A New Model To Describe Extrinsic Protein Binding to Phospholipid Membranes of Varying Composition: Application to Human Coagulation Proteins†

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Appendix: A New Model To Describe Extrinsic Protein Binding to Phospholipid Membranes of Varying Composition: Quantitative Development

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ABSTRACT: We introduce here a new model to describe the binding of extrinsic membrane proteins to acidic lipid membranes. In this view, macroscopic binding affinity is determined by two processes: nonspecific adsorption of protein to the membrane surface and association of acidic lipids with specific sites on the bound protein. We apply this model here to compare the binding of human prothrombin and factor X/X_a to phosphatidylglycerol (PG)- and phosphatidylserine (PS)-containing small unilamellar vesicles measured via relative light scattering. This comparison was undertaken because model membranes containing PS are much more effective in supporting thrombin formation than are membranes containing PG. Analysis of binding isotherms in terms of a traditional membrane binding model gave apparent dissociation constants systematically varying from 0.1 to 10 μM over a range of 8–65 mol % negatively charged phospholipid. With our new description of membrane binding, the dependence of binding data on the acidic lipid surface concentration revealed that only two or three acidic lipid molecules were associated with each surface-bound factor X/X_a or prothrombin molecule. Assuming four independent and equivalent acidic lipid binding sites per protein, it was possible to adjust the values of only the nonspecific adsorption equilibrium constant and the equilibrium constant describing binding of each species of acidic lipid to individual sites on the protein and thereby obtain a good simulation of log-linear binding isotherms for the full range of acidic lipid surface concentrations. The protein-associated binding sites had a greater affinity for PS than for PG; i.e., a lower surface concentration of PS was required to fill the binding sites. The assumption that 20 acidic lipids, i.e., a sufficient number to form a "pool" or "domain", would associate with each bound protein produced a significantly poorer simulation of the experimental binding isotherms. We conclude that the simple model proposed here offers a physically reasonable, alternative description of coagulation protein binding that avoids the apparently inappropriate assumption of protein-induced phospholipid domains. This model also may be applicable to the binding of other similar extrinsic membrane proteins.

Protein–Lipid Interactions in Blood Coagulation. Crucial proteolytic steps in the blood coagulation cascade are catalyzed by multiprotein complexes that assemble on platelet-derived membranes (Nelsestuen, 1978; Mann, 1987). The protein components of these proteolytic complexes are thought to bind to the platelet membrane via interactions with negatively charged phospholipids. Upon stimulation of human platelets, the requisite negatively charged phospholipids are thought either to "flip" from the cytoplasmic to the extracytoplasmic surface of the platelet plasma membrane (Bevers et al., 1983) or to be exposed on the surface of membranous vesicles released from stimulated platelets (Bode et al., 1985; Sandberg et al., 1985). One such enzyme complex, the prothrombinase complex, catalyzes the proteolytic conversion of prothrombin to thrombin. The enzyme, factor X_a, associates with its cofactor, factor V_a, on the surface of a platelet or phospholipid vesicle to accelerate the conversion.

Factor X, the zymogen of factor X_a, and prothrombin (factor II) are vitamin K dependent proteins containing doubly negatively charged γ-carboxyglutamic acid residues. Binding of these proteins to membranes containing negatively charged phospholipids requires Ca^{2+} and has been proposed to occur via calcium bridging of γ-carboxyglutamic acid residues to negatively charged phospholipids in the membrane (Lim et al., 1977; Dombrose et al., 1979; Wei et al., 1982). Analysis of the binding of these proteins to membranes has utilized a binding model that assumes the existence of protein "binding sites" on a membrane (Lim et al., 1977). The binding site is proposed to be a local pool or "domain" of negatively charged phospholipid that condenses under the surface-bound protein (Lim et al., 1977; Dombrose et al., 1979; Mayer & Nelsestuen, 1981). This model for the formation of domains of negatively charged phospholipids has been suggested for the binding of other extrinsic membrane proteins as well (Birrell & Griffith, 1976; Hartmann et al., 1977; Wiener et al., 1985).

