy & Mann, 1983; van de Waart et al., 1983), and the EDTA-dependent release of Vh (but not Vl) from the membrane surface (Higgins & Mann, 1983; Tracy & Mann, 1983; Pusey & Nelsestuen, 1984; Isaacs et al., 1986) indicate that Vh is associated with the membrane solely through its interaction with Vl. A similar subunit arrangement, with one subunit noncovalently bound to a second subunit that is partially embedded in the lipid core of the

bilayer, has been observed, for example, both with the major histocompatibility complex (β_2-microglobulin plus a heavy chain; Goldman et al., 1979; Coligan et al., 1981) and with the sucrase–isomaltase complex (Spiess et al., 1982).

The nature of the interactions between the factor Va subunits and either prothrombin or factor Xa has not yet been clearly established. Guinto and Esmon (1984) used affinity chromatography to show that Vh, by itself, bound to prothrombin. Tracy and Mann (1983) found that Vl alone stimulated factor Xa binding to the platelet surface, and both subunits of platelet-bound factor Va are cleaved by factor Xa (Tracy et al., 1983). Tucker et al. (1983) observed that Vl-specific monolconal antibodies blocked factor Xa binding to membrane-bound factor Va. Both van de Waart et al. (1984) and Pusey and Nelsestuen (1984) found that intact factor Va, but not Vl alone, was required for high-affinity factor Xa binding to phospholipid vesicles. Guinto and Esmon (1984) found that both subunits of factor Va and Ca^{2+} were required to observe factor Va binding to immobilized factor Xa. The data presented in this paper provide additonal information on the association of these proteins and demonstrate the utility of lipophilic photoreagents as probes of the topography of multicomponent complexes. Since the TID photoreaction with Vh is reduced substantially by both factor Xa and prothrombin (Table III and accompanying text), it appears that factor Xa and prothrombin each cover hydrophobic surfaces on the larger subunit of factor Va when the proteins associate on a phospholipid surface. It therefore seems likely that the topographical arrangement of the prothrombinase complex on the membrane surface includes a direct interaction both between Vh and factor Xa and between Vh and prothrombin. Furthermore, these interactions would appear to be mediated through the association of nonpolar surfaces on the macromolecules. In contrast, the presence of factor Xa or prothrombin only slightly decreased the photolabeling of Vl. This approach therefore does not indicate whether or not Vl interacts directly with either prothrombin or factor Xa. However, our results do suggest that any association between Vl and either prothrombin or factor Xa does not involve significant nonpolar domains.

References

Bayley, H. (1983) in *Photogenerated Reagents in Biochemistry and Molecular Biology* (Work, T. S., & Burdon, R. H., Eds.) Elsevier, Amsterdam.

Bayley, H., & Knowles, J. R. (1980) *Biochemistry 19*, 3883–3892.

Bloom, J. W., Nesheim, M. E., & Mann, K. G. (1979) *Biochemistry 18*, 4419–4425.

Brunner, J., & Semenza, G. (1981) *Biochemistry 20*, 7174–7182.

Brunner, J., Franzusoff, A. J., Luscher, B., Zugliani, C., & Semenza, G. (1985) *Biochemistry 24*, 5422–5430.

Coligan, J. E., Kindt, T. J., Uehara, H., Martinko, J., & Nathenson, S. G. (1981) *Nature (London) 291*, 35–39.

Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964–973.

Farley, R. A., Goldman, D. W., & Bayley, H. (1980) *J. Biol. Chem. 255*, 860–864.

Fujikawa, K., & Davie, E. W. (1976) *Methods Enzymol. 45*, 89–95.

Goldman, D. W., Pober, J. S., White, J., & Bayley, H. (1979) *Nature (London) 280*, 841–843.

Guinto, E. R. (1983) Ph.D. Dissertation, University of Oklahoma Health Sciences Center, Oklahoma City, OK.

Guinto, E. R., & Esmon, C. T. (1982) *J. Biol. Chem. 257*, 10038–10043.

Guinto, E. R., & Esmon, C. T. (1984) *J. Biol. Chem. 259*, 13986–13992.

Higgins, D. L., & Mann, K. G. (1983) *J. Biol. Chem. 258*, 6503–6508.

Hoppe, J., Brunner, J., & Jorgensen, B. B. (1984) *Biochemistry 23*, 5610–5616.

Isaacs, B. S., Husten, E. J., Esmon, C. T., & Johnson, A. E. (1986) *Biochemistry 25*, 4958–4969.

Jackson, C. M., & Nemerson, Y. (1980) *Annu. Rev. Biochem. 49*, 765–811.

Kahan, I., & Moscarello, M. A. (1985) *Biochemistry 24*, 538–544.

Krebs, J., Buerkler, J., Guerini, D., Brunner, J., & Carafoli, E. (1984) *Biochemistry 23*, 400–403.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lampe, P. D., Pusey, M. L., Wei, G. J., & Nelsestuen, G. L. (1984) *J. Biol. Chem. 259*, 9959–9964.

Laue, T. M., Yphantis, D. A., Esmon, C. T., & Johnson, A. E. (1982) *Circulation 66*, II-51.

Laue, T. M., Johnson, A. E., Esmon, C. T., & Yphantis, D. A. (1984) *Biochemistry 23*, 1339–1348.

Laura, R., Robinson, D. J., & Bing, D. H. (1980) *Biochemistry 19*, 4859–4864.

Lentz, B. R., Alford, D. R., Jones, M. E., & Dombrose, F. A. (1985) *Biochemistry 24*, 6997–7005.

Lim, T. K., Bloomfield, V. A., & Nelsestuen, G. L. (1977) *Biochemistry 16*, 4177–4181.

Mayer, L. D., Pusey, M. L., Greip, M. A., & Nelsestuen, G. L. (1983) *Biochemistry 22*, 6226–6232.

Nemerson, Y., & Furie, B. (1980) *CRC Crit. Rev. Biochem. 9*, 45–85.

Nesheim, M. E., Taswell, J. B., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 10952–10962.

Owen, W. G., Esmon, C. T., & Jackson, C. M. (1974) *J. Biol. Chem. 249*, 594–605.

Pusey, M. L., & Nelsestuen, G. L. (1984) *Biochemistry 23*, 6202–6210.

Pusey, M. L., Mayer, L. D., Wei, G. J., Bloomfield, V. A., & Nelsestuen, G. L. (1982) *Biochemistry 21*, 5262–5269.

Spiess, M., Brunner, J., & Semenza, G. (1982) *J. Biol. Chem. 257*, 2370–2377.

Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.

Tracy, P. B., & Mann, K. G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2380–2384.

Tracy, P. B., Nesheim, M. E., & Mann, K. G. (1983) *J. Biol. Chem. 258*, 662–669.

Tucker, M. M., Foster, W. B., Katzmann, J. A., & Mann, K. G. (1983) *J. Biol. Chem. 258*, 1210–1214.

van de Waart, P., Bruls, H., Hemker, H. C., & Lindhout, T. (1983) *Biochemistry 22*, 2427–2432.

van de Waart, P., Hemker, H. C., & Lindhout, T. (1984) *Biochemistry 23*, 2838–2842.

Weber, G., & Laurence, D. J. R. (1954) *Biochem. J. 56*, 31P.