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Prothrombin–Membrane Interaction. Effects of Ionic Strength, pH, and Temperature†

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ABSTRACT: The effects of ionic strength, pH, and temperature on three separate aspects of prothrombin–phospholipid membrane binding were studied. The three parameters include a calcium-dependent protein transition, a calcium–membrane interaction, and, finally, the binding of calcium-saturated protein to a calcium-saturated phospholipid membrane. The results are consistent with calcium binding to carbonyl groups in the protein and to phosphate in the phospholipids. These interactions show the expected pH profiles and sensitivity to ionic strength. Temperature effects indicate a small negative enthalpy change for each process. The binding of calcium-saturated protein to calcium-saturated membrane shows very little variation between pH 6 and pH 9, is accompanied by no detected enthalpy change, and is relatively insensitive to ionic strength. These latter results indicate that ionic calcium bridging between the protein and membrane is not important. A chelation model for prothrombin–membrane binding is proposed where the two interacting species have no net charge; ligands on the protein complete the coordination sphere of membrane-bound calcium and vice versa.

The binding of vitamin K dependent proteins to membranes is dependent on the presence of γ-carboxyglutamic acid residues. There are 10 of these residues in the amino-terminal region of prothrombin [see Stenflo & Suttie, (1977) and Suttie & Jackson (1977) for reviews]. Several lines of evidence have shown that native protein structure is required for tight calcium binding to prothrombin (Henriksen & Jackson, 1975; Nelsestuen et al., 1975) and for prothrombin–membrane interaction (Nelsestuen, 1976). In other words, the 10 γ-carboxyglutamic acid residues do not act independent of a larger

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protein segment. Further studies have indicated that the equilibria shown in reaction 1 represent the minimum processes required to describe prothrombin–membrane binding at saturating calcium concentrations (Nelsestuen & Lim, 1977):

$$\begin{aligned} P + x\mathrm{Ca} &\overset{K_1}{\rightleftharpoons} P_{x\mathrm{Ca}} \overset{K_2}{\rightleftharpoons} P'_{x\mathrm{Ca}} \\ &\quad + \overset{K_4}{\rightleftharpoons} P'\cdot PL_{(x+y)\mathrm{Ca}} \qquad (1) \\ PL + y\mathrm{Ca} &\overset{K_3}{\rightleftharpoons} PL_{y\mathrm{Ca}} \end{aligned}$$

P is protein, P′ is the protein after undergoing a calcium-dependent protein transition, PL is a phospholipid membrane expressed as the molar concentration of protein binding sites, and P′·PL is the protein–phospholipid complex. Equilibrium 2 is actually a subset of equilibrium 1; the protein transition requires only 3 or 4 of the 10 calcium sites on prothrombin (Nelsestuen, 1976). The dissociation constant K_4 contains no calcium term and is measured at saturating calcium concentrations. Where calcium is not saturating (between 1 and 10 mM calcium), a dissociation constant for prothrombin–membrane binding can be measured but varies with calcium concentration. The calcium added to the complex under these conditions probably represents completion of the x and y sites.

Dissociation constants for prothrombin–membrane binding were obtained for different phospholipids and calcium concentrations (Nelsestuen & Broderius, 1977). Other vitamin K dependent plasma proteins have also been examined (Nelsestuen et al., 1978). Independent studies on prothrombin fragment 1 and phosphatidylglycerol utilized very different methods but gave comparable results (Dombrose et al., 1979). For example, membranes of phosphatidylglycerol–phosphatidylcholine (1:1 at ~5 mM calcium) gave dissociation constants of ~1 μM in both studies. Dombrose et al. reported a somewhat higher protein-binding capacity for the membranes and the existence of two classes of binding sites. These differences may arise from the use of prothrombin fragment 1 vs. prothrombin or from the fatty acid distributions used.

Prendergast & Mann (1977) and Jackson et al. (1979) have shown a calcium-dependent change in the sedimentation coefficient of prothrombin fragment 1 which appears to be due to protein self-association. This phenomenon was much less pronounced for the parent molecule, prothrombin (Jackson et al., 1979). The concentration dependence of aggregation indicates that it is minimal at the protein concentrations used to measure K_2 and K_4 (~0.1 mg of protein per mL), and this process is not included in reaction 1.

