# A Domain of Membrane-Bound Blood Coagulation Factor Va Is Located Far from the Phospholipid Surface. A Fluorescence Energy Transfer Measurement†

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ABSTRACT: The larger subunit of blood coagulation factor Va was covalently labeled with iodoacetamido derivatives of fluorescein and rhodamine without loss of functional activity, as measured by either the one-stage clotting assay or the ability to accelerate prothrombin activation in a purified system. The spectral properties of the dyes were not altered by the presence or absence of the smaller subunit of factor Va, $Ca^{2+}$, prothrombin, factor Xa, or phosphatidylcholine/phosphatidylserine (PC/PS, 4:1) vesicles. When fluorescein-labeled protein (factor VaF) was titrated with PC/PS vesicles containing either octadecylrhodamine or 5-(*N*-hexadecanoylamino)eosin, fluorescence energy transfer was observed between the protein-bound donor dyes and the acceptor dyes at the outer surface of the phospholipid bilayer. The extent of energy transfer correlated directly with the extent of protein binding to the vesicles monitored by light scattering. The distance of closest approach between the fluorescein on factor Va and the bilayer surface averaged 90 Å for the two different acceptors. Association of factor VaF with factor Xa on the phospholipid surface reduced this separation by 7 Å, but association with prothrombin did not alter the distance between the labeled domain on factor VaF and the surface. The efficiency of diffusion-enhanced energy transfer between rhodamine-labeled factor Va and terbium dipicolinate entrapped inside PC/PS vesicles was less than 0.01, consistent with the location of the dye far above the inner surface of the vesicle. Thus, a domain of membrane-bound factor Va is located a *minimum* of 90 Å above the phospholipid surface. If the factor Va molecule is elongated, as suggested by sedimentation data [Laue, T. M., Johnson, A. E., Esmon, C. T., & Yphantis, D. A. (1984) *Biochemistry 23*, 1339-1348], it then appears likely that the long axis of the protein projects more or less perpendicularly out of the plane of the surface when bound to a phospholipid bilayer, whether or not the factor Va molecule is associated with factor Xa or prothrombin.

The conversion of prothrombin to thrombin during blood coagulation is accomplished by the serine protease factor Xa (Jackson & Nemerson, 1980; Nemerson & Furie, 1980). Maximal rates of thrombin generation are achieved in the presence of a protein cofactor (factor Va), a phospholipid surface, and calcium ions (Nesheim et al., 1979; Rosing et al., 1980), which together with factor Xa constitute the prothrombinase complex. Phospholipid vesicles that contain acidic phospholipids such as phosphatidylserine have been used extensively to study the structure and function of the prothrombinase complex and its components [e.g., see Higgins & Mann (1983), van de Waart et al. (1984), and Lampe et al. (1984)], and these vesicles have been shown to support prothrombinase activity as well as activated platelets (Nesheim et al., 1979).

The nature of the protein–phospholipid interactions and the arrangement of the prothrombinase macromolecules on the phospholipid surface have been examined in several studies using a variety of techniques. In the case of factor Va, a two-subunit glycoprotein that has no demonstrable enzymatic activity of its own (Colman, 1976; Nesheim et al., 1979; Rosing et al., 1980), binding of the protein to membrane surfaces is mediated by the smaller subunit (Vl)[1] (Higgins & Mann, 1983; van der Waart et al., 1983; Tracy & Mann, 1983; Pusey & Nelsestuen, 1984). Light scattering (Bloom et al., 1979; Pusey et al., 1982; Higgins & Mann, 1983; Lampe et al., 1984), surface pressure changes (Mayer et al., 1983), fluorescence (Pusey & Nelsestuen, 1984), centrifugation [e.g., see van de Waart et al. (1983, 1984)], and electron microscopy (Lampe et al., 1984) have been employed to characterize the interaction of factor Va with the phospholipid surface.

Light-scattering results suggest that factor Va increases the vesicle radius by 46–63 Å when it binds to the surface (Pusey et al., 1982; Lampe et al., 1984). On the basis of our ultracentrifugation studies (Laue et al., 1984), factor Va has a length near 220 Å and a diameter near 42 Å when modeled as a prolate ellipsoid. The light-scattering data therefore suggest that factor Va either lies "flat" on the vesicle surface or has a large portion of the molecule embedded in the bilayer

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[1] Abbreviations: Vl and Vh, light and heavy chains of factor Va, respectively; VhF, fluorescein-labeled Vh; VhR, rhodamine-labeled Vh; factor VaF or factor VaR, proteins reconstituted with unmodified Vl and either VhF or VhR, respectively; IAF, 5-(iodoacetamido)fluorescein; IAR, (iodoacetamido)tetramethylrhodamine; OR, octadecylrhodamine B chloride; HAE, 5-(*N*-hexadecanoylamino)eosin; $Me_2SO$, dimethyl sulfoxide; DPA, dipicolinic acid; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; *p*-APMSF, *p*-(amidinophenyl)methanesulfonyl fluoride; PC, phosphatidylcholine (dioleoyl form); PS, phosphatidylserine; QAE-Sephadex, quaternary aminoethyl-Sephadex; DIFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; TPCK, tosylphenylalanine chloromethyl ketone.

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if the long axis of the protein is oriented approximately perpendicularly to the surface. The latter possibility is not supported by the surface pressure data (Mayer et al., 1983), while the data from different groups on the ionic strength dependence of factor Va binding to membranes have led to conflicting conclusions about the extent to which the association is hydrophobic or electrostatic in nature (van de Waart et al., 1983; Mayer et al., 1983; Higgins & Mann, 1983; Pusey & Nelsestuen, 1984). The electron microscopy studies showed that some of the factor Va molecules were lying flat, while some were seen to be projecting out of the surface (Lampe et al., 1984). Hence, the nature of the factor Va interaction with the phospholipid surface remains unclear.

Another approach to examining the molecular architecture of membrane-bound proteins such as those in the prothrombinase complex is to use singlet–singlet energy transfer. Recently, two variations of the fluorescence energy transfer technique have been used successfully to determine the distance from a protein-bound dye to a surface of the phospholipid bilayer (Baird et al., 1979; Fleming et al., 1979; Cerione et al., 1983; Holowka & Baird, 1983b; Snyder & Hammes, 1984; Kleinfeld & Lukacovic, 1985; Thomas & Stryer, 1982; Kouyama et al., 1983). In this paper, we report our results utilizing both of these techniques to investigate the orientation of factor Va bound to a phospholipid surface.

## Experimental Procedures

*Chemicals.* 5-(Iodoacetamido)fluorescein (IAF), octadecylrhodamine B chloride (OR), and 5-(*N*-hexadecanoylamino)eosin (HAE) were obtained from Molecular Probes, Inc., Junction City, OR. (Iodoacetamido)tetramethylrhodamine (IAR) was purchased from Research Organics, Cleveland, OH. All fluorescent dyes were used without further purification. Terbium chloride ($TbCl_3 \cdot 6H_2O$) was purchased from Alfa, Danvers, MA. Dimethyl sulfoxide ($Me_2SO$; gold label) and benzamidine hydrochloride were purchased from Aldrich. Dipicolinic acid (DPA), tris(hydroxymethyl)-aminomethane (Tris), 2-(*N*-morpholino)ethanesulfonic acid (MES), synthetic dioleoylphosphatidylcholine (PC), and 98% bovine brain phosphatidylserine (PS) were obtained from Sigma Chemical Co. Bing reagent, *p*-(amidinophenyl)-methanesulfonyl fluoride (*p*-APMSF), was purchased from Cal-Med, South San Francisco, CA. S2238 (Kabi Diagnostica) was purchased from Helena, Beaumont, TX.

*Proteins.* Bovine factor Va and its subunits were prepared as described by Esmon (1979). The purified factor Va or subunit (Vl or Vh) fractions were dialyzed and stored in buffer A [0.1 M NaCl, 0.02 M Tris·HCl (pH 7.5), 2 mM $CaCl_2$, and 1 mM benzamidine] at 4 °C. Protein homogeneity was examined by polyacrylamide gel electrophoresis using a 7.5% resolving gel and 0.1% sodium dodecyl sulfate (Laemmli, 1970).

Bovine factor Xa was isolated from bovine plasma as described by Guinto (1983). Bovine prothrombin was isolated according to Owen et al. (1974) and was further purified from its activation products by gel filtration on Sephadex G-200. The proteins were stored at 4 °C in 100 mM NaCl, 5 mM MES-HCl (pH 6.0), and 5 mM benzamidine for factor Xa or in 100 mM NaCl and 20 mM Tris·HCl (pH 7.5) for prothrombin. Prior to use, each was dialyzed for 12 h at 4 °C against two changes of buffer A (1:1000 v/v); any unused material was then stored in buffer A at 4 °C.

The protein concentrations were determined by using the following molecular weights and $E^{1\%}_{1cm}$ extinction coefficients at 280 nm, respectively: factor Xa, 45300 and 12.4 (Fujikawa & Davie, 1976); prothrombin, 72000 and 14.4 (Owen et al., 1974); and from Guinto and Esmon (1982), factor Va, 174000 and 15.0; factor Vl, 82000 and 18.0; factor Vh, 93000 and 12.7.