Our laboratory has questioned the concept of negative lipid domain formation in response to binding of extrinsic membrane proteins, in particular the γ-carboxyglutamic acid containing proteins prothrombin and factor X. On the basis of the shape of membrane phase diagrams, we have failed to find any evidence to support the formation of extensive domains in phosphatidylserine (PS)[1]/phosphatidylcholine (PC) or phos-

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[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; bov PS, bovine brain phosphatidylserine; DOPG, dioleoylphosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; K_d, equilibrium dissociation constant; N/n, phospholipid to protein stoichiometric ratio; $[M_2/M_1]_{sat}$, the ratio of the molecular weight of the protein–vesicle complex to the molecular weight of the vesicle; EDTA, disodium ethylenediaminetetraacetic acid; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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phatidylglycerol (PG)/PC membranes saturated with bovine prothrombin or its γ-carboxyglutamic acid containing fragment 1 region (Lentz et al., 1985; Tendian & Lentz, 1989). In addition, studies with pyrene-labeled phospholipids have shown that if negative phospholipid domains do form in response to prothrombin fragment 1 binding, they must contain less than 15–20 lipid molecules (Jones & Lentz, 1986).

Application of New Binding Model to Blood Coagulation Proteins. On the basis of analysis and simulation of binding isotherms in terms of the model presented in the Appendix (see Figure A1 of the Appendix), we propose that there exist at least four binding sites for negatively charged phospholipid molecules on a molecule of prothrombin or factor X/X_a. The extent of occupancy of these sites is viewed as determined by the law of mass action; i.e., it depends on the surface concentration of negatively charged phospholipids in the target membrane. The assumption that a domain of 20 negatively charged phospholipids associates with bound prothrombin or factor X/X_a produced a significantly poorer fit to the data. Simulation of observed binding isotherms in terms of our model also has led us to conclude that one or more of the acidic lipid binding sites on factor X/X_a has a decreased affinity for PG as compared to PS molecules.

Materials and Methods

N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) was purchased from Research Organics, Inc. (Cleveland, OH). Whatman DE52 DEAE-cellulose was purchased from American Scientific Products (Charlotte, NC). Heparin–Sepharose was prepared (Kohn & Wilchek, 1981) from commercially available heparin (Sigma Chemical Co., St. Louis, MO). Bovine brain PS (bov PS), dioleoylphosphatidylglycerol (DOPG), and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). All solvents were of HPLC grade; all chemicals were of ACS reagent grade or the highest available purity.

Protein Isolation. Human prothrombin and human factor X/X_a were isolated from plasmapheresis waste plasma donated by North Carolina Memorial Hospital. Upon receipt, 25 mg of soybean trypsin inhibitor was added per liter of plasma; Tris-HCl (Bethesda Research Products, Gaithersburg, MD), pH 7.4, was added to a concentration of 50 mM; sodium citrate, pH 7.4, to 20 mM; and benzamidine hydrochloride to 5 mM. The plasma was frozen at −20 °C and thawed immediately before use. All steps were performed at 4 °C, and all solutions contained 1 mM benzamidine hydrochloride. Dry poly(ethylene glycol) 8000 (Thomas Scientific, Atlanta, GA) was added to 3.3% (w/v) and the resulting suspension allowed to stir for 20 min and centrifuged (Sorval RC3B with H-6000 rotor, 4000 rpm, 20 min). One molar barium chloride (10% of the original plasma volume) was added slowly to the supernatant and the suspension stirred for 20 min. The centrifuged precipitate was washed three times with 0.1 M $BaCl_2$ and once with distilled water. Ammonium sulfate (276 g/L, 10% of the original plasma volume) was added to the washed barium citrate precipitate and the suspension stirred for 20 min. The resulting supernatant was dialyzed against 12 L of 50 mM sodium phosphate buffer, pH 6.3/0.1 M NaCl, with two changes. The dialyzed protein was batch adsorbed to Whatman DE52 DEAE-cellulose; the gel was washed with 2–3 L of the above buffer by vacuum filtration and packed into a glass column, 2.5 cm in diameter. Proteins were eluted by a linear salt gradient (0.1–0.55 M NaCl) in sodium phosphate buffer. Fractions containing protein were pooled and dialyzed against 8 L of 50 mM Tris-HCl, 20 mM sodium citrate, 0.1 M NaCl, pH 7.4, with two changes. The dialyzed protein was loaded onto a heparin–Sepharose affinity column, prothrombin was collected in the column wash, and factors IX and X were eluted with a linear salt gradient (0.1–0.75 M) in Tris–citrate buffer. The purity of the isolated proteins was estimated by SDS–PAGE (Wycoff et al., 1977), using the Bio-Rad (Richmond, CA) Minigel system. Protein concentration was determined by optical density at 280 nm corrected for scattering at 320 nm (Donovan, 1969), using extinction coefficients of 1.44 (mL mg^{-1} cm^{-1}) (prothrombin; Mann, 1976) and 1.16 (factor X; DiScipio et al., 1977).

