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Structure of the Prothrombin- and Blood Clotting Factor X-Membrane Complexes†

T. K. Lim,‡ Victor A. Bloomfield, and Gary L. Nelsestuen*,§

ABSTRACT: The configuration of the prothrombin- and factor X-membrane complexes was investigated by the technique of quasielastic light scattering. It is concluded that the fragment 1 region of prothrombin is located at one end of the prothrombin molecule and that the membrane binding site is at the tip of the fragment 1 region. Prothrombin binds to the surface of the membrane with no detected penetration into the lipophilic region of the membrane. The remainder of the prothrombin molecule extends radially from the membrane surface with maximum protrusion into solution. Factor X also binds to the membrane at one end of the molecule and extends into solution. Based on the evidence presented here and in other communications [Nelsestuen, G. L., and Lim, T. K. (1977), *Biochemistry 16,* and Nelsestuen, G. L., and Broderius, M. (1977), *Biochemistry 16* (respectively the first and second in a series of three papers in this issue)] a model for prothrombin-membrane interaction is given. Quasielastic light scattering appears to be a valuable new method for studying protein-membrane interactions.

Membrane-associated proteins are often referred to as intrinsic or extrinsic membrane proteins. A more recent terminology which appears closely related is the terms "integral" and "peripheral" membrane proteins (Singer, 1974). Proteins are categorized largely on the ease by which they can be separated from the membrane. Proteins which bind tightly to the membrane and require detergents for release are considered integral or intrinsic proteins and are thought to be buried in the lipid region of the membrane. Those which are released by chelating agents or high salt concentrations are considered extrinsic or peripheral proteins and are thought to bind to the membrane through ionic forces or to integral membrane proteins. While the available evidence supports these conclusions, they have not been established for a large number of proteins.

Since prothrombin and factor X require calcium ions and acidic phospholipid for membrane interaction (Papahadjopoulos and Hanahan, 1964) and are always in rapid equilibrium between the bound and free state (Nelsestuen et al., 1976; Nelsestuen and Lim, 1977), the current model for protein-membrane interaction would predict that these proteins bind to the membrane surface through ionic forces only. This is merely an expectation, however, and does not eliminate considerable penetration of these proteins into the lipophilic region of the membrane. Direct experimental evidence is required to support this expectation.

The technique of quasielastic light scattering (Berne and Pecora, 1976) can be used to rapidly determine the diffusion constant of a molecule or complex in solution. From the diffusion constant and knowledge of the molecular shape, the dimensions of the particle can be calculated. This communication utilizes quasielastic light scattering for determination of the configuration of the prothrombin- and factor X-membrane complexes. The results provide evidence about the structure of the protein as well as that of the protein-membrane complex. When combined with results presented in other communications (Nelsestuen and Lim, 1977; Nelsestuen and Broderius, 1977), a model containing considerable detail about the prothrombin- and factor X-membrane complexes can be drawn. The technique of quasielastic light scattering appears to be a valuable new tool in the study of protein-membrane structure.

Materials and Methods

Diffusion Constant Measurement. Quasielastic laser light scattering (QLS) spectroscopy was used to determine the diffusion coefficients for the phospholipid vesicles and protein-phospholipid vesicle complexes. The QLS apparatus used in this experiment has been described previously by Benbasat and Bloomfield (1975). The concentration of sample was kept at 2 to 5 mg/mL, giving an average distance between the particles of ca. 1600 to 1200 Å. With an ionic strength 0.13 for all samples (Debye shielding length < 100 Å), charge interaction between the particles was negligible. No dependence

† From the Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108. *Received March 17, 1977.* Supported in part by Grants GM 17855 to V.A.B. and HL 15728 to G.L.N. from the National Institutes of Health.
‡ A Minnesota Heart Association Postdoctoral Fellow.
§ An Established Investigator of the American Heart Association.

of the diffusion coefficient on the concentration was observed.