*Active-Site-Blocked Factor Xa.* To prevent enzymatic cleavage of the other coagulation proteins present in the in vitro prothrombinase incubation, *p*-APMSF (Laura et al., 1980) at 5.5 mM in methanol was titrated at room temperature into a 1.0-mL solution of factor Xa (35 μM) in buffer A. Successive 30-μL additions were made at 10-min intervals until the activity of factor Xa was less than 0.1% of its original activity as measured by the one-stage clotting assay using factor X deficient plasma. The final concentration of *p*-APMSF was typically 50-fold higher than the factor Xa concentration. The inactivated factor Xa was dialyzed for a total of 15 h at 4 °C against three changes of buffer A (1:1000 v/v) and then stored in buffer A at 4 °C.

*Fluorescein Labeling of Factor Va.* A typical reaction contained 20 mg of factor Va in 25 mL of buffer A. Aliquots of IAF (0.63 mg in 180 μL of $Me_2SO$) were added every hour for 6 h to give a final IAF concentration of 240 μM, a 60-fold molar excess over factor Va. During the reaction and all subsequent procedures, the sample was protected from possible photodegradation by covering all vessels and columns with aluminum foil. The reaction mix was stirred for 2 h at 4 °C following the final addition of reagent and then dialyzed once overnight against 4 L of buffer A at 4 °C to remove most of the unreacted excess dye. The protein was further purified from free dye by using gel filtration chromatography at room temperature with Bio-Gel P6 (75 cm × 2.5 cm i.d.) equilibrated in buffer A. The factor Va protein peak was pooled, dialyzed once against 4 L of buffer B [50 mM NaCl, 20 mM Tris·HCl (pH 8.0), 5 mM EDTA, and 1 mM benzamidine], and applied to a QAE-Sephadex A-50 ion-exchange column (20 cm × 1.5 cm i.d.) equilibrated in buffer B. The column was developed at 4 °C with a linear salt gradient from 0.05 to 0.5 M NaCl to separate the Vl and Vh subunits of factor Va (Esmon, 1979). Only the fractions containing the Vh subunit had measurable absorbance at 490 nm. Labeled Vh (designated VhF) was stored at 4 °C in the fractions from the ion-exchange separation, and individual fractions were dialyzed into buffer A prior to use.

*Rhodamine Labeling of Factor Va.* Factor Va was treated as described above, except that IAR was used in place of IAF. After the overnight dialysis to remove most of the free dye, the gel filtration was done at room temperature using a Sephadex G-100 column (60 cm × 1.5 cm i.d.) equilibrated in buffer A. After the separation of subunits by ion-exchange chromatography at 4 °C as described above in the preceding section, the absorbance at 550 nm was found only in fractions containing the Vh subunit. The rhodamine-labeled Vh subunit (designated VhR) was stored as described above.

*Enzymatic Digestion of VhF.* VhF (350 μg) in 0.5 mL of 0.2 M Tris·HCl, 0.35 M NaCl, 5 mM EDTA, 1 mM benzamidine hydrochloride, and 6 M guanidine hydrochloride was titrated to pH 8.0 with $NH_4OH$ and incubated for 2 h with 1 mM dithiothreitol. The sample was then made 2.4 mM in iodoacetic acid, titrated to pH 8.5 with $NH_4OH$, reacted in the dark for 2 h at 22 °C, and then dialyzed against 0.1 M $NH_4HCO_3$ (pH 8.2). Tryptic digests were obtained by adding $^1/_{100}$th volume of TPCK-treated trypsin (1 mg/mL in 0.1 N HCl; Worthington Biochemicals) to the carboxymethylated VhF sample, incubating for 8–12 h at 37 °C, and drying the samples in a Savant Speed-vac for analysis by HPLC or TLC. Total digests of the carboxymethylated VhF (350 μg) were done by using procedures described previously (Johnson et al.,

1976). Total digestion of the fluorescent peptides purified by HPLC was accomplished by drying the appropriate fractions, resuspending the material in 0.5 mL of 0.1 M $NH_4HCO_3$ (pH 7.5), and incubating with 2 units of leucine aminopeptidase M (Sigma) at 37 °C for 6 h. Two more units of aminopeptidase M were then added to the sample, and the incubation was continued for another 12 h before evaporation prior to analysis by TLC or HPLC.

*HPLC.* HPLC separations were done by using a Gilson gradient HPLC system interfaced to an Apple II+ microcomputer and equipped with a Chromanetics C-18 reversed-phase column (4.6 × 250 mm). Samples were typically dissolved in 0.5 mL of solvent A [0.1% (v/v) trifluoroacetic acid in HPLC-grade water] for injection. Following an isocratic elution with 100% solution A for 5 min, samples were eluted with a 60-min linear gradient from 0% to 65% solution B [0.1% (v/v) trifluoroacetic acid in acetonitrile]. Absorbance was monitored at 206 nm with an LKB Uvicord SII detector using a 2.5-mm path-length flow cell. Fluorescence was detected by using a Gilson Spectra/glo equipped with a 485-nm interference filter on the excitation light path and a 520–650-nm band-pass filter on the emission light path.

*Preparation of Phospholipid Vesicles.* Dioleoylphosphatidylcholine (PC) (1.0 mg) and phosphatidylserine (PS) (0.25 mg) were mixed to give 4:1 PC/PS in a 13 × 100 mm Pyrex test tube, and the chloroform solvent was evaporated in a nitrogen stream. Nitrogen-purged buffer A (0.5 mL) was then added and vortexed for 30 s. The resultant dispersion was sonicated to clarity in a bath sonicator.

Vesicles containing OR or HAE were made as described above, except that an appropriate amount of OR or HAE (5–40 μg) in ethyl acetate was added to the tube containing the lipid and the ethyl acetate was volatilized in a nitrogen stream prior to the addition of buffer A. The total concentration of OR or HAE in a vesicle preparation was determined by using extinction coefficients of $9.54 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 564 nm for OR and $1.19 \times 10^5$ $M^{-1}$ $cm^{-1}$ at 528 nm for HAE, respectively (Cerione et al., 1983). The surface density in acceptors per square angstrom was calculated by assuming that the dyes at the phospholipid surface were distributed uniformly, that the molecular weights of PS and PC are 786, and that each phospholipid molecule occupies 70 $Å^2$ of surface area (Huang & Mason, 1978).

Phospholipid vesicles for diffusion-enhanced energy transfer were prepared by using 2 mg of PS and 8 mg of PC (10 mg of PC in the case of 100% PC vesicles) in 2.0 mL of buffer A which contained 10 mM $TbCl_3$ and 50 mM dipicolinate. Sonication was carried out in a Heat Systems W225 cell disrupter with a microtip probe, on ice and under a nitrogen stream, for 45 min at 60% duty cycle and output control level 3. After sonication, the vesicle preparation was diluted to 4.5 mL with buffer A and centrifuged for 30 min at 100000*g* in an SW65-Ti rotor at 4 °C. After centrifugation, 0.5 mL of the top 2 mL of supernatant was applied to a Sephadex G-25 gel filtration column (1.2 cm i.d. × 32 cm) to remove extravesicular $Tb(DPA)_3^{3-}$. The column was equilibrated and eluted in buffer A at room temperature. The absorbance at 280 nm, the absorbance maxima of $Tb(DPA)_3^{3-}$, was measured for each fraction (1.25 mL) to determine the elution profile. The peak fraction of the vesicle preparation was used in the experiments. Vesicles for samples without the $Tb(DPA)_3^{3-}$ donor were prepared as described above except that $Tb(DPA)_3^{3-}$ was omitted.

*Functional Assays.* The activities of the fluorescent analogues and their unlabeled precursors were examined by using both the one-stage clotting assay (Esmon, 1979) and a two-stage prothrombin activation assay (Nesheim et al., 1979). In the latter assay, thrombin concentration was measured by using Spectrozyme TH (H-D-hexahydrotyrosyl-L-Ala-L-Arg-*p*-nitroanilide; American Diagnostica, Greenwich, CT). Vl was first recombined with Vh, VhF, or VhR to form factor Va, factor VaF, or factor VaR, respectively, by incubation at 37 °C in buffer A for 30 min. The resultant factor Va was then incubated (0.5-mL final volume) in 0.1 M NaCl, 0.02 M Tris·HCl (pH 7.5), 2 mM $CaCl_2$, and 0.01% (w/v) gelatin at 22 °C with factor Xa and PC/PS (4:1) at final concentrations of 2 nM, 15 nM, and 115 μM, respectively. After the addition of prothrombin (to 1.4 μM) to the reaction mixture, aliquots were removed and analyzed as described by Nesheim et al. (1979). In our assays, factor Va was not in excess, but was the limiting component. This permitted a direct comparison of the activities of the different factor Va species.

To evaluate the effect of OR and HAE on the prothrombinase activity, the Spectrozyme TH assay was repeated, but in this case, the concentration of PC/PS vesicles (0.58 μM) containing OR ($3.4 \times 10^{-4}/Å^2$) or HAE ($4.2 \times 10^{-4}/Å^2$) was limiting. The factor Va, factor Xa, and prothrombin concentrations were 1.9 nM, 2.0 nM, and 1.4 μM, respectively.

*Spectral Measurements.* Fluorescence measurements were made by using a Spex Fluorolog I spectrofluorometer, a photon-counting instrument equipped with a 450-W xenon lamp, and double monochromators in both the excitation and emission light paths. The Spex was interfaced to an Apple II+ microcomputer. Slits were kept closed except during scans to avoid photodegradation of the sample. Temperature control was maintained with a Lauda K2R circulating bath attached to the cell holder. All spectral measurements were done at 25 °C in buffer A in 1 cm × 1 cm cuvettes. Emission intensities were corrected and recorded at 1-nm intervals. Correction factors were determined by using a standard lamp (Optronics). The band-pass was 10 nm during the polarization measurements and 5 nm at all other times.