The question of protein structures which are important to the overall processes outlined above can be studied from several standpoints. This paper deals with effects of pH, ionic strength, and temperature on the individual equilibria (K_2, K_3, and K_4 of reaction 1).

Materials and Methods

Bovine prothrombin was purified by a modification (Nelsestuen & Suttie, 1973) of the method of Ingwall & Scheraga (1968). Prothrombin fragment 1 was produced by the action of thrombin on prothrombin, followed by isolation as described by Heldebrant & Mann (1973). The proteins were quantitated by the absorbance at 280 nm with $E_{280}^{1\%}$ values of 14.1 for prothrombin (Cox & Hanahan, 1970) and 10.1 for fragment 1 (Heldebrant & Mann, 1973).

Phospholipids (bovine brain phosphatidylserine, egg yolk phosphatidylcholine, and phosphatidic acid formed from egg yolk phosphatidylcholine) were purchased from Sigma Chemical Co. and are reported to be >95% pure.[1] Single bilayer vesicles containing mixtures of phospholipids were prepared essentially according to Huang (1969) as described in detail previously (Nelsestuen & Lim, 1977). The composition of the vesicles is given as a percent value based on the acidic phospholipid with the remaining constituent being phosphatidylcholine. Phospholipid concentrations were estimated by organic phosphate measurement (Chen et al., 1956) using a phospholipid/phosphorus weight ratio of 25.

Calcium titration of the protein transition (equilibrium 2, reaction 1) was studied with prothrombin fragment 1. The method involves incubating the protein with a known concentration of calcium, followed by additon of excess EDTA (Nelsestuen, 1976). The value of F/F_0 is then obtained (F is the fluorescence intensity in the presence of calcium; F_0 is the fluorescence intensity after EDTA addition). Duplicate samples were run for each determination.

Previous studies have indicated that equilibrium 3 (reaction 1) is a calcium–membrane interaction that may be related to lateral phase separation of acidic phospholipids (Nelsestuen & Lim, 1977). Calcium titration of protein–membrane binding in the presence of manganese appears to be a function of this equilibrium. The manganese functions in the protein transition (equilibrium 2) but will not cause protein–membrane binding. Since this is a model interpretation of the results and we do not know the total effect of the added manganese, conclusions about the properties of equilibrium 3 are tentative.

The amount of protein bound to the membrane vesicles was calculated from the relationship

$$\frac{I_{s_2}}{I_{s_1}} = \left(\frac{\partial n_2/\partial c_2}{\partial n_1/\partial c_1}\right)^2\left(\frac{M_2}{M_1}\right)^2$$

where I_{s_2} is the 90° light scattering intensity from the protein–phospholipid vesicle complex measured at 320 nm in a Hitachi Perkin-Elmer Model 44A fluorescence spectrophotometer, I_{s_1} is the light scattering intensity from the initial phospholipid vesicles, $\partial n_2/\partial c_2$ is the refractive index increment for the protein–phospholipid complex, $\partial n_1/\partial c_1$ is the refractive index increment for the phospholipid alone, M_2 is the molecular weight of the protein–phospholipid complex, and M_1 is the molecular weight of the vesicles alone. Derivation of the equation and more detailed description of the calculations have been described previously (Nelsestuen & Lim, 1977).

Dissociation constants for prothrombin–membrane binding were obtained by the same relative scattering technique. The M_2/M_1 ratio was determined at known protein and phospholipid concentrations and compared to the M_2/M_1 ratio anticipated if all of the protein were bound to the membrane. This provides a method of estimating free and bound protein. These data were analyzed by double-reciprocal plots as illustrated under Results. Complete reversibility of binding was shown in all cases by addition of EDTA at the end of the experiment with return of the light scattering intensity to the level of free phospholipid vesicles plus free protein. This implies that irreversible vesicle fusion has not occurred. Previous studies using quasi-elastic light scattering demonstrated that reversible fusion of the protein–membrane complexes is not a difficulty (Lim et al., 1977).