The scattering cell is of cylindrical shape of 7-mm inner diameter and 7.5 cm long (Pyrex No. 9820 test tube). A sample volume of 1 mL was placed in the cell and centrifuged for an hour at 9000 rpm in the Beckman JA20 rotor to remove dust particles. The cell is then carefully transferred to the light-scattering chamber for the measurement of the translational diffusion coefficient. When the sample is a solution of single size spherical particles, the photocurrent autocorrelation function decays exponentially with decay constant proportional to the diffusion coefficient of the scatterer, $D = kT/6\pi\eta R$, where η is the viscosity of solution and R is the hydrodynamic radius of the particle (Tanford, 1961). When the system is polydisperse in size, the calculated diffusion coefficient will be a Z-average diffusion coefficient (Koppel, 1972). In subsequent stoichiometric consideration, the molar properties are the pertinent ones. Therefore, the number average diffusion coefficient rather than the Z-average is required.

The Z average diffusion coefficient, $\overline{D}_Z$, obtained from the cumulant analysis of the correlation function is, therefore, corrected to the number average diffusion coefficient (Selser and Yeh, 1976), $\overline{D}_N$, before the size of the vesicle is calculated ($\overline{D}_N = \overline{D}_Z(1 + 3\delta_Z)$, where δ_Z is a normalized standard deviation of $\overline{D}_Z$). The diffusion coefficients and sizes of the vesicles and vesicle–protein complexes are given in Table I below. Measured diffusion coefficients, and therefore hydrodynamic radii derived from them, are uncertain by ±2%.

The average size of the phospholipid vesicles, prepared as described in an accompanying communication (Nelsestuen and Lim, 1977), increased with time for some preparations. For example, one preparation of 50% phosphatidylserine gave a 10% change in average vesicle radius in 13 h. The diffusion coefficient of the vesicles was always measured as a reference before measurement of the diffusion coefficient for the protein–vesicle complex. The membrane compositions are expressed as a percentage of acidic phospholipid. The remaining membrane weight is phosphatidylcholine.

Electrophoresis. The electrophoretic mobility of membrane vesicles was measured by the method described by Lim et al. (1977). The cell is a glass tube with a 5-mm inner diameter and 11-cm length. This tube stands upright and both ends are connected to buffer chambers in which the electrodes are placed. A band of sample (~1 mm thick) is layered at the bottom of the tube and a sucrose gradient of 3% sucrose/in. is established along the tube to stabilize the band. As the electric field is applied along the cell, the sample moves upward and the position of the band is monitored as a function of time by monitoring the intensity of scattered light from the sample.

The electrophoretic mobility is obtained from the velocity of band movement and the electric field applied. The electroosmosis effect was minimized to less than 0.1×10^{-4} cm^2 V^{-2} s^{-1} by treating the cell surface with Siliclad. The system was calibrated with red blood cells ($\mu_{20,w} = 1.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 7.5) and hemoglobin ($\mu_{20,w} = 0.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 6.8) (Pauling et al., 1949). The measured electrophoretic mobility of monomer vesicles of 50% phosphatidylserine was 4.8×10^{-4} cm^2 V^{-1} s^{-1}.

All other conditions, materials, and methods are as described in an accompanying communication (Nelsestuen and Lim, 1977).

Results

The questions being addressed are: (a) The number of protein molecules bound to a vesicle; (b) the maximum packing density of the protein on the membrane; (c) the degree of penetration, if any, of the protein into the membrane; and (d) the configuration of the protein on the surface of the membrane.

The number of prothrombin molecules bound to a monomer vesicle was calculated from light-scattering intensity measurements and the estimated molecular weight of the vesicles. The molecular weight of 50% phosphatidylserine vesicles of radius 163 Å, assuming a membrane thickness of 50 Å (Shipley, 1973) and density of 0.9 ± 0.1 gm/cm^3 is calculated to be 1.09×10^7. This value is consistent with the molecular weight estimated by the electrophoretic mobility.

The electrophoretic mobility of these vesicles was $(-4.8 \pm 0.4) \times 10^{-4}$ cm^2 V^{-1} s^{-1}, from which the ζ potential (Tanford, 1961) is calculated to be 78 mV. The surface charge density estimated (Barton, 1968) from this potential is 7×10^4 esu/cm^2 which corresponds to an average area of 68 Å^2 per univalent charge. The number of univalent charges on the surface of the membrane vesicle (of radius 163 Å) will then be about 4730. Each phosphatidylserine contributes one univalent charge and has a molecular weight of about 770. For a membrane vesicle of 50% phosphatidylserine, the molecular weight of a vesicle is calculated to be 1.07×10^7. This value agrees with that calculated above from the estimated dimensions and density of the vesicles.

The maximum protein binding capacity of these vesicles is 1.2 g of prothrombin per g of phospholipid (Nelsestuen and Broderius, 1977) which gives protein bound of 1.31×10^7 daltons or 182 molecules of prothrombin per vesicle (prothrombin mol wt = 72 000 (Ingwall and Scheraga, 1969)). The ratio of phosphatidylserine to bound prothrombin is estimated from this binding capacity to be about 40. Since about 67% of the membrane surface is exterior, a similar percentage of phosphatidylserine will be on the exterior surface of the membrane and the number of exposed phosphatidylserine residues per prothrombin molecule is 27. Since the minimum number of phosphatidylserine residues per prothrombin is 8 to 10 (Nelsestuen and Lim, 1977), the factor that limits protein binding to the membrane appears to be the protein packing density on the membrane surface.

A packing density of 182 prothrombin molecules per vesicle of 163 Å radius gives an area of 1830 Å^2 per prothrombin molecule. With hexagonal close packing, the minimum area required per spherical molecule can be calculated from area $= 2\sqrt{3}r^2$. Assuming spherical shape, the diameter of the fragment 1 region of prothrombin is 38 Å and would require 1250 Å^2 per molecule. From these values we conclude that, at maximum binding, the membrane surface is almost filled (68%) with prothrombin molecules. Studies on the configuration of the protein on the membrane surface given below substantiate this conclusion.

The Configuration of the Prothrombin–Phospholipid Binding. When small particles bind to the surface of a large particle forming a new envelope, the effective size of the complex is not the outermost surface of the small particle envelope. Bloomfield et al. (1967) calculated the effective radius or Stokes radius of the fully packed particle when both the large and small particles are spherical:

$$R_{\text{eff}} = R(1 + 0.25\beta)$$

where β is the ratio of the small to large particle radius and R is the radius of the large particle. This result was confirmed analytically by McCammon et al. (1975). The effective radius decreases further if the surface of the vesicle is not fully packed. The Stokes radius obtained from the diffusion coefficient as a function of protein/membrane mixing ratio was measured

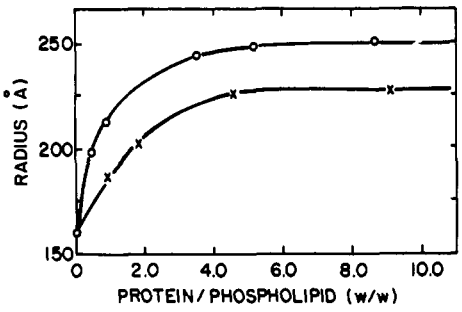

FIGURE 1: Changes in particle radius as protein binds to the vesicle surface. The phospholipid is 50% phosphatidylserine at a concentration of 0.24 mg/mL in 1 mM calcium ion. Prothrombin (O) or factor X (X) was added and the effective particle radius measured by the quasielastic light-scattering procedure given in Materials and Methods.

TABLE I: Radius Changes for Protein-Vesicle Complexes.[a]

Protein	Radius of vesicle (Å)	Radius of complex[b] (Å)	Δr (Å)	Δr_{theor}[c] (Å)
Prothrombin	163	248	85	85
Fragment 1	173	201	28	24
Factor X	163	237	74	

[a] Determined by quasielastic light scattering as given in Materials and Methods. [b] Determined at protein/phospholipid ratios giving about 90% saturation binding. [c] Calculated as outlined in the text for the protein-membrane configurations shown in Figure 3.

and is shown in Figure 1 for both prothrombin and factor X. The change in radius as the protein to membrane ratio is increased corresponds to the expected changes (Bloomfield et al., 1967) when a small particle (prothrombin) binds randomly to the surface of a large particle (phospholipid vesicle). At low protein packing densities the changes in diffusion coefficient are large and near saturation the changes in diffusion coefficient and Stokes radius are very small (see Figure 1). For measuring the configuration of proteins bound to the membrane surface, it is therefore important to measure the diffusion coefficient at high protein packing density and to correct for both incomplete protein packing and the irregular surface effects.