All absorbance measurements were made on a Varian DMS-100 spectrophotometer interfaced to a Varian DS-15 data station.

*Anisotropy.* Steady-state anisotropy was measured in the Spex using Glan-Thompson prism polarizers in both the excitation and emission beams and a depolarizing scrambler plate in the emission beam after the polarizer. The emission intensity was designated as $I_{VH}$ when a sample was excited by vertical plane-polarized light and the emission was detected through a horizontal polarizer. $I_{HH}$, $I_{HV}$, and $I_{VV}$ were defined analogously. The component intensities of a dye-free (blank) sample were subtracted from the appropriate component intensities of a dye-containing sample to obtain the emission intensities of the fluorescent-labeled Vh subunit. The fluorescence anisotropy was calculated as

$$\bar{A} = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where the grating factor $G = I_{HV}/I_{HH}$. The anisotropy of VhF was measured by using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. For VhR, the excitation wavelength was 550 nm, and the emission wavelength was 577 nm. The excitation and emission wavelengths were 564 and 590 nm for OR and 528 and 550 nm for HAE.

*Collisional Quenching.* Samples of VhF or VhR (0.12 μM) in buffer A in the presence and absence of Vl (0.12 μM) and/or PC/PS vesicles (7.6 μM) were titrated with KI or KCl in buffer A containing 0.1 mM sodium thiosulfate. A sample

of IAR in buffer A was also titrated with KI. Emission intensities were recorded following a 10-min wait to ensure equilibration after the addition of iodide ions and were corrected for dilution due to the addition of titrant.

*Quantum Yield.* The quantum yield of the fluorescein-labeled Vh subunit (VhF) was determined by using the relationship:

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (2)$$

where $Q_1$ is the quantum yield of VhF in buffer A (Parker & Rees, 1966). The quantum yield of disodium fluorescein in 0.1 M NaOH, $Q_2$, was assumed to be 0.92 (Weber & Teale, 1957). $F_1$ and $F_2$ are the integrated areas of the corrected emission spectra of the VhF and $Na_2F$ samples, respectively, in those solvents, while $A_1$ and $A_2$ are the absorbances at the exciting wavelength of 490 nm for the VhF and $Na_2F$ samples, respectively.

*Calculation of $R_0$.* $R_0$ for each donor–acceptor pair was calculated by using the equation:

$$R_0^6 = (8.79 \times 10^{-5})\kappa^2 n^{-4} Q_D J_{DA} \quad (3)$$

where $R_0^6$ is in Å$^6$, $J_{DA}$ is the spectral overlap integral, $Q_D$ is the quantum yield of the donor dye in the absence of energy transfer, $n$ is the refractive index of the medium between the donor and acceptor dyes, and $\kappa^2$ is a geometric factor that depends upon the relative orientation of the donor and acceptor. The absorbance spectra of the OR and HAE acceptors were recorded at 1-nm intervals in the presence of unmodified factor Va and PC/PS in buffer A. $J_{DA}$ was calculated, by using the corrected emission spectrum of VhF, $f_D(\lambda)$, as follows:

$$J_{DA} = \int_0^\infty f_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda / \int_0^\infty f_D(\lambda) \, d\lambda \quad (4)$$

where $J_{DA}$ is in $M^{-1}$ cm$^{-1}$ nm$^4$ and $\epsilon_A$ is the molar extinction coefficient of the acceptor dyes at the vesicle surface. For each donor–acceptor pair, the same $J_{DA}$ values (within 3%) were obtained by using the acceptor excitation spectra instead of its absorbance spectra.

*Protein to Phospholipid Surface Energy Transfer Measurements.* Four separate samples were prepared in parallel for each experiment: sample D (donor) contained VhF·Vl and PC/PS vesicles without acceptors; sample DA (donor + acceptor) contained VhF·Vl, PC/PS vesicles, and either OR or HAE; sample A (acceptor) contained unlabeled Vh·Vl, PC/PS vesicles, and either OR or HAE; and sample B (blank) contained unlabeled Vh·Vl and PC/PS vesicles without acceptors. In each case, subunits were added first to the cuvettes in a molar ratio of Vl to Vh or VhF of 1.1:1. The final concentration of VhF or Vh was 0.1 μM and was assumed to yield a 0.1 μM final concentration of factor Va after a 25 °C, 45-min incubation. This was sufficient to allow the subunits to recombine and recover full activity in the one-stage clotting assay.

The fluorescence emission intensities of the reconstituted (and active) factor Va or VaF samples were determined and designated $F_0$. The fluorescein donor was excited at 490 nm in all experiments. The corrected emission intensity was integrated from 510 to 550 nm at 1-nm intervals in experiments using OR as the acceptor, and from 505 to 520 nm in experiments using HAE as the acceptor. Prior to integration of the emission intensity over these ranges, the signal of the blank sample (B) was always subtracted from the signal of sample D, DA, or A. The blank signal never exceeded 1% of the signal of D or DA. Vesicles were then added to the cuvettes, either in successive small aliquots or in a single addition, to a final phospholipid concentration of 8 μM. The concentration was in excess of that needed to bind all the factor Va present in a sample, as judged by the results of our light-scattering experiments. After each addition of vesicles, stable integrated intensities were obtained in less than 10 min, suggesting that the binding of protein to vesicles was completed within 10 min. This was supported by the results of the light-scattering experiments which showed that the binding was completed in less than 7 min. However, the emission spectra of samples D, DA, A, and B were recorded only after 15–20 min to ensure equilibration. To compensate for any signal in the DA sample due to the acceptor, the net donor emission in the DA sample was obtained by subtracting the signal of the A sample from that of the DA sample. $Q_D/Q_{DA}$ was therefore given by $I_D/(I_{DA} - I_A)$, where $I$ is the net emission intensity of a sample. At the end of some experiments, the solutions were made 5 mM in EDTA to dissociate the subunits. At the end of other experiments, a 10-fold molar excess of factor Va, reconstituted from unmodified Vh and Vl, was added to each cuvette.

In experiments to evaluate the effects of factor Xa and prothrombin on the observed energy transfer, active-site-blocked Xa and/or prothrombin was added to each cuvette to a final concentration equimolar to that of factor Va or VaF (0.1 μM). These additions were made after the emission spectra in the presence of vesicles had been recorded.

For each set of samples (D, DA, A, and B) prepared with PC/PS vesicles, an equivalent set was prepared by using 100% PC vesicles and then examined as above. Also, a set of samples was prepared without Vl and then examined with PC/PS vesicles as above.

The reproducibility of emission intensity measurements was better than ±0.5%. For the experiments reported here (see Results), this corresponds to an uncertainty of ±2 Å in the distances measured.

*Diffusion-Enhanced Energy Transfer Experiments.* Each experiment consisted of four parallel samples: sample D (donor) and sample DA (donor + acceptor) both contained PC/PS vesicles at 5 μM with $Tb(DPA)_3^{3-}$ inside; sample A (acceptor) and sample B (blank) contained PC/PS vesicles at 5 μM without $Tb(DPA)_3^{3-}$. The $Tb(DPA)_3^{3-}$ complex was excited at 285 nm, and the corrected emission intensity was scanned from 530 to 560 nm. The background signal of the blank solution was always subtracted from that of samples D, DA, and A before integration of the emission intensity over the scanned wavelengths. An Oriel 5215 cutoff filter was placed in the emission light path to block any second-order light.

Following a determination of the emission intensities of the samples in the absence of acceptor ($F_0$), reconstituted rhodamine-labeled factor Va was added to samples DA and A, either in successive small aliquots or in a single addition, to give a final concentration of 0.28 μM. Reconstituted unmodified factor Va was similarly added to samples D and B. This protein concentration was far in excess of that needed to saturate factor Va binding sites on the surface of the vesicles, based on the results of the light-scattering experiments. To ensure equilibration, the $Tb(DPA)_3^{3-}$ emission was scanned only after a 15–20-min wait following each addition of protein. At the end of each experiment, the solutions were made 5 mM in EDTA to check for leakage of the donor from inside the vesicle (Thomas & Stryer, 1982).

In some experiments, either active-site-blocked factor Xa or prothrombin was added to samples containing the recon-

stituted factor Va species and the vesicles. The final concentrations of factor Xa and prothrombin were equimolar to that of factor Va or factor VaR.

*Light Scattering.* Relative 90° light-scattering measurements were made by using the Spex with both excitation and emission monochromators set at 320 nm. Before each experiment, all buffer solutions were centrifuged for 7 min in a Brinkmann microfuge to remove dust particles. Parallel samples containing either buffer A or 0.11 μM protein (factor Va or VaF) in buffer A were titrated with 1.25 mg/mL PC/PS (4:1) vesicles in buffer A. Ten minutes after each addition of titrant, the intensity of the scattered light was measured for each sample. The relative light-scattering intensity was therefore given by the difference in the signals of these two samples. The initial signals of the protein and buffer A samples were the same and did not change when titrated with buffer A instead of vesicles.