The functional activity of the purified proteins was verified by using synthetic, chromogenic polypeptide substrates (S2222, S2238; Helena Laboratories, Beaumont, TX). Thrombin was generated from purified prothrombin digested with *Echis carinatus* viper venom coupled to agarose beads. The activation mixture was diluted with prewarmed (37 °C) 0.15 M Tris-HCl, pH 7.4, and the reaction was initiated by the addition of 200 μL of S2238. Rate analysis of hydrolysis, monitored by *p*-nitroaniline absorbance at 405 nm, was correlated to prothrombin concentration. Factor X_a was generated by activation of purified factor X with a purified factor X activating fraction from Russell's viper venom coupled to agarose beads (Jesty & Nemerson, 1976). The concentration of functionally active factor X_a was determined by rate analysis of the proteolytic cleavage of S2222 (Jones et al., 1985). Dilute protein solutions were concentrated on a Pharmacia (Norwalk, CT) Mono Q column using a Perkin-Elmer IsoPure LC system.

Membrane Preparation. Small, unilamellar, phospholipid vesicles were prepared by mixing the appropriate amounts of the phospholipids (1.5–3.5 mg total) in chloroform, drying under a stream of argon, redissolving in benzene, and lyophilizing. The dried phospholipid was suspended in 2 mL of buffer (10 mM TES, 0.1 M NaCl, 25 μM EDTA, pH 7.96) and sonicated to clarity in a Heat Systems, Inc. cup horn (Barrow & Lentz, 1980). The suspension, after sonication, was centrifuged for 25 min at 70000 rpm in a Beckman TL100 centrifuge (TLA-100.3 rotor) to obtain a homogeneous suspension of vesicles (Barenholz et al., 1977). Due to their established instability (Lentz et al., 1987), vesicles were used for binding isotherms within 24 h of their preparation. Concentrations of vesicle suspensions following fractionation were determined by scintillation counting of [^{14}C]DMPC (Amersham, Arlington Heights, IL) incorporated into all phosphatidylcholine stocks. Total phosphate concentrations were determined by using the assay of Chen et al. (1956). In all cases, phospholipid composition is expressed as the molar ratio of phospholipids present before sonication and centrifugation.

Relative Molecular Weight Determination by Light Scattering. Relative 90° light-scattering measurements (Nelsestuen & Lim, 1977) were made at 37 °C in an SLM Model 4800 or 48000 fluorometer. For each isotherm, 1 mL of a phospholipid suspension was equilibrated at 37 °C in the cuvette at a concentration such that the number of vesicle binding sites for protein approximated the anticipated K_d. All solutions were centrifuged for 2 min in a Beckman Microfuge B at 10000 rpm and subsequently filtered through a 0.22-μm GS filters (Millipore Corp., Bedford, MA) to remove dust particles. Proteins were preequilibrated in 7 mM $CaCl_2$ prior to their addition to phospholipid suspensions in the cuvette for binding measurements. Binding of prothrombin to DOPG/POPC and PS/POPC vesicles and of factor X/X_a[2] to

DOPG/POPC was measured in the presence of 5 mM calcium ion; binding of factor X/X_a to PS/POPC vesicles was measured in the presence of 3 mM calcium ion due to an irreversible change in light scattering of the vesicle suspension observed at 5 mM calcium ion.

Light-scattering intensities from the protein–phospholipid complex were corrected for the scattering of unbound protein and otherwise analyzed by the method of Nelsestuen and Lim (1977). All isotherms were carried out to apparent vesicle surface saturation. The addition of disodium EDTA following titration of vesicles returned the light-scattering intensity to the level predicted for unassociated vesicles plus protein, thus guaranteeing the reversibility of the binding. Data were plotted in the Hildebrand (Klotz, 1986) and Scatchard formats (Scatchard, 1949) from which apparent dissociation constants were obtained. For some isotherms, the first one, two, or three points of the isotherms showed evidence of apparent positive cooperativity in binding. Such points were not used in determination of the best-fit linear regression for Hildebrand or Scatchard analysis. Apparent outer leaflet stoichiometries of binding were calculated from saturation values of the relative molecular weight ($[M_2/M_1]_{sat}$), as

$$\text{phospholipid/protein} = 2(\text{MW of protein})/3\{\text{MW of PL} \times ([M_2/M_1]_{sat} - 1)\}$$

This expression is based on the observation that, in small vesicles, approximately two-thirds of the phospholipid molecules are in the outer leaflet (Barenholz et al., 1977). Stoichiometries obtained from the Hildebrand or Scatchard analyses were less reproducible between experiments, possibly due to heavy weighting given by the Hildebrand analysis to the first few, and least well determined, points in a binding isotherm.

Results

Traditional Analysis of Extrinsic Protein Binding. Three papers have reported binding or dissociation constants of bovine coagulation proteins as a function of negative phospholipid content (Lim et al., 1977; Nelsestuen & Broderius, 1977; Dombrose et al., 1979). Although the sum of these three papers provides an extensive and useful set of data, this data set was inadequate for a variety of reasons (range of acidic lipid concentrations considered, specific lipid species treated, specific proteins or peptides treated, different methods used) for the thermodynamic analysis presented in the Appendix. In addition, it was of interest to us to compare the binding properties of human and bovine coagulation proteins. The convenient and sensitive relative light scattering method introduced by Nelsestuen and Lim (1977) allowed us to obtain an extensive data set on the binding properties of human prothrombin and factor X/X_a over the requisite range of acidic lipid surface concentrations.

Both for the purpose of comparison with literature results and for the purpose of providing a useful set of empirical binding parameters, our results have been analyzed by a traditional binding model that assumes the existence of protein binding sites or domains in the plane of the target membrane [see Appendix, also Nelsestuen and Lim (1977)]. Figure 1 presents a binding isotherm and Hildebrand transformation for factor X/X_a binding to 20 mol % PS-containing vesicles. Apparent dissociation constants and stoichiometries (total outer leaflet lipids per bound protein) obtained from such data (see Materials and Methods) are summarized in Table I.

[2] The binding of factor X_a was indistinguishable from the binding of factor X to vesicles of the same composition; therefore, we do not distinguish between the binding constants for the zymogen and the active enzyme.

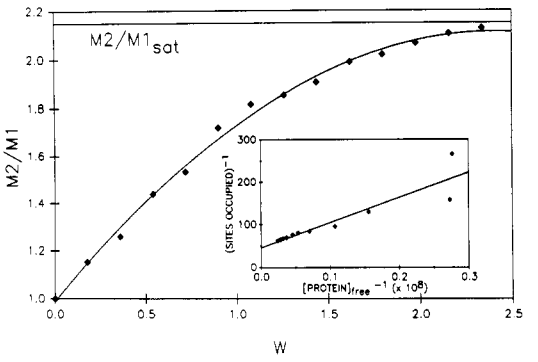

FIGURE 1: Factor X/X_a binding to 20/80 PS/POPC small vesicles prepared as described in the text. Data were generated at 37 °C from 90° relative light scattering measurements to obtain values for the molecular weight of the complex relative to that of the vesicle alone (M_2/M_1). Calcium ion concentration was 3 mM. Factor X/X_a was preincubated in 7 mM calcium ion. The abscissa is plotted as the mass of protein added per mass of phospholipid (W). (Inset) Hildebrand analysis of the binding data was used to obtain K_d as the ratio of the slope to the intercept according to the standard Hildebrand form $1/\nu = (1/P_f)(K_d/n) + 1/n$, where ν is molecules of protein bound per vesicle, n is the stoichiometry of binding in molecules of protein per vesicle, and P_f is the concentration of free protein.

Table I: Experimental Parameters[a] for Prothrombin and Factor X/X_a Binding to Model Membranes

lipid composition	protein	K_d (μM)	N/n[b]
25/75 DOPG/POPC	factor X/X_a	0.68 ± 0.41	422 ± 158
35/65 DOPG/POPC	factor X/X_a	0.30 ± 0.95	179 ± 48
45/55 DOPG/POPC	factor X/X_a	0.22 ± 0.79	113 ± 10
65/35 DOPG/POPC	factor X/X_a	0.05 ± 0.02	65 ± 9
8/92 PS/POPC	factor X/X_a	1.31 ± 0.32	187 ± 66
10/90 PS/POPC	factor X/X_a	1.47 ± 0.85	170 ± 54
12/88 PS/POPC	factor X/X_a	1.03 ± 0.37	113 ± 15
15/85 PS/POPC	factor X/X_a	0.56 ± 0.40	76 ± 33
20/80 PS/POPC	factor X/X_a	0.15 ± 0.08	42 ± 5
8/92 DOPG/POPC	prothrombin	5.33 ± 3.4	292 ± 107
15/85 DOPG/POPC	prothrombin	3.67 ± 1.22	319 ± 54
25/75 DOPG/POPC	prothrombin	3.92 ± 2.25	163 ± 72
35/55 DOPG/POPC	prothrombin	0.92 ± 0.50	81 ± 28
45/65 DOPG/POPC	prothrombin	0.20 ± 0.17	48 ± 13
65/35 DOPG/POPC	prothrombin	0.04 ± 0.02	74 ± 28
8/92 PS/POPC	prothrombin	3.64 ± 3.14	246 ± 57
10/90 PS/POPC	prothrombin	2.56 ± 1.04	182 ± 21
15/85 PS/POPC	prothrombin	1.66 ± 0.21	91 ± 2
20/80 PS/POPC	prothrombin	0.50 ± 0.02	51 ± 8

[a] Values and standard deviations are based on between four and eight independent determinations. [b] Stoichiometry given as the ratio of total outer leaflet lipid per bound protein.

For both phospholipids, the stoichiometry steadily decreased at increasing mole percent negative phospholipid. The stoichiometry of human prothrombin binding to DOPG/POPC membranes reached a limiting value of roughly 48 outer leaflet phospholipids per protein before increasing to even larger values at high DOPG content. This may represent a surface packing limit (Lim et al., 1977). This value corresponds to roughly twice the projected area previously estimated to be occupied by bound bovine prothrombin fragment 1 (Dombrose et al., 1979), whose molecular weight is roughly one-third that of prothrombin. The stoichiometries of binding to PS-containing vesicles were consistently less than for DOPG-containing vesicles but also appeared to approach a limiting value at high PS content. This limiting value could not be established as unambiguously as for PG-containing vesicles since PS vesicles demonstrate irreversible changes in light scattering at PS contents greater than 20 mol %. Nonetheless, our

extrapolated value of 42 for PS-containing membranes is consistent with our estimates from literature data [36 for human prothrombin binding to 50/50 bovine PS/bovine PC membranes (on the basis of a reasonable value of membrane lipid packing density) (Torbet & Freyssinet, 1987); 52 for bovine prothrombin to 40/60 PS/PC (Lim et al., 1977)].

We note that the uncertainties associated with the stoichiometries in Table I are rather large. It is notoriously difficult to obtain precise binding stoichiometries for loosely associating systems. For this reason, it is not surprising that our results for human prothrombin differ in a fundamental way from results reported by Dombrose et al. (1979) for bovine prothrombin fragment 1. These authors reported an *increase* in the number of outer leaflet acidic lipids per bound peptide as the surface concentration of acidic lipid (PG) increased. From the rate of this change, they concluded that 19 PG molecules were required to form the binding site for 1 fragment 1 peptide. We, on the other hand, have found a *decrease* in the outer leaflet acidic lipid stoichiometry with increasing acidic lipid surface concentration for each of the four protein–lipid systems we have examined here. This is consistent with our picture of extrinsic protein binding, which predicts low surface occupany at low acidic lipid surface concentration, since acidic lipid binding sites on an adsorbed protein must be occupied according to the laws of mass action in order that the protein remain surface associated. However, it is probably inappropriate for us or for Dombrose et al. to base interpretations on subtle trends in such ill-defined values.

Although differences exist in the methodologies used and in the specific lipid and protein systems studied, the apparent dissociation constants reported by Dombrose et al. (1979) for bovine prothrombin fragment 1 binding and by Nelsestuen and Broderius (1977) for bovine prothrombin binding to DOPG-containing vesicles are quite similar to those obtained here for human prothrombin binding to PG- or PS-containing vesicles, and all decreased with increasing acidic lipid content (Table I).

The dependence of apparent dissociation constants on PS and DOPG content is shown in Table I. Dissociation constants obtained at low acidic lipid concentration must be considered "apparent dissociation constants" because of the failure to achieve complete surface saturation under our experimental conditions. Nonetheless, binding at low acidic surface concentration probably does reflect saturation of the acidic lipid component of the membrane and must be reported to compare our results to those in the literature as well as to provide an empirical basis for description of these binding events. The most remarkable aspect of our results was that binding could not be detected for factor X/X_a to membranes containing less than 25 mol % DOPG. Nonetheless, at greater than 25 mol % DOPG, factor X/X_a bound to the membranes with a K_d that was comparable to that observed for prothrombin binding at the same DOPG content. Both this and the anomalously large apparent lipid stoichiometries observed at low acidic lipid surface concentration will find possible explanations in terms of our new model for extrinsic protein binding (see Discussion).

New Analysis To Determine the Number of Acidic Lipids Involved in Extrinsic Protein Binding. It is evident from the traditional analysis presented above that this type of analysis is not likely to allow us to draw conclusions about the number of acidic lipids involved in binding one protein molecule. However, it is reasonable to presume that the dependence of the binding event on acidic lipid surface concentration must contain information on this issue. The model outlined in the introduction and treated quantitatively in the Appendix deals directly with this issue without making any assumptions as to the exact number of acidic lipids involved in the binding event.

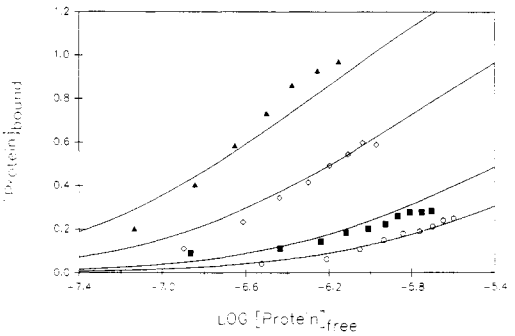

FIGURE 2: Experimental and modeled Klotz Plots of the bound protein concentration versus the logarithm of free protein concentration for the binding at 37 °C of prothrombin to PS/PC membranes containing 0.2 (solid triangles), 0.15 (open diamonds), 0.1 (solid squares), and 0.08 (open circles) mole fraction PS. Data were generated as described under Materials and Methods. The lines drawn through the data were calculated with the simplified version of our model (i.e., independent and equivalent sites) described in the Appendix, using parameters summarized in Table IV.

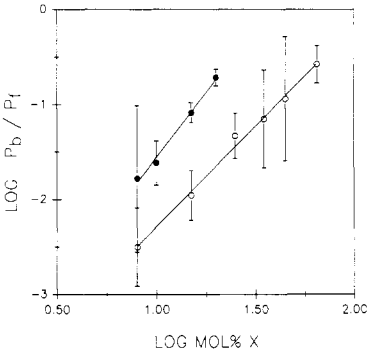

FIGURE 3: Plots of the logarithm of the partition coefficient between bound and free prothrombin (Γ in eq A9 of the Appendix) versus the logarithm of surface concentration of DOPG (open circles) or PS (solid circles). The slope of the plot at any given free protein concentration yields the average number of binding sites on the protein occupied by acidic phospholipid molecules (see Appendix).

Figure 2 shows, in a semilogarithmic representation, data obtained for prothrombin binding to bovine PS/POPC membranes at four different surface concentrations of PS. In the Appendix, we show that the variation, at constant $[P_f]$, of the partition coefficient of bound to free protein, $([P_b]/[P_f])$, with acidic lipid surface concentration gives the average occupancy of protein binding sites by acidic lipid molecules (eq A10). This partition coefficient for bovine prothrombin binding to bov PS/PC and DOPG/POPC membranes is plotted in logarithmic fashion versus the mole fraction of acidic lipid (x) in Figure 3. Similar plots were prepared for the binding of factor X/X_a to bovine PS/POPC and DOPG/POPC membranes (not shown). In all cases, the data were well described by straight lines, the slopes of which are equal to the average number of protein sites occupied by ligand (in this case the ligand is anionic phospholipid). These slopes are summarized in Table II. In the limit of low surface occupancy (i.e., low free protein concentration), the slopes approached a value of 3. It would appear that both prothrombin and factor X/X_a have at least three sites for binding acidic phospholipids, although this analysis does not preclude there being more sites that are not occupied at the surface acidic lipid concentrations we have used. This is consistent with our previous estimate of 10 or fewer acidic phospholipids associated with bound prothrombin fragment 1 (Jones & Lentz, 1986), but inconsistent with the existence of phase-separated lateral domains

Table II: Slopes of ln ([P_b]/[P_f]) vs ln x

protein	phospholipid	[P_f] (μM)	slope
prothrombin	DOPG/POPC	0.1	2.9 ± 1.2
		0.2	2.4 ± 1.2
		0.4	2.1 ± 0.4
		0.8	2.0 ± 0.4
		1.6	1.4 ± 1.1
	bov PS/POPC	0.4	3.0 ± 0.7
		1.6	1.4 ± 1.0
factor X/X_a	DOPG/POPC	0.1	2.4 ± 1.0
		0.4	1.8 ± 0.7
	bov PS/POPC	0.4	2.4 ± 0.6

Table III: Variation of Fit with m, the Number of Lipid Binding Sites on Protein

	variance (s^2)[a] ($\times 10^{16}$)		
m	all data	prothrombin	X/X_a
1	5.3	5.7	4.8
2	1.6	1.9	1.3
3	0.79	0.88	0.66
4	0.67	0.40	0.95
5	1.2	0.65	1.8
6	1.4	0.81	2.0
20	2.8	1.7	4.0

[a] The variance of the fit is a measure of both the goodness of fit and the mean square variance of the parent population of data (Bevington, 1969).

(Mayer & Nelsestuen, 1981) or patches containing 19 acidic lipid molecules (Dombrose et al., 1979).

Simulation of Binding Isotherms Using Our Model. To obtain greater insight into the mechanism of prothrombin and factor X/X_a binding, we simulated the observed binding behavior in terms of our binding model. To do so, we assumed that the protein has *m independent and equivalent sites*, each with a site binding constant, k, for association with acidic phospholipid. For the sake of generality, protein is assumed to adsorb nonspecifically to any membrane surface with a stoichiometric binding constant $K_a°$. This simplified version of our model contains four parameters: m