The changes in the average radius of vesicles of 50% phosphatidylserine when prothrombin, prothrombin fragment 1, and factor X are bound are given in Table I. From the dissociation constant for prothrombin (and presumably prothrombin fragment 1) and factor X binding to vesicles of this composition (Nelsestuen and Broderius, 1977), the binding sites on the membrane are estimated to be 90% saturated under the conditions used in all cases.

The shape of the prothrombin molecule is a prolate ellipsoid with semiaxes a = 18 Å and b = 55 Å (Lamy and Waugh, 1953). Prothrombin consists of three regions designated prothrombin fragment 1, prothrombin fragment 2, and prethrombin 2 with molecular weights of 23 000, 12 000 and 40 000, respectively (Heldebrant et al., 1973; Stenn and Blout, 1972; Owen et al., 1974). Prethrombin 2 is the precursor of thrombin and fragment 1 contains the γ-carboxyglutamic acid residues and membrane binding site. Assuming that each of these regions is spherical and has similar specific volumes, one arrangement giving the correct overall dimensions is shown in Figure 2. The diffusion coefficient calculated from this configuration by the method of Bloomfield et al. (1967), $D_{cal} = 6.12 \times 10^{-7}$ cm^2/s, agrees well with the experimental data ($D_{exp} = 6.24 \times 10^{-7}$ cm^2/s, Lamy and Waugh, 1953).

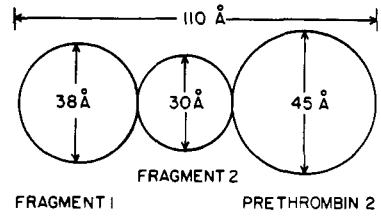

FIGURE 2: Proposed configuration of the prothrombin molecule.

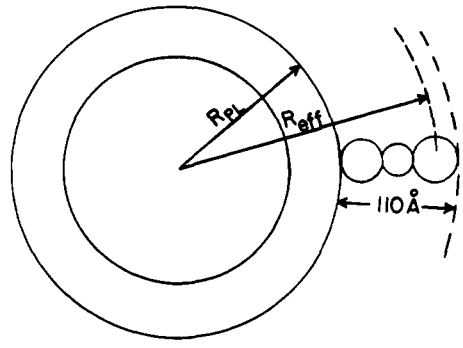

FIGURE 3: Configuration of the prothrombin-membrane complex. The radius of the phospholipid vesicle alone is given by R_{PL}. The effective radius of protein-membrane particle when the vesicle surface is saturated with protein molecules is given by R_{eff}.

If a protein with the dimensions given in Figure 2 binds to a vesicle in the configuration shown in Figure 3, the theoretical effective diameter change of the vesicle would be 85 Å. This value has been corrected for both incomplete surface packing and surface irregularity produced by the protein molecules. The outer edge of the protein envelope will be only 45% filled at 90% of maximum binding. The excellent agreement with the observed effective radius change (Table I) indicates that prothrombin binds to the membrane at one tip of the molecule with no detectable penetration into the membrane as shown in Figure 3.

In a separate experiment, the prothrombin and fragment 1 binding capacities of vesicles of 40% Folch fraction III were determined by relative light scattering intensity to be 0.019 and 0.023 μmol per mg of phospholipid, respectively. This may indicate that the failure of prothrombin to totally saturate the membrane surface is due to repulsion between groups in the prethrombin 2 region of adjacent molecules. Upon binding of fragment 1 to the membrane, the increase in the effective radius is 28 ± 3 Å compared with a theoretical change of 24 Å for 85% surface saturation with a spherical protein molecule of diameter 38 Å (Table I). This result more clearly indicates that there is essentially no penetration of prothrombin into the membrane.

The change in effective vesicle radius when factor X was bound is 74 Å (Table I). The shape of the factor X molecule is not accurately defined and we cannot draw firm conclusions regarding its degree of membrane penetration. However, based on a model similar to that drawn for prothrombin in Figure 3, and assuming similar protein density and no membrane penetration, the factor X molecule would be expected to be 93 Å in length and bind to the membrane at one end of the molecule.

Size or shape changes of protein when calcium is added can be a problem in interpretation of our data. However, the diffusion constants of prothrombin with and without calcium ions in the solution were measured to be the same. There is no detected change in the shape or size of protein which would affect

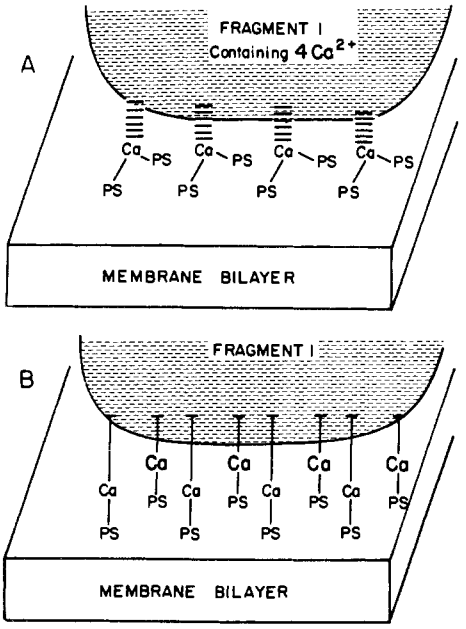

FIGURE 4: Proposed structures for the prothrombin-membrane interaction site showing the minimum observed calcium requirements. PS represents phosphatidylserine in the membrane bilayer, solid lines indicate ionic interactions and dashed lines represent an unspecified number of coordination bonds. The calcium ions in heavy type (Figure 4B) are derived from protein-bound calcium ions which have also been involved in catalysis of the protein transition necessary for phospholipid binding (Nelsestuen, 1976).

our data interpretation. Both prothrombin (Cox and Hanahan, 1970) and prothrombin fragment 1 (Prendergast and Mann, 1977) have been reported to undergo aggregation. Studies with human prothrombin indicate that this is an end-to-end dimerization with probable interaction between the prethrombin 2 regions (Agarwal et al., 1977). If a few such end-to-end prothrombin structures were formed on the membrane, the effective radius of the particle would increase dramatically. The change should also be protein concentration dependent and the maximum radius change observed would be up to 150 Å. Prothrombin dimerization does not appear to contribute to our observations.

Dimerization of fragment 1 is calcium dependent but the observed calcium requirement (Prendergast and Mann, 1977) is different from that of either the protein transition (Nelsestuen et al., 1976) or for the protein–membrane interaction. This dimerization is strongly concentration dependent. Based on the results obtained by relative light-scattering intensity and the radius changes observed when fragment 1 binds to the membrane vesicles, this dimerization does not appear to be occurring on the membrane surface.

Another potential problem in interpretation of our radius change would arise if protein were catalyzing a secondary fusion or association of the vesicles. The result would be a larger radius change than that due to protein binding. It appears that this problem does not exist. First of all, when EDTA was added to vesicle–protein–calcium complex, a complete reversal to the original value of vesicle radius was obtained. If vesicle association, or fusion were occurring, the result would have been a very heterogeneous particle distribution with a larger average size. This phenomenon was not observed. The standard deviation of the particle size distribution obtained from analysis of the light-scattering intensity autocorrelation function (Koppel, 1972), varied from ±5% for vesicles of 25% phosphatidylserine to ±10% for vesicles of 100% phosphatidylserine. The standard deviation of the particle size distribution for the vesicle–protein complex was the same as that for the vesicles. This indicates that the vesicles and the vesicle–protein complexes formed a reasonably homogeneous solution.

Discussion

The Structure of the Protein–Phospholipid Interaction. The combination of results presented here and in accompaning communications (Nelsestuen and Lim, 1977; Nelsestuen and Broderius, 1977) allows the construction of a model containing features of the most probable protein–phospholipid interactions. The evidence indicates that the protein does not penetrate into the lipid region of the membrane and Figure 4 shows two possibilities which involve ionic and coordination bonding only. Figure 4A depicts the interaction consisting of 8 phosphatidylserine residues (Nelsestuen and Broderius, 1977) and 4 calcium ions with coordination of the protein (perhaps through γ-carboxyglutamic acid residues) by an unspecified number of interactions to the calcium ions. In addition to the 4 calcium ions forming the protein–membrane bridge in Figure 4A, 3 or 4 other calcium ions are required for maintaining the correct protein conformation (the protein transition (Nelsestuen, 1976)). Since the number of phosphatidylserine residues per prothrombin molecule is 8 to 10 (Nelsestuen and Broderius, 1977), an equally valid model would involve 10 phosphatidylserine residues with interactions similar to those drawn in Figure 4A.

The model in Figure 4B indicates 8 phosphatidylserine residues which are each bridged to sites in the protein (perhaps γ-carboxyglutamic acid residues) through ionic bonding with calcium ions. In this model it would be necessary that essentially all of the calcium ions of the prothrombin–membrane complex be involved in this ionic bridge since 9 ± 1.2 calcium ions are sufficient to complete the prothrombin–membrane complex (Nelsestuen and Lim, 1977). Also, in Figure 4B, half of the calcium ions must be derived from the protein-bound calcium ions (indicated by those in heavier print) and these must be the same ones which catalyzed the protein transition. As for Figure 4A, an equally valid structure would involve 10 phosphatidylserine residues and 10 calcium ions. The actual protein–membrane interaction site may involve both ionic and coordinate bonding and be a hybrid of the structures shown in Figure 4.

While factor X–membrane binding would be expected to generally resemble those structures given in Figure 4, the details of the factor X–phospholipid binding differ somewhat from those of prothrombin–phospholipid binding. Only 4 to 6 phosphatidylserine residues are involved in factor X–membrane interaction but the binding affinity is greater (Nelsestuen and Broderius, 1977). In addition, the factor X–membrane interaction appears to require a larger number of membrane-bound calcium ions to form the "nucleation" structure which the protein can recognize (Nelsestuen and Lim, 1977). Continued comparison of the membrane binding properties of prothrombin and factor X, and studies on the other vitamin K dependent proteins, may allow construction of more detailed models for these protein–membrane interactions.

To our knowledge, these studies represent the first application of quasielastic light scattering to protein–membrane interactions, although other workers (Uzgaris and Fromageot, 1976) have studied protein binding to polystyrene latex spheres. The results would indicate that this technique could be applied to many protein–membrane interactions. The data obtained provide information about the protein structure which otherwise could only be obtained by x-ray crystallographic studies. For example, the results indicate that the peptide portion of prothrombin which is referred to as prothrombin

fragment 1 is located at one end of the elliptical molecule. The fact that fragment 1 is derived from the amino-terminal region of prothrombin (Heldebrant et al., 1973) makes this arrangement likely but does not in any way establish the location of this peptide in the overall protein structure. The information presented also establishes that the area of fragment 1 which interacts with the membrane (presumably the γ-carboxyglutamic acid-containing region) is positioned across from the prethrombin 1 region. Since γ-carboxyglutamic acid residues are found in the light chain of factor X (Howard and Nelsestuen, 1975), the information presented here indicating that factor X binds to the membrane at one tip of the molecule provides evidence for the configuration of the light chain in the factor X molecule as well.

The specific configuration of prothrombin and factor X on the membrane surface is undoubtedly of considerable importance in aligning factor Xa and prothrombin for optimal proteolytic action in the prothrombinase reaction. This aligning function of the membrane would also be of major importance in the other membrane-associated blood-clotting reactions which involve the other vitamin K dependent proteins (factors IX and X). Thorough application of the methods presented in this and accompanying communications should go a long way in enabling construction of realistic molecular models of the blood-clotting reactions.

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