## RESULTS

*Labeling of Factor Va.* A 6-h reaction with IAF or IAR at 4 °C and pH 8.0, as detailed under Experimental Procedures, was used for the covalent modification of factor Va with the fluorescent dyes. An increase in reaction time to 10 h did not improve the extent of labeling significantly. Since Vl and Vh, the two subunits of factor Va, are each thought to possess one free cysteine residue (Guinto, 1983), it was expected that dyes would be covalently attached to each subunit. However, after gel electrophoresis in the presence of sodium dodecyl sulfate, only Vh was found to be fluorescent. Very weak fluorescence was occasionally associated with lower molecular weight degradation products, but no fluorescence was ever observed at the position of Vl in the gels. The putative SH group in the Vl subunit is apparently inaccessible to the dye reagent. The extent of labeling of Vh was the same in the presence or absence of Vl, while no fluorescent labeling of Vl was observed, even in the absence of Vh.

Purification of the fluorescent proteins by both dialysis and gel filtration chromatography did not remove all of the noncovalently bound dyes from the sample. Hence, chromatography on QAE-Sephadex A-50 was used to remove the remaining free dyes. The fluorescent Vh subunit was eluted from the ion-exchange column at 0.35 M NaCl. The fluorescein or rhodamine dyes which were not attached to the protein could be eluted from the QAE-Sephadex only with 2.0 M NaCl in the presence of 2% Triton X-100 (v/v). The fluorescent-labeled Vh exhibited properties indistinguishable from those of the unmodified Vh in terms of both its elution off the ion-exchange column and its electrophoretic mobility in the presence of sodium dodecyl sulfate.

To reconstitute functional factor Va molecules in our experiments, we used only Vl subunits which had never been exposed to the fluorescent reagent. This was done to eliminate the possibility that any spectral change was due to a dye attached to Vl.

*Extent of Labeling.* The molar extinction coefficient of the conjugate between IAF and cysteine was determined to be 73 000 $M^{-1}$ $cm^{-1}$ at 490 nm in buffer A. When this value was used, the average number of dyes covalently attached to each Vh polypeptide was found to range from 0.23 to 0.31 in our VhF preparations. Similarly, on the basis of an extinction coefficient at 550 nm of 30 000 $M^{-1}$ $cm^{-1}$ for the IAR–cysteine conjugate in buffer A, the average number of rhodamine dyes per Vh ranged from 0.78 to 1.1 in our preparations.

*Site of Labeling.* To determine the number of fluorescent-labeled peptides in Vh, the tryptic digest of VhF was examined by using HPLC as detailed under Experimental Procedures. Three major fluorescent peaks were eluted from the C-18 reversed-phase column, at 35%, 40%, and 42% solution B. In five separate tryptic digests, the peak at 40% B contained 65–85% of all fluorescence detected, while the peak at 42% contained 12–14% of the total fluorescence intensity. The remaining fluorescent material (1–21% of the total fluorescence detected) eluted at the same concentration of solution B (35%) as did the conjugate prepared from Cys and IAF. When the fluorescent material in the three peaks was analyzed by using silica gel thin-layer chromatography, each had an $R_f$ value of 0 using benzene/ethyl acetate/acetic acid (32:36:7) solvent. This ruled out the possibility that this material was free dye (free IAF eluted at 43% B and had an $R_f$ value of 0.8) and suggested that the fluorescein in these peaks was attached to peptides. This was confirmed when the material in the 40% B and 42% B peaks was treated with leucine aminopeptidase M to digest totally any peptides present and then rechromatographed over the HPLC column. In each case, the fluorescent material then eluted as a single peak at 35% B. Thus, we conclude that IAF reacted only with Cys in the Vh polypeptide and that the labeling occurred primarily at one site. It remains to be determined whether the material in the three fluorescent peaks of the tryptic digest was derived from a single fluorescein-labeled peptide that was cleaved and truncated at different sites.

Table I: Activity of Factor Va Species[a]

| protein added | one-stage clotting assay[b] (units/mg of protein) | prothrombin activation [mol of thrombin $min^{-1}$ (mol of factor Va)$^{-1}$] |
|---|---|---|
| none | – | 1.4 |
| factor Va | 1470 | 684 |
| factor VaF | 1510 | 741 |
| factor VaR | 1460 | 730 |

[a] Assays were done as described under Experimental Procedures. [b] Bovine plasma is defined as containing 1 unit of factor V/mL.

When older and less active samples of factor Va have been reacted with IAF, we have found more than two fluorescent peptides and have occasionally seen two overlapping fluorescent peaks eluting near 35% B in a total enzymatic digest of VhF. Hence, to minimize heterogeneity in the VhF samples, only fresh and undegraded protein should be labeled with fluorescein.

*Functional Activity of Labeled Factor Va.* Both the fluorescein- and rhodamine-labeled Vh subunits recombined readily with Vl in the presence of 2 mM $CaCl_2$ to form fully active factor Va. Following reconstitution, the modified Vh subunits were as active as the unlabeled subunits in both the one-stage clotting assay and the chromogenic substrate detected activation of prothrombin (Table I).

*Protein Stability.* A 30% loss in activity in the one-stage clotting assay was typically observed after 2 months at 4 °C in buffer A, and a complete loss of activity after storage for 5–6 months. The modified proteins were therefore used within a month of preparation. The integrity of the fluorescent analogues was monitored regularly by their clotting activity, by electrophoresis in the presence of sodium dodecyl sulfate, and by measuring their fluorescent anisotropies.

The anisotropy of VhF and VhR fluorescence decreased during prolonged storage, presumably because a cleavage(s) in the Vh polypeptide allowed the dye to rotate freely. Consistent with this interpretation, digestion of a sample of VhF with trypsin (2 mg/mL) and proteinase K (2 mg/mL) at 37 °C in buffer A at pH 8.0 for 11 h reduced the anisotropy from

Table II: Spectral Parameters of Vesicle-Bound Donor–Acceptor Pairs[a]

| donor | emission max (nm) | $Q_D$ | $\bar{A}_D$ | acceptor | absorption max (nm) | $\bar{A}_A$ | $R_0$[b] (Å) | $L$ (Å) |
|---|---|---|---|---|---|---|---|---|
| factor VaF | 521 | 0.13 | 0.25 | OR | 564 | 0.14[d] | 45.6 | 88 |
| | | | | HAE | 528 | 0.14[e] | 45.2 | 92 |
| $Tb(DPA)_3^{3-}$ | 546 | 0.44[c] | | factor VaR | 555 | 0.26 | 47.3 | >80[f] |

[a] Parameters were $^2/_3$ as detailed under Experimental Procedures. [b] Values of $^2/_3$ and 1.4 were assumed for $\kappa^2$ and $n$, respectively. [c] From Thomas & Stryer (1982). [d] At a surface density of $1.1 \times 10^{-4}$ OR/Å$^2$. [e] At a surface density of $1.0 \times 10^{-4}$ HAE/Å$^2$. [f] Diffusion-enhanced energy transfer measurements indicate that the distance of closest approach between $Tb(DPA)_3^{3-}$ and rhodamine is greater than 130 Å. The value given assumes a bilayer thickness of 50 Å.

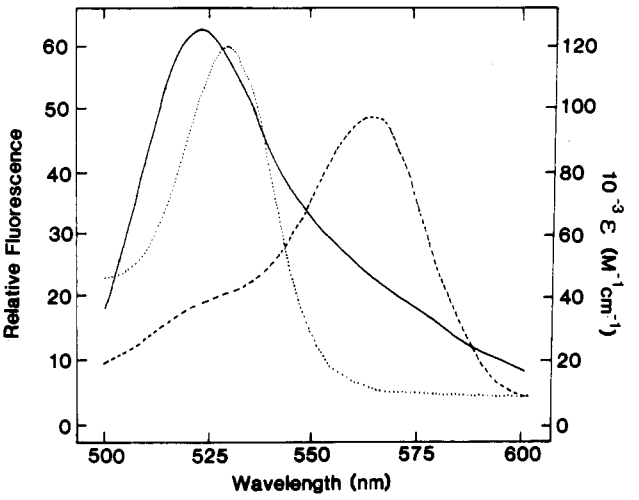


FIGURE 1: Spectral overlaps. The corrected emission spectrum of VhF (—), the absorbance spectrum of OR (---), and the absorbance spectrum of HAE (···) were each recorded in buffer A at room temperature in the presence of 4:1 PC/PS vesicles.

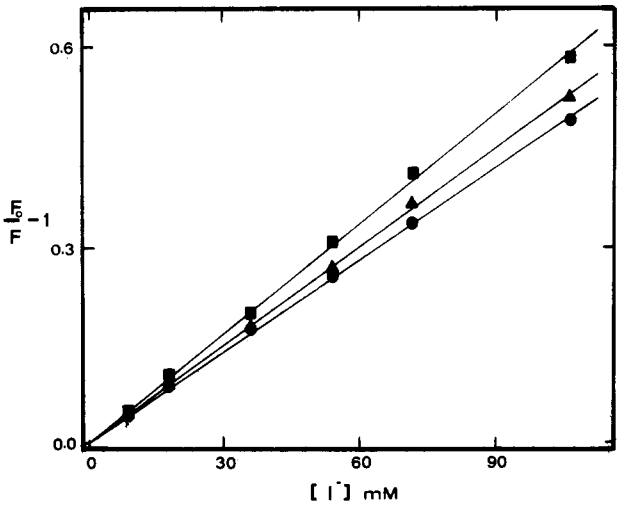


FIGURE 2: Iodide ion quenching of factor VaF fluorescence. Stern–Volmer plots of the quenching in the presence of buffer A only (■), in the presence of Vl (▲), and in the presence of both Vl and PC/PS vesicles (●). Details are given under Experimental Procedures.

0.28 to 0.04, close to that of the free dye (0.038).

*Spectral Properties of Fluorescent Factor Va.* Selected spectral parameters of the labeled proteins are listed in Table II. The emission spectra (Figure 1), emission intensities, and anisotropies of VhF and VhR were not altered by the presence or absence of $Ca^{2+}$ ions, PC/PS vesicles, Vl, prothrombin, or active-site-blocked factor Xa. Thus, the dye is located at a site on Vh which does not allow it to serve as a reporter probe for Vh interactions with phospholipid surfaces, $Ca^{2+}$ ions, or other proteins. Although it would have been useful if the dye had been sensitive to one or more of the interactions between Vh and the other components of the prothrombinase complex, the insensitivity of the dye to the composition of an in vitro incubation is an advantage in interpreting energy transfer experiments.

*Collisional Quenching of Dyes on Vh.* Collisions between fluorescent chromophores and certain solutes, such as iodide ions, result in a quenching of fluorescence (Lehrer & Leavis, 1978). Thus, the exposure of a particular fluorophore to the solvent and dissolved solute can be examined by measuring the emission intensity as a function of quencher concentration. For steady-state collisional quenching of fluorescence, a linear plot is obtained when data are analyzed according to the Stern–Volmer law:

$$F_0/F = K_{SV}[Q] + 1 \qquad (5)$$

where $F_0$ is the fluorescence intensity in the absence of quencher, $F$ is the fluorescence intensity in the presence of quencher at concentration [Q], and $K_{SV}$ is the Stern–Volmer quenching constant.

The accessibilities of the fluorescein and rhodamine dyes on Vh to solvent were examined by using iodide ions as the collisional quenchers. The Stern–Volmer plots were linear in all experiments (Figure 2), indicating that the intensity decrease resulted from the collisional quenching of a homogeneous sample in each case. The observed decrease in fluorescence was not an ionic strength effect because the emission intensity did not change when KCl was added to the solution instead of KI. The $K_{SV}$ for free IAF, 9.8 $M^{-1}$, is consistent with a diffusion-controlled interaction between dye and quencher (Adkins et al., 1983). The similarity of the $K_{SV}$ values for free and protein-bound dyes (Table III) indicates that the fluorescein and rhodamine dyes are exposed to the solvent and are not buried in the protein or in the vesicle. A slightly reduced $K_{SV}$ value would be expected for a protein-bound dye because the presence of the macromolecule would sterically inhibit iodide ion access to the dye to some extent; however, the local electrostatic environment of the dye will also influence $K_{SV}$. Since Vl has little effect on the quenching rate, the dye on Vh does not directly interact with the Vl subunit. Also, the dye appears to be located far from the phospholipid charged surface because there is only a small reduction in $K_{SV}$ in the presence of the net negative charge at the bilayer surface.

Table III: Collisional Quenching of VhF and VhR Fluorescence[a]

| | $K_{SV}$ ($M^{-1}$) | |
|---|---|---|
| | fluorescein | rhodamine |
| free dye[b] | 9.8[c] | 14.3 |
| Vh[d] | 5.5 | 8.8 |
| Vh[d] + Vl | 5.0 | 8.8 |
| Vh[d] + Vl + PC/PS vesicles | 4.6 | 8.0 |

[a] Experiments were done in buffer A described under Experimental Procedures. [b] IAF or IAR. [c] From Adkins et al. (1983). [d] VhF or VhR.

The difference in $K_{SV}$ values for VhF (5.5 $M^{-1}$) and VhR (8.8 $M^{-1}$) is most likely explained by the fact that rhodamine, at pH 7.5, has a positive charge, while fluorescein is negatively

Table IV: Effect of OR and HAE on the Chromogenic Substrate Detected Prothrombinase Activation of Prothrombin[a]

| vesicles added | mol of thrombin $min^{-1}$ (mol of factor Va)$^{-1}$ |
|---|---|
| none | 6.4 |
| PC/PS | 431 |
| PC/PS–OR | 394 |
| PC/PS–HAE | 431 |

[a] Details given under Experimental Procedures.

charged. Hence, electrostatic considerations would dictate that the negatively charged iodide ions at a particular concentration would collide more frequently with a rhodamine dye than with a fluorescein dye at the same site.

*Effect of OR and HAE upon the Prothrombinase Activity.* The energy acceptors in the protein to surface energy transfer experiments, HAE and OR, each possess a long hydrocarbon tail that partitions into the nonpolar region of a phospholipid vesicle bilayer in aqueous solution. However, the dye portion of each of these molecules is charged at the pH of the experiments (7.5) and hence is located at the surface of the phospholipid vesicle (Cerione et al., 1983). It was therefore important to ascertain whether or not the presence of these dyes affected the binding of factor Va, factor Xa, and prothrombin to the phospholipid surface, and the subsequent assembly of the prothrombinase complex. The results presented in Table IV show that insertion of OR or HAE into vesicles did not inhibit thrombin generation.

*Protein to Phospholipid Surface Energy Transfer.* When the reconstituted factor VaF was titrated with PC/PS vesicles containing OR, the fluorescein emission intensity was decreased (Figure 3A). This decrease in intensity reached a maximum value and plateaued at the concentration of phospholipid required for saturation of the vesicle surface with factor Va. In contrast, no fluorescence change was seen when factor VaF was titrated with vesicles lacking OR (Figure 3A). Hence, the decrease in fluorescein emission intensity required the presence of OR and was presumably due to singlet–singlet energy transfer between fluorescein donors and OR acceptors.

When factor VaF was titrated with 100% PC vesicles containing OR at the same concentration as in the above experiment, no decrease in fluorescence was observed (Figure 3B). Since factor Va does not bind to PC vesicles (Bloom et al., 1979; van de Waart et al., 1983), the spectral change shown in Figure 3A requires, in addition to OR, the binding of factor VaF to the vesicles.

Equivalent results were obtained when HAE was substituted for OR in the above experiments.

*Light Scattering.* A sample of factor VaF at the same concentration as in Figure 3A and a sample containing only buffer were each titrated with PC/PS vesicles. The relative right-angle light scattering increased as factor VaF bound to the PC/PS vesicles until it reached a constant value when all factor VaF molecules were bound to the vesicle surface (Figure 4). Most important, the light-scattering results correlated precisely with the fluorescence data (Figure 4). Therefore, two techniques provide independent evidence of the binding of factor VaF to vesicles in this system. The data in Figure 4 also show that the binding of fluorescent and unmodified factor Va molecules to PC/PS vesicles is equivalent.

As expected, when factor VaF was titrated with PC vesicles, there was no change in the relative light-scattering signal (data not shown). This confirms that there was no binding of the protein to PC vesicles in the energy transfer experiments.

*Subunit and $Ca^{2+}$ Dependence.* VI binds to a PC/PS surface in the absence of $Ca^{2+}$, but Vh requires both $Ca^{2+}$ and VI to

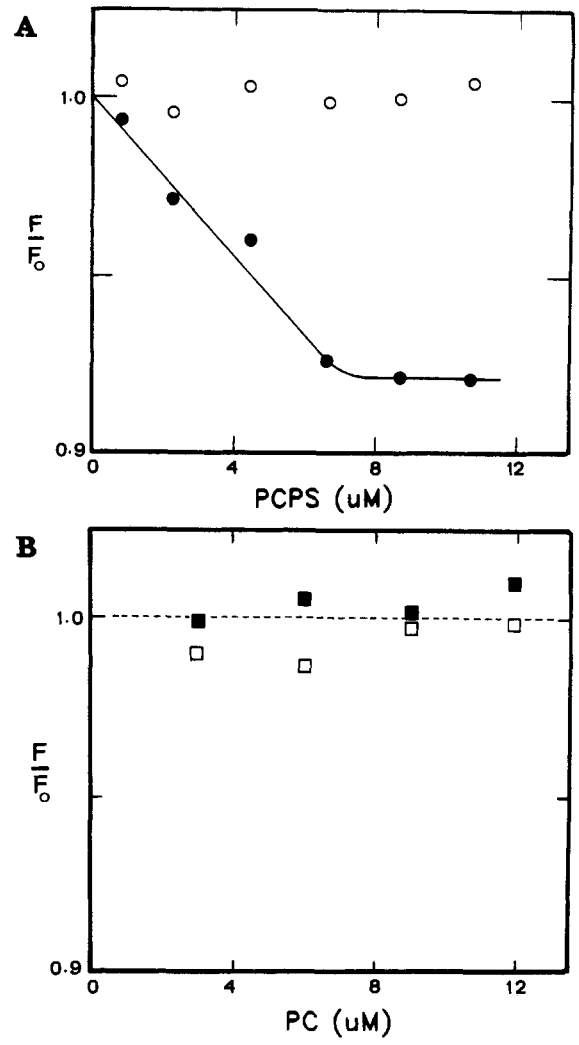


FIGURE 3: Fluorescence energy transfer between protein-bound dyes and dyes at the surface of the phospholipid bilayer. Details are given under Experimental Procedures. (A) Titration of factor VaF with PC/PS (○) and PC/PS–OR (●) vesicles at an acceptor density of $3.28 \times 10^{-4}$ acceptor/Å$^2$. (B) Titration of factor VaF with PC (□) and PC–OR (■) vesicles as in (A).

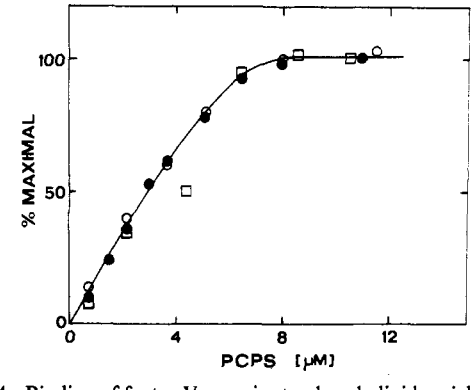


FIGURE 4: Binding of factor Va species to phospholipid vesicles. Direct comparison of light-scattering data obtained with factor Va and PC/PS (●) or factor VaF and PC/PS (○) with the energy transfer data from Figure 3 (□). Details are given under Experimental Procedures; concentrations were the same as in Figure 3, except that the acceptor density in the factor VaF–PC/PS–OR light-scattering experiment was $1.3 \times 10^{-3}$/Å$^2$.

bind to a phospholipid surface (van de Waart et al., 1983; Higgins & Mann, 1983; Tracy & Mann, 1983; Pusey & Nelsestuen, 1984). The energy transfer shown in Figure 3A also required $Ca^{2+}$ and Vh. When 5 mM EDTA was added

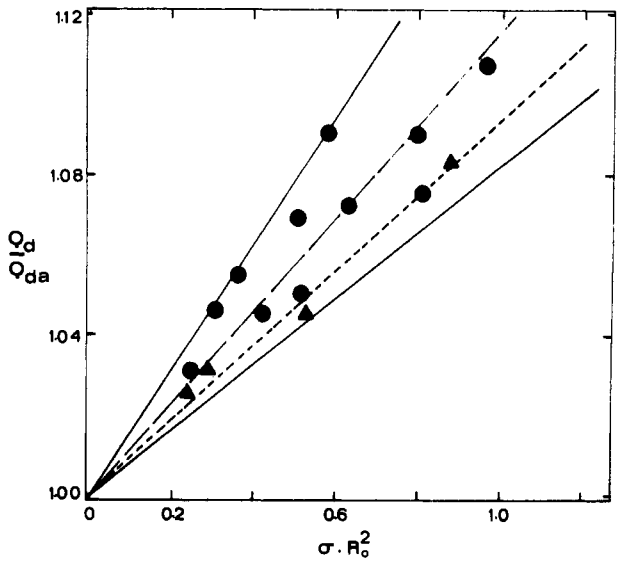


FIGURE 5: Dependence of emission intensity on acceptor density. Ratio of quantum yield in the absence and presence of acceptor ($Q_D/Q_{DA}$) vs. $C$ ($=\sigma R_0^2$) for factor VaF in the presence of either PC/PS–OR (●) or PC/PS–HAE (▲). The solid lines represent the theoretically expected efficiencies of transfer as a function of density for two different ratios of $L/R_0$ (see text). The long-dash line (– –) represents the linear least-squares best-fit line for the 11 OR experiments; the short-dash line (- - -) represents the best-fit line for the 4 HAE experiments.

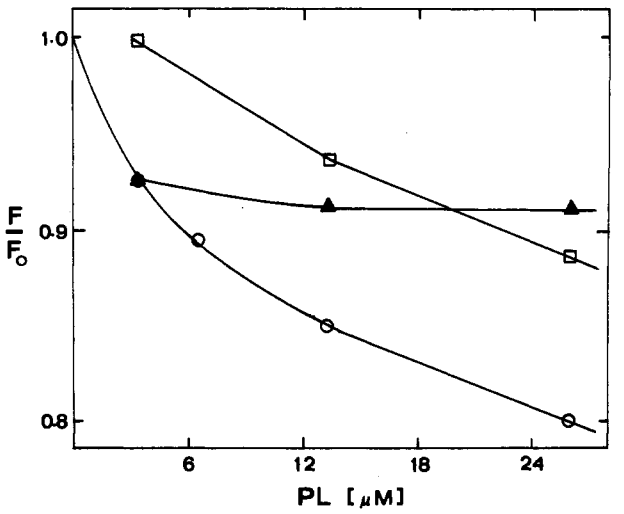


FIGURE 6: Energy transfer at high acceptor density. Titration of factor VaF with PC/PS–OR (○) and PC–OR (□) vesicles at identical acceptor densities of $5.2 \times 10^{-4}$ acceptor/Å$^2$. (▲) represents the difference between the fluorescence changes observed with PC/PS–OR and PC/OR.

to the sample of Figure 3A at the end of the titration, the emission intensity returned to its original value. This was anticipated because $Ca^{2+}$ ion chelation causes the VhF to dissociate from the Vl (Laue et al., 1984). The dissociated VhF would then be too far from the PC/PS surface for significant energy transfer to occur.

Also, when VhF was titrated with PC/PS–OR vesicles in the absence of Vl so that VhF could not bind to the vesicle surface, there was no change in fluorescein emission intensity (data not shown).

*Sensitivity to Unlabeled Factor Va.* An 11-fold excess of unlabeled factor Va was added to a sample of factor VaF which had been titrated with PC/PS vesicles containing OR at an acceptor density of $1.85 \times 10^{-4}$ acceptor/Å$^2$. This addition returned the fluorescein emission back to its value prior to the addition of any vesicles (data not shown). Hence, the dissociation of factor VaF from the surface and its replacement by the unlabeled factor Va eliminated the fluorescence energy transfer.

*Dependence upon Acceptor Density.* The efficiency of energy transfer is a function of, among other things, the density of acceptor dyes at the phospholipid surface. Data from 11 separate experiments with OR and 4 experiments with HAE are shown in Figure 5, with the ratio of the donor (factor VaF) quantum yields in the absence and presence of acceptor, $Q_D/Q_{DA}$, plotted as a function of $C$, the product of $R_0^2$ and $\sigma$, the surface density of acceptor in angstroms squared.

At high acceptor concentrations (>$4 \times 10^{-4}$ acceptor/Å$^2$), there was a fluorescence decrease when factor VaF was titrated with PC–OR vesicles (Figure 6). Since the protein did not bind to PC–OR vesicles, this spectral change did not result from energy transfer between membrane-bound factor VaF and OR. When factor VaF was titrated with PC/PS–OR at the same acceptor densities, the emission intensity did not reach a plateau (Figure 6). However, subtraction of the PC–OR spectral change from the PC/PS–OR spectral change yielded a net emission change (Figure 6) that closely resembled the saturable fluorescence change observed at low acceptor density (Figure 3A). Thus, for acceptor densities greater than 0.0004/Å$^2$, only the phospholipid binding-dependent emission decrease ($\Delta F_{PC/PC-OR} - \Delta F_{PC-OR}$) was considered to be due to energy transfer. Consistent with this conclusion, the addition of EDTA at the end of a titration to a PC/PS–OR sample such as that in Figure 6 did not restore the emission intensity to its value in the absence of OR, as it did in samples of lower acceptor density. Instead, the emission intensity was increased only to the level found in a parallel PC–OR sample.

*Calculation of $L$, the Distance of Closest Approach.* The energy transfer between randomly and uniformly distributed donors in one plane and randomly and uniformly distributed acceptors in a parallel infinite plane has been considered by several groups, including Shaklai et al. (1977), Wolber and Hudson (1979), Estep and Thompson (1979), Koppel et al. (1979), Dewey and Hammes (1980), and Snyder and Freire (1982). When the extent of energy transfer is small, as it is in this case, the first term in the approximate series solution of Dewey and Hammes (1980) can be used to solve for the distance $L$:

$$Q_D/Q_{DA} = 1 + (\pi\sigma R_0^2/2)(R_0/L)^4 \quad (6)$$

where $L$ is the distance of closest approach between the protein-bound donors and the acceptors at the phospholipid surface.

With eq 6, the value of $L$ found in the 11 OR experiments ranged from 82 to 94 Å, with an average of 88 Å. When the data in Figure 5 were analyzed by using linear least-squares regression analysis, a value of 88 ± 3.8 Å was calculated for $L$. The data points cluster about the calculated best-fit line (upper dashed line in Figure 5), and there is no systematic deviation from this line.

The average value found in the four HAE experiments was 93 Å, with a range from 91 to 96 Å. The value of $L$ obtained by using linear least-squares analysis was 92 ± 1.8 Å for this donor–acceptor pair.

The data in Figure 5 were also analyzed by using the Monte Carlo method of Snyder and Freire (1982). Theoretical curves of $Q_D/Q_{DA}$ vs. $C$ (defined above) were generated by using a program generously supplied to us by Drs. Brian Snyder and Gordon Hammes. The solid lines in Figure 5 were obtained by using values of 82 and 96 Å for $L$, and hence, all of our experiments yielded a value of $L$ between those limits. The

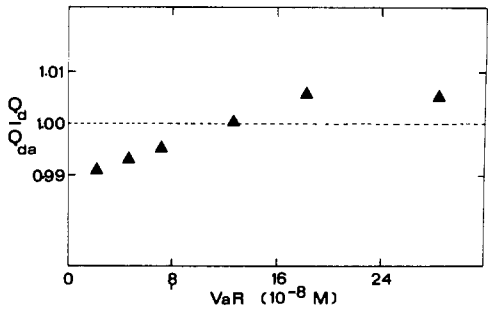


FIGURE 7: Titration of PC/PS (4:1) vesicles containing $Tb(DPA)_3^{3-}$ with factor VaR. Details are given under Experimental Procedures.

theoretical curves obtained by using the above best-fit values of $L$ overlaid the best-fit lines in Figure 5 for each donor–acceptor pair. Thus, on the basis of the average of the two best-fit $L$ values, the fluorescein dye on membrane-bound factor VaF is located 90 Å away from the acceptor dyes at the surface of the phospholipid vesicle. The data are summarized in Table II.

*Effect of Factor Xa and Prothrombin on the Observed Energy Transfer.* At the end of four titration experiments (cf. Figure 3A), factor Xa, freshly treated with *p*-APMSF to block its active site, was added to the cuvettes in amounts equimolar to that of factor VaF. This resulted in a decrease in the fluorescein emission intensity that ranged from 1.2% to 4.5%, depending upon OR density. This small, but significant (see Experimental Procedures), change in intensity corresponded to an average decrease in $L$ of 7 Å (range over four experiments was 6–10 Å). The fluorescence of the sample did not change during the period from 10 to 50 min following the addition of the active-site-blocked factor Xa, indicating that the emission change did not result from proteolysis of the VhF.

The addition of prothrombin (treated with DIFP just before use to inactivate any thrombin contaminant) did not alter the donor emission intensity in a sample containing factor VaF and PC/PS–OR (cf. Figure 3A). Also, the fluorescence of a sample containing factor VaF, PC/PS–OR, and active-site-blocked factor Xa was not affected by the addition of prothrombin.

*Diffusion-Enhanced Energy Transfer.* The distance of closest approach between the fluorescent-labeled domain on the Vh subunit and the inside surface of the phospholipid bilayer was measured by using factor VaR, reconstituted from unmodified Vl and rhodamine-labeled Vh, as the energy acceptor. The donor, a complex of $Tb^{3+}$ and dipicolinate (DPA), was entrapped inside phospholipid vesicles as described under Experimental Procedures, and the extravesicular $Tb(DPA)_3^{3-}$ was then removed by gel filtration. No leakage of the $Tb(DPA)_3^{3-}$ was observed during any of the 2–3-h-long experiments.

When factor VaR was added to PC/PS vesicles containing $Tb(DPA)_3^{3-}$, there was less than a 1% decrease in the terbium emission and hence very little measurable energy transfer between the donor and acceptor dyes (Figure 7). No fluorescence change was observed when factor VaR was added to a sample of PC vesicles containing $Tb(DPA)_3^{3-}$ (data not shown).

Terbium emission was not altered when either active-site-blocked factor Xa or prothrombin was added to samples that contained factor VaR bound to vesicles in which $Tb(DPA)_3^{3-}$ was trapped. Thus, it appears that the distance between $Tb(DPA)_3^{3-}$ donors and the rhodamine acceptors is so large that not even a 7-Å movement (see above) is sufficient to increase the diffusion-enhanced energy transfer significantly.

## Discussion

The covalent attachment of fluorescein or rhodamine dyes to the larger subunit of blood coagulation factor Va had no apparent effect upon the protein: its functional activity (Table I) and its phospholipid binding properties (Figure 4) were the same before and after modification. The spectral insensitivity of the attached dyes to Vh interaction with Vl, $Ca^{2+}$, factor Xa, prothrombin, and phospholipid vesicles suggests that the attachment site is spatially distinct and conformationally uncoupled from the Vh domains involved in those interactions. The collisional quenching data show that the dyes are located on the surface of the Vh subunit, and the similarity of the bimolecular collisional constants in the presence and absence of PC/PS vesicles indicates that the extrinsic dyes are not located close to the phospholipid surface (Figure 2, Table III).

The distance of closest approach, $L$, between the fluorescein dyes on vesicle-bound factor Va and the outer surface of the phospholipid bilayer was determined directly by using fluorescence energy transfer, and the average value of $L$ obtained by using two different amphipathic acceptor dyes was 90 Å. In these experiments, the dependency of the fluorescence change upon both factor VaF binding to the phospholipid vesicles and the presence of acceptor dyes demonstrated that the spectral change resulted from energy transfer between the dyes attached to membrane-bound proteins and the dyes at the outer phospholipid surface. Because the acceptor dyes at the inner surface of the 50-Å-thick bilayer are located so far from the fluorescein on factor VaF, they do not contribute significantly to the observed energy transfer.

The 90-Å value for $L$ was calculated by assuming that $\kappa^2$ was $^2/_3$. Since the dyes at the phospholipid surface and on the protein do not rotate with complete freedom, there is uncertainty in the acceptor–donor dipole orientation during the excited-state lifetime of the donor. The *maximum* uncertainty in $R_0$ amounts to ±25% (Dale et al., 1979) when the steady-state anisotropy values in Table II are used. However, because the acceptors are oriented randomly at the phospholipid surface (Fung & Stryer, 1978; Holowka & Baird, 1983a), a more reasonable estimate of the uncertainty in $R_0$ is ±10% (Stryer, 1978; Fung & Stryer, 1978; Cerione et al., 1983). It is also important to emphasize that nearly the same value of $L$ was obtained by using two different donor–acceptor pairs. This indicates that there are no unusual orientation problems.

The calculation of the distance of closest approach also assumed that the acceptor dyes were uniformly distributed. There was no evidence of acceptor aggregates around the protein because the absorptivities and emission intensities of positively charged OR or negatively charged HAE in PC/PS vesicles were not altered by the binding of factor Va to the vesicle surface. In addition, the prothrombinase complex functioned equally well in the presence and absence of OR and HAE (Table IV), and light-scattering data showed that the presence of OR did not interfere with the binding of factor VaF to vesicles (Figure 4).

As noted under Results, three fluorescent peaks were found in the tryptic digests of Vh. If these peptides did in fact originate from different regions of the Vh polypeptide, and fluorescein dyes were located at two or more different sites on Vh, the observed energy transfer would be a weighted average [see Holowka & Baird (1983b)] of the energy transfer from the sites. Hence, if one site were located less than 90 Å from the surface, a second site would have to be located more than 90 Å from the surface. Thus, the 90-Å distance of closest approach that we have measured represents the *minimum* distance between a dye attached to a domain of

factor Va and the dyes at the phospholipid surface.

The data obtained at acceptor densities greater than $4 \times 10^{-4}$ acceptor/Å$^2$ were corrected for fluorescence changes that were not related to factor VaF binding to vesicles (see Results). The cause of this $Ca^{2+}$- and PS-independent fluorescence change is not understood. It cannot be due to an inner filter effect, because the absorbances at the excitation and emission wavelengths were less than 0.01 in these experiments. One possibility is that it originates from an interaction between factor VaF and the acceptors in the aqueous phase. Although the acceptors partition strongly into the lipid phase (Holowka & Baird, 1983a), the aqueous concentration may become high enough, at high acceptor densities, that the acceptor molecules bind weakly to a site(s) either close enough to the donor dye to participate in energy transfer or conformationally coupled so that the binding of the acceptor affects the environment and quantum yield of the donor. Such an effect is more noticeable, of course, in systems where the low efficiency of energy transfer requires that experiments be done at high concentrations of acceptor. A similar effect has also been observed by Holowka and Baird (1983b) in their system. Whatever the source of the PS-independent fluorescence decrease, it did not require factor VaF binding to the phospholipid surface, and hence was not due to energy transfer from membrane-bound factor VaF to acceptor dyes at the bilayer surface.

It is conceivable that the binding of factor Va to the phospholipid surface creates an area, termed the "excluded area" by Wolber and Hudson (1979), which lacks acceptor molecules. In such a situation, the distance of closest approach between the donor and acceptor dyes is not equal to the perpendicular distance of the donor dye above the plane of the phospholipid surface. For example, if factor Va is shaped like a disk, the sedimentation data of Laue et al. (1984) indicate that factor Va would have a major axis near 234 Å and a minor axis near 40 Å. Then if this disk bound flat on the surface and excluded OR, the fluroescein dye could be located near the middle of the disk, only a few angstroms above the surface, and still be 90 Å from the nearest OR.

To examine this possibility, energy transfer in the rapid-diffusion limit was measured between $Tb(DPA)_3^{3-}$ entrapped inside vesicles and factor VaR bound to the outer vesicle surface. Almost no energy transfer was observed, indicating that the rhodamine dye on factor Va was located very far from the inner surface of the phospholipid vesicle. Negatively stained electron micrographs showed that the vesicles in our preparations were unilamellar and had an average diameter of 750 Å. Assuming a bilayer thickness of 50 Å, 1.1 dyes per factor VaR, and 2400 Å$^2$ per factor VaR (Higgins & Mann, 1983), and using eq 8 in Thomas and Stryer (1982) to calculate the transfer efficienty, $E$, an $E$ value of 0.009 would be predicted if the rhodamine dye were located 90 Å above the outer surface of the vesicle. If the rhodamine dye were located 70, 40, or 20 Å above the outer bilayer surface, the expected $E$ values would be 0.015, 0.032, and 0.065, respectively. Thus, the diffusion-enhanced energy transfer results (Figure 7) are consistent with the fluorophore on the Vh subunit being located a minimum of 80 Å above the outer surface of the phospholipid vesicle. This in turn indicates that the excluded area, if it exists at all, is small.

The large frictional coefficient of factor Va shows that the protein is not spherical (Laue et al., 1984), and the results described in this paper demonstrate that a domain of factor Va is located a minimum of 90 Å above the phospholipid surface. Numerous molecular shapes for factor Va could accommodate both of these results without conflict. Also, the overall dimensions of factor Va, as visualized in electron microscope studies (Lampe et al., 1984; Dahlbäck, 1985; Mosesson et al., 1985), are compatible with a domain located 90 Å above the surface. However, as noted earlier, protein molecules were seen both lying "flat" and projecting out of the surface in the electron microscopic studies of membrane-bound factor Va (Lampe et al., 1984).

In the absence of a clear indication of the shape of membrane-bound factor Va, it is useful to consider what the energy transfer measurements suggest about the orientation of an *elongated* factor Va molecule bound to a membrane surface. If we assume factor Va is a prolate ellipsoid of axial ratio 5:1, length 220 Å, and diamter 42 Å (Laue et al., 1984), the extremes of the possible orientations of membrane-bound factor Va are lying flat, projecting radially, or embedded and projecting out of the surface. Because the 90-Å distance is considerably larger than the 42-Å hydrodynamic diameter of a prolate factor Va, it would appear that the protein does not lie flat on the surface of the vesicle. Thus, fluorescence energy transfer rules out the first possibility. The essential difference between the other two models is that the protein is embedded in the lipid bilayer in one case, and not in another. Recent experiments using two different lipophilic photoreagents suggest that only a small domain of Vl is exposed to the lipid bilayer (U. C. Krieg, B. S. Isaacs, S. S. Yemul, C. T. Esmon, H. Bayley, and A. E. Johnson, unpublished results).

The minimum angle between the long axis of factor Va and the plane of the surface is 24°. This could occur only if the protein bound to the surface at one end and only if the dye is located at the other end of the protein. Any other location of the dye on the protein would increase the angle between the long axis of factor Va and the plane of the surface. Also, an increased extent of insertion of Vl into the lipid bilayer will tend, in general, to increase this angle. Because the probability of the dye being at one end is small, it seems most likely that the long axis of the elongated factor Va is oriented more or less perpendicularly to the membrane surface (Figure 8).

Light-scattering data indicate that factor Xa and prothrombin, two proteins that associate with factor Va on a phospholipid surface, each project radially out of the surface of the vesicle (Lim et al., 1977). Therefore, it is reasonable for factor Va to bind to a membrane so that it also projects more or less radially out of the surface. The prothrombinase complex would then consist of three elongated proteins (enzyme, cofactor, and substrate) associated with each other to form a rodlike complex that is bound to a membrane at one end and projects out of the surface. Such an arrangement would be advantageous in that it would take a minimal amount of membrane surface area. The rate of blood coagulation would then be less likely to be limited by the amount of free, uncovered phospholipid available on cell surfaces. The projection into the blood may also help factor Va to recruit and interact with the other macromolecular components (factor Xa and prothrombin) needed for blood coagulation.

A particularly interesting observation made in this work is that the active-site-blocked factor Xa decreased $L$ by 7 Å. Thus, the association of factor Xa with factor VaF on the vesicle surface moves a domain of Vh 7 Å closer to the surface, either by causing a conformational change in the factor Va molecule or by altering the orientation of the entire molecule with respect to the surface. However, association with the substrate of the prothrombinase complex, prothrombin, did not alter the distance between the labeled domain on factor VaF and the phospholipid surface.

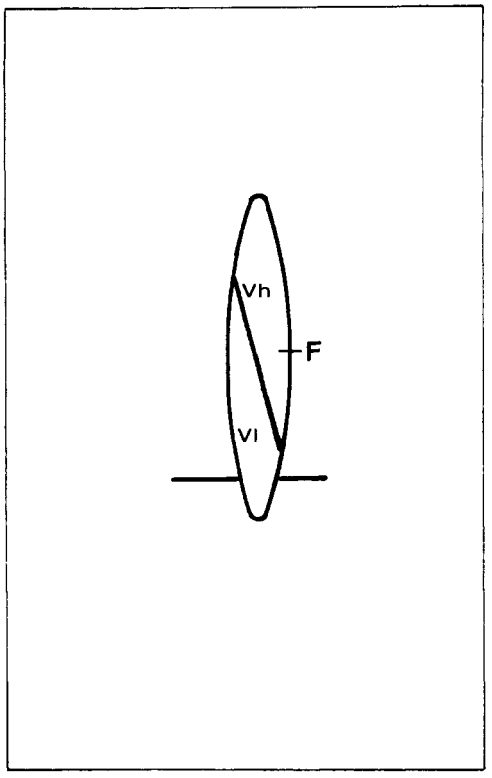


FIGURE 8: Model of an elongated factor Va molecule bound to a phospholipid surface. This orientation is compatible with the results discussed in the text.

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**Registry No.** DOPC, 4235-95-4; blood coagulation factor Va, 65522-14-7; blood coagulation factor Xa, 9002-05-5.

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# Structural and Functional Differences between the Two Intrinsic Zinc Ions of *Escherichia coli* RNA Polymerase†

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ABSTRACT: DNA-dependent RNA polymerase (RPase) from *Escherichia coli* contains 2 mol of intrinsic Zn(II)/mol of core enzyme ($\alpha_2\beta\beta'$). In techniques analogous to those employed with the Zn(II) metalloenzyme aspartate transcarbamoylase [Hunt, J. B., Neece, S. H., Schachman, H. K., & Ginsberg, A. (1984) *J. Biol. Chem. 259*, 14793–14803], we show that titration of core or holoRPase with 10 or 16 equiv, respectively, of the sulfhydryl reagent *p*-(hydroxymercuri)benzenesulfonate (PMPS) results in the facile release of 1 mol of Zn(II) [B-site Zn(II)] in a reaction totally reversible with the addition of excess thiol provided no metal chelator is present. If ethylenediaminetetraacetic acid (EDTA) is present, reversal of the PMPS–enzyme complex results in formation of a $Zn_1$ RPase [A-site Zn(II)]. This enzyme retains full transcriptional activity relative to $Zn_2$ RPase on both calf thymus (nonspecific) and T7 ($\sigma$-dependent, specific) DNA templates. If the core enzyme–PMPS complex is incubated with a large excess of another metal such as Cd(II) followed by thiol treatment, a hybrid $Zn_ACd_B$ RPase is formed. Direct treatment of the enzyme with excess Cd(II) also gives rise to a hybrid $Zn_ACd_B$ RPase. Transcription by these enzymes is also comparable to that of the starting $Zn_2$ enzyme. Isolation of in vivo synthesized $Co_2$ RPase and $Cd_2$ RPase and treatment of either enzyme with PMPS/EDTA results in formation of a $Co_A$ and $Cd_A$ enzyme, respectively. $Co(II)_A$ and $Cd(II)_A$ enzymes show 123 and 76%, respectively, of the elongation rates on T7 DNA observed for the Zn(II) enzyme. Visible absorption spectroscopy of the $Co_2$ enzyme exhibits four d–d transition bands positioned at 760 ($\epsilon$ = 800), 710 ($\epsilon$ = 900), 602 ($\epsilon$ = 1500), and 484 ($\epsilon$ = 4000) nm. In addition, two charge-transfer bands are found at 350 ($\epsilon$ = 9600) and 370 ($\epsilon$ = 9500) nm. Only the Co(II) ion bound at site A is associated with this unique set of intense d–d transitions. The positions and intensities of both the visible and charge-transfer bands of $Co(II)_A$ RPase approximate those shown by Co(II)-substituted metalloenzyme sites where the ligands are four S rather than mixed S,N or S,O sites.

DNA-dependent RNA polymerase (RPase)[1] from *Escherichia coli* is a complex, multisubunit Zn(II) metalloenzyme ($M_r \approx 450000$) consisting of the subunit arrangement of $\alpha_2\beta\beta'\sigma$ [review by von Hippel et al. (1984)]. Subunit $\sigma$ confers upon the catalytically competent core enzyme promoter specific recognition in the transcription of RNA chains (Chamberlin, 1974). More limited information exists concerning the role that each of the subunits plays in the transcription process with the possible exception of the $\beta$ subunit (Yura & Ishihama, 1979; Zillig et al., 1976). Mutant forms of the enzyme insensitive to the initiation inhibitor rifamycin without exception contain base changes in the coding sequence for the $\beta$ subunit (Rabussay & Zillig, 1969; Iwakura et al., 1973). Thus, the $\beta$ subunit almost certainly plays a role in the initiation of RNA chains. The $\beta'$ subunit, on the other hand, appears to contribute to template binding due perhaps to its strongly positive charge at neutral pH (Zillig et al., 1970). Topological experiments by Meares and co-workers suggest that the path of the growing RNA chain occurs proximate to both $\beta$ and $\beta'$ subunits with the involvement of $\sigma$ subunit only through two to three rounds of nucleotide incorporation (Hanna & Meares, 1983a,b). Chemical cross-linking experiments between RPase subunits (Hillel & Wu, 1977) and between the enzyme and promoter-containing DNA (Park et al., 1980) are consistent with this picture. None of the individual subunits alone, nor a $\beta$–$\beta'$ heterodimer, possesses significant transcriptional activity (Yarbrough & Hurwitz, 1974).

The precise role, structural or catalytic, played by the two intrinsic Zn(II) ions in the transcription process is not well-defined. Limited data provided by complete denaturation of RPase, isolation of denatured subunits, and renaturation and assessment of Zn(II) binding by individual subunits suggest that one Zn(II) is associated with the $\beta'$ subunit, while the other is either bound by the $\beta$ subunit or present at the $\beta$–$\beta'$ interface (Wu et al., 1977; Miller et al., 1979; Chatterji &

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[1] Abbreviations: PMPS, *p*-(hydroxymercuri)benzenesulfonate; PAR, 4-(2-pyridylazo)resorcinol; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; TNG buffer, 10 mM Tris-HCl, pH 7.9, 0.2 M NaCl, and 5% glycerol; RPase, RNA polymerase; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; M, metal.

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