Circumstances which alter the light scattering intensity of the phospholipids were avoided. For example, salt concentrations in excess of those used here (as well as higher calcium concentrations and greater pH variations) alter the light scattering properties of the vesicles. Such changes imply that

[1] The high degree of reproducibility observed for prothrombin–membrane binding with different batches of phospholipid also suggest a high phospholipid purity. For example, binding constants and membrane binding capacities reported here are all within 20% of those previously reported (Nelsestuen & Broderius, 1977). This would correspond to the expected experimental error (Nelsestuen & Lim, 1977).

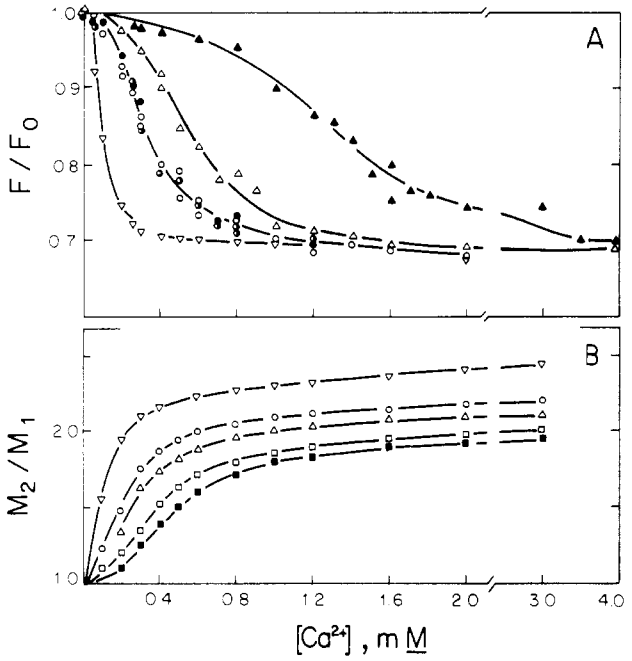

FIGURE 1: Calcium titration of protein fluorescence (A) and protein–membrane binding (B) at varying ionic strength. The prothrombin fragment 1 protein concentration used in (A) was 0.1 mg/mL. Protein fluorescence emission at 344 nm (excitation at 280 nm) was determined in calcium solution (F) and after addition of excess EDTA (F_0). The solutions contained 0.05 M Tris buffer (pH 7.5) plus additional salts as follows: (▽) none; (○) 0.1 M NaCl; (●) 0.1 M LiCl; (◑) 0.1 M KCl; (△) 0.2 M NaCl; (▲) 0.5 M NaCl. (B) gives the calcium titration of prothrombin–membrane binding in the presence of 0.4 mM $MnCl_2$. This concentration elicited the maximum protein fluorescence change in all ionic strengths. Single bilayer vesicles of 25% phosphatidylserine (0.096 mg/mL) and prothrombin (0.247 mg/mL) were incubated in the presence of calcium, buffer, and salts. The molecular weight ratios of the protein–phospholipid complex to that of the phospholipid alone (M_2/M_1) are plotted as a function of total calcium concentration. The symbols used for the different salts are as in (A). Additional symbols: (□) and (■) represent 0.3 and 0.4 M NaCl, respectively.

the constants (e.g., refractive index increment) used in the calculations may not be valid.

Results

Ionic Strength Studies. Three aspects of prothrombin–membrane binding were studied. These are given under Materials and consist of (1) effects on the calcium dependence of the protein transition (K_2, reaction 1), (2) effects on the calcium titration of protein–membrane binding in the presence of manganese (K_3, reaction 1), and (3) effects on the dissociation constant for protein–membrane interaction at saturating calcium (K_4 of reaction 1).

The raw data for titration of the protein transition at four ionic strengths are shown in Figure 1A. The data were analyzed by a Hill plot constructed as previously described (Nelsestuen, 1976) and the midpoints are plotted in Figure 2. The results show a linear relationship between ionic strength and calcium requirement. Extrapolation shows an intercept at zero. This is anticipated for an ionic interaction. There was no selectivity for the different monovalent cations used (Figure 1). Studies obtained from solutions of sodium or potassium chloride should be directly comparable.

Figure 1B gives some of the raw data for calcium titration of prothrombin–membrane binding in the presence of manganese. This titration is thought to measure primarily equilibrium 3 (reaction 1). As indicated by the maximum value of M_2/M_1, the maximum amount of protein bound varies with ionic strength. This reflects changes in K_4 (which alter the

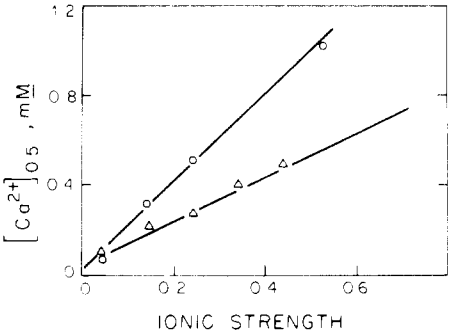

FIGURE 2: Midpoints of the calcium titrations shown in Figure 1. The data in Figure 1 were analyzed by Hill plots as described previously (Nelsestuen, 1976; Nelsestuen & Lim, 1977), and the midpoints (given as $[Ca^{2+}]_{0.5}$) were determined. The ionic strength is calculated from the buffer and added salts; contributions from calcium chloride are not included. The data for fragment 1 protein fluorescence change (○) and for prothrombin–membrane binding in the presence of manganese (△) are shown. Three manganese concentrations (0.2, 0.4, and 0.8 mM) were used at each ionic strength. These were indistinguishable so that an averaged value is plotted.

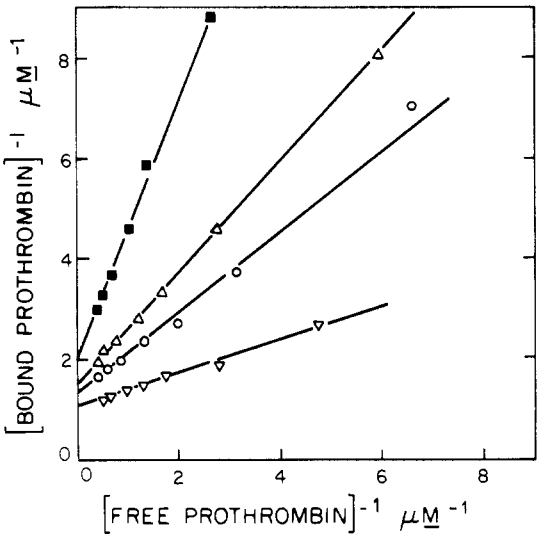

FIGURE 3: Double-reciprocal plots of prothrombin–membrane binding at varying ionic strengths. Free and bound prothrombin concentrations were estimated as described under Materials and Methods. Single bilayer membranes of 20% phosphatidylserine (40 μg/mL) in 4 mM $CaCl_2$–0.05 M Tris buffer (pH 7.5) were used. The plots show data obtained in the presence of additional salt. The additional salts were as follows: (▽) none; (○) 0.1 M NaCl; (△) 0.2 M NaCl; (■) 0.4 M NaCl.

distribution of free and bound protein) and changes in the actual binding capacity of the membranes (see below). In each case the midpoint of the titration was estimated from a Hill plot of the data. These midpoints are plotted in Figure 2. The results show a large effect of ionic strength on the calcium requirement of equilibrium 3. This change was similar for the different monovalent cations used (lithium, potassium, and sodium; data not shown). As expected, the interaction of calcium with the membrane appears to be primarily an ionic process.

The effects of ionic strength on the dissociation constant for prothrombin–membrane binding are shown in Figures 3 and 4. Figure 3 presents double-reciprocal plots obtained at nonsaturating calcium. The maximum protein-binding capacity of the vesicles varies somewhat as indicated by the intercepts. This variation corresponds to somewhat higher capacity at low ionic strength. The slopes of the plots vary considerably, especially at nonsaturating calcium concentrations (Figure 3). This is due primarily to different dissociation constants. The parameters which should be compared, how-

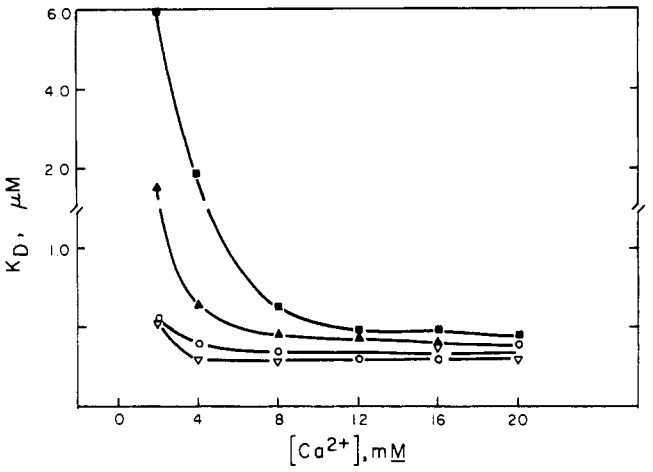

FIGURE 4: Summary of dissociation constants for prothrombin–membrane binding at different ionic strengths and calcium concentrations. The dissociation constants (K_D) estimated from double-reciprocal plots (e.g., Figure 3) are plotted vs. calcium concentration. Contributions to ionic strength come from buffer, calcium chloride, and added salts. The added salts are as follows: (▽) none; (○) 0.1 M NaCl; (▲) 0.2 M NaCl; (■) 0.4 M NaCl. The phospholipid concentrations and composition are as given in Figure 3.

ever, are the values of K_4 (reaction 1). This dissociation constant is obtained at saturating calcium concentrations. The results in Figure 4 above 10 mM calcium demonstrate that ionic strength has a measurable but small effect on K_4. The observed differences could arise from parameters other than ionic shielding. For example, the degree of lateral phase separation may be altered at high ionic strength. As a minimum, the results indicate that the interaction of calcium-saturated prothrombin (P_{xCa}, reaction 1) with calcium-saturated phospholipid (PI_{yCa}) contains only a small ionic contribution.

Dombrose et al. (1979) reported that ionic strength had a large effect on the binding constant for prothrombin fragment 1–membrane interaction. Their values, however, were obtained at 4.5 mM calcium and assumed a constant protein-binding capacity for the membranes. This calcium concentration is not saturating at the higher ionic strengths (e.g., see Figure 4) so that dissociation constants obtained would not represent K_4.

pH Studies. The midpoint for calcium titration of the intrinsic protein fluorescence change was determined at varying pH. The method of procedure and appearance of the raw data are essentially as given in Figure 1A. The quantum yield was relatively insensitive to pH changes over the range studied. In addition, the maximum protein fluorescence change brought about by calcium was similar at the pH values given in Figure 5A.

The data show that the midpoint of the calcium titration is insensitive to pH between about 6 and 9. Below pH 6 there is a dramatic increase in calcium requirement. These results are consistent with involvement of an ionizable carboxyl group in the calcium binding responsible for the protein transition. The reported pK values for the γ-carboxyl groups of γ-carboxyglutamic acid are 4.4 and 2.0 (Sperling et al., 1978). The results in Figure 5A suggest that protonation of the first γ-carboxyl may be causing the changes.

These results differ from those of a recent report (Scott et al., 1979) where a large pH effect at 7.5 was observed in addition to the change at acid pH. An earlier report on calcium binding to prothrombin also contained a pH effect in this range (Nelsestuen & Suttie, 1972). Other studies (Nelsestuen & Suttie, 1973; Benson & Hanahan, 1975) observed no alkaline pH effect. The reasons for these differences

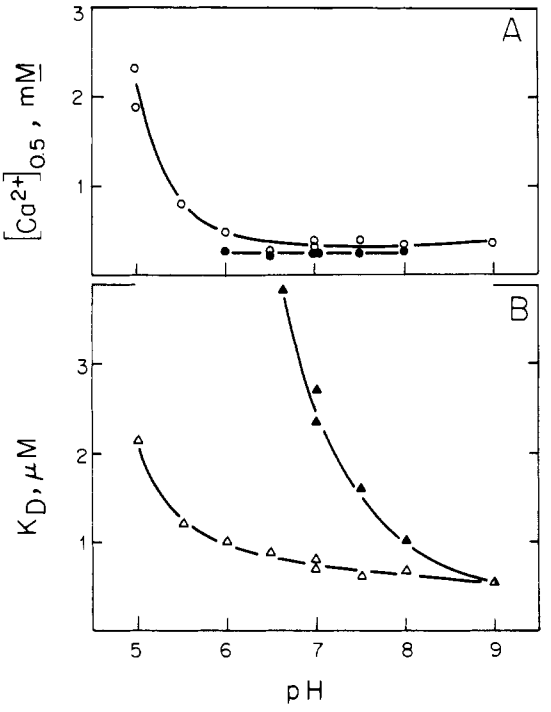

FIGURE 5: Effect of pH on the parameters of prothrombin–membrane binding. (A) gives the midpoints of calcium titration, $[Ca^{2+}]_{0.5}$, of (1) the prothrobmin fragment 1 protein fluorescence change (○) and (2) prothrombin–membrane binding in the presence of 0.4 mM manganese (●). The procedures and method of data analysis are essentially as given in Figures 1 and 2. Exceptions are that membranes of 20% phosphatidylserine were used at concentrations of 48 μg/mL. Buffers used were 0.05 M Tris–0.1 M NaCl (pH values 7–9) and 0.05 M sodium cacodylate–0.1 M NaCl (pH values 5–7). Duplicate determinations at pH 7 represent results from the two buffers. (B) shows the dissociation constants for prothrombin–membrane binding at varying pH. The buffers used were the same as in (A) at a constant calcium concentration of 2 mM. The two membrane compositions are 20% phosphatidylserine [(△) 48 μg/mL] and 20% phosphatidic acid [(▲) 77 μg/mL]. The data analysis is similar to that shown in Figure 3.

are not known. A number of attempts to demonstrate a change at pH 7.5 have been without success (unpublished data). The results in Figure 5A indicate that the ionizable groups involved in calcium binding to the protein consist of carboxyl groups.

The effects of pH on calcium titration of prothrombin–membrane binding in the presence of manganese (K_3 of reaction 1) give a similar pH profile. Data collection and the method of analysis are similar to those in Figure 1B. The midpoints of the titration curves are shown in Figure 5A. Ionizable groups with pK values between about 5.5 and 9 are not important in equilibrium 3 (reaction 1). This is expected from the ionization groups found in phosphatidylserine.

The dissociation constant for prothrombin–membrane binding was determined from pH 5 to pH 9. The values plotted in Figure 5B were obtained at nonsaturating calcium concentrations. These dissociation constants are nevertheless closely related to K_4 (Nelsestuen & Lim, 1977). The results for phosphatidylserine show very minor changes between pH 6 and pH 9. The increase below pH 6 may be due to higher calcium requirements of equilibrium 2 (reaction 1) rather than to changes in K_4. The results allow the conclusion that there are no groups which ionize between pH 5 and pH 9 involved in the actual protein–phospholipid interaction (K_4, reaction 1). In addition, the protein-binding capacity of the membrane vesicles did not vary in this pH range (data not shown).

The results for phosphatidic acid are also given in Figure 5B. In this case, pH has a major effect on the binding affinity. The maximum affinity achieved at high pH is similar to that

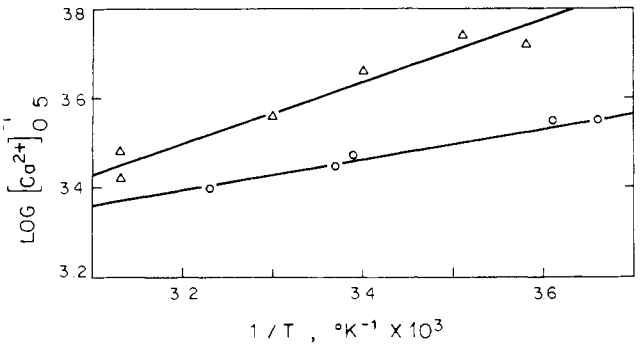

FIGURE 6: Temperature dependence of calcium titration of the fragment 1 protein fluorescence change (○) and prothrombin–membrane binding in the presence of 0.4 mM manganese (△). The buffer used was 0.05 M Tris–0.1 M NaCl. The method of data analysis is given in Figures 1 and 2. Prothrombin–membrane binding was measured with 86 μg of 20% phosphatidylserine plus 264 μg of prothrombin per mL. The data are presented in a form related to a van't Hoff plot. The recipocal of the titration midpoint, $[Ca^{2+}]_{0.5}$, is used as an estimate of the association constant (see the text).

of phosphatidylserine. The decreased affinity at neutral and acidic pH is probably related to protonation of the phosphate group at pH ~6.7. The capacity of these membranes, however, did not change over this pH range (data not shown). We conclude that divalent phosphatidic acid is similar to monovalent phosphatidylserine in prothrombin–membrane binding. Monovalent phosphatidic acid still functions, but in a reduced capacity. The results plotted in Figure 5B were not obtained at saturating calcium. Further studies are required to determine whether the reduced efficacy of monovalent phosphatidic acid is due to a change in K_4 or in K_3 (reaction 1).

Temperature Effects. We have previously reported that temperature has relatively little effect on the calcium dependence of the intrinsic protein fluorescence change (Nelsestuen, 1976). The actual data are plotted in Figure 6 in the form of a van't Hoff plot. The data indicate an enthalpy change of −1.84 kcal/mol. Analysis of a titration midpoint in this manner assumes that it represents a dissociation constant. The validity of this calculation must therefore be viewed with caution.

Temperature also has a small effect on the titration of prothrombin–membrane binding in the presence of manganese (equilibrium 3, reaction 1). The analysis given in Figure 6 indicates an enthalpy change of −3.3 kcal/mol. This value must, once again, be used cautiously because of the potential complexities of this measurement where the total effect of manganese is still unknown.

Temperature effects on the prothrombin–membrane dissociation constant (K_4, reaction 1) were determined by the usual method outlined in Figure 3. The values were indistinguishable over the range of 4–40 °C and are not plotted. We conclude that K_4 (reaction 1), the interaction of calcium-saturated protein with calcium-saturated phospholipid membrane, is not accompanied by enthalpy changes (≤1 kcal/mol).

Discussion

The protein–membrane interactions involving vitamin K dependent proteins are often viewed as ionic processes where calcium ions bridge between negatively charged protein and negatively charged phospholipids [e.g., see Dombrose et al. (1979) and Lim et al. (1977)]. Previous results have suggested that the proteins do not penetrate into the lipid region of the membrane (Lim et al., 1977; Hanahan et al., 1969). The results presented here are consistent with ionic binding of calcium to protein carboxyl groups and to phosphate in the membrane. These observations are consistent with, but do not establish, an ionic bridging model for the actual protein–membrane interaction.

An important study relating to ionic bridging is the effect of ionic strength on protein–phospholipid interaction in the presence of saturating calcium. This measures K_4 (reaction 1). This interaction shows only a small sensitivity to ionic strength (Figure 4 above). A small or negligible sensitivity to ionic strength is usually considered to be indicative of hydrophobic interactions. Other interpretations of these results may also be considered. We have previously discussed interaction through nonionic calcium coordination (Lim et al., 1977). The interaction of calcium-saturated protein with a calcium-saturated membrane may involve two neutral species. Sites on the protein may complete the coordination sphere of membrane-bound calcium ions and vice versa. In this way, major competition for the protein-binding site on the membrane would come from coordination ligands such as water.

A coordination model for this interaction is also consistent with the cation specificity of prothrombin–membrane binding. Calcium, strontium, and barium all function in this process while magnesium functions very poorly (Dombrose et al., 1979) or not at all (Nelsestuen et al., 1976). Nevertheless, magnesium does interact with phospholipids (Portis et al., 1979) as does manganese (Puskin & Martin, 1979), another divalent cation which does not support prothrombin–membrane interaction. Magnesium and manganese generally have lower coordination numbers than calcium, strontium, or barium. The ineffective cations also do not appear to be able to promote lateral phase separations in the membrane (Ito et al., 1975). Thus, while hydrophobic contacts between prothrombin and the membrane cannot be eliminated on the basis of our data, we prefer to view prothrombin–membrane binding by calcium coordination.

The nonionic nature of prothrombin–membrane interaction is also consistent with other observations. Ionic bridging between two negatively charged structures should be inhibited by high ionic strength, even when derived from the bridging ion. A simple example is given by reactions 2 and 3:

$$P^- + Ca^{2+} + PL^- \rightleftarrows P{\cdot}Ca{\cdot}PL \quad (2)$$

$$P^- + \text{excess } Ca^{2+} + PL^- \rightleftarrows (P{\cdot}Ca)^+ + (PL{\cdot}Ca)^+ \quad (3)$$

An optimal calcium concentration allows complex formation (reaction 2) while high calcium blocks the ionic site needed for bridging and inhibits interaction (reaction 3). Since elevated calcium does not result in such inhibition (see Figure 4), the ionic bridging model appears unlikely.

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Structure, Function, and Assembly in the Hemocyanin System of the Scorpion *Androctonus australis*†

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ABSTRACT: The interactions between structurally and functionally distinct subunit types in the assembly and function of heteropolymers can be subjected to detailed investigations using the high molecular weight copper proteins known as hemocyanins. Hemocyanin of the scorpion *Androctonus australis* is a 34S heteropolymer which contains 24 subunits. Eight distinct types of subunits can be isolated when the oligomer is dissociated. Reassembly can be accomplished by two-step dialysis of an equimolar mixture of the isolated subunits or a similar dialysis of the unfractionated subunit mixture. In both cases the reassembled aggregate is similar to the native 24-mer, as evidenced by electron microscopy, but differs from the native molecule in subunit composition. The functional properties of reassembled forms which lack subunit 3A suggest that this subunit plays an important role in stabilizing a conformation of low oxygen affinity. At pH 7.5 the native molecule binds oxygen with a $P_{1/2}$ of 27 Torr and a high degree of cooperativity. The Hill coefficient is pH sensitive and reaches a maximum value of 9.25 at pH 7.8. Strong pH-dependent homotropic interactions are also evident in the time course of oxygen dissociation. At pH 8.2 the apparent first-order rate constant has an initial value of 2.6 s^{-1} and increases to 20 s^{-1} as the reaction proceeds. Interactions between subunits markedly decrease at low pH where a low affinity form is stabilized. Thus, at pH 6.5 the Hill coefficient is 3.0 and the $P_{1/2}$ is 60.25 Torr. Sodium chloride acts as an allosteric effector that also brings about a stabilization of the low affinity conformation. When the native molecule is dissociated, the unfractionated mixture of subunits shows no cooperativity and $P_{1/2}$ values range from 4.29 to 4.17 Torr in the pH range from 7.5 to 8.9. The kinetics and equilibria of oxygen binding by isolated subunits were studied. Functional diversity at the subunit level is indicated by differences in oxygen affinities, oxygen dissociation rate constants, and sodium chloride sensitivities.

Hemocyanins are high molecular mass copper proteins which reversibly bind oxygen. These proteins are found freely dissolved in the hemolymph of arthropods and mollusks. The hemocyanins of these two phyla are easily distinguished on the basis of their structure. Mollusk hemocyanins are very high molecular mass aggregates having sedimentation coefficients ranging from 60 to 130 S. They can be dissociated into subunits which are uncommonly large polypeptide chains. These polypeptides have multiple oxygen binding domains (Gielens et al., 1977). On the other hand, arthropod hemocyanins are assembled from 70 000 to 95 000 M_r[1] subunits possessing single oxygen binding sites. The native molecules encountered in different arthropod species can be considered to be built by successive dimerizations of building blocks containing six subunits, often designated as hexamers (Schutter et al., 1977; Bonaventura et al., 1977; Bijlholt et al., 1979). Thus, for instance, hexamers are observed in the hemolymph of the shrimp *Penaeus setiferus* (Brouwer et al., 1978a), dodecamers in the freshwater crayfish *Cherax destructor* (Jeffrey et al., 1978), tetracosamers (24 subunits) in the scorpion *Buthus occitanus* (Wibo, 1966), and a 48-mer in the horseshoe crab *Limulus polyphemus* (Van Holde & van Bruggen, 1971). The reversible dissociation of hemocyanin oligomers into halves and subfragments has long held the interest of researchers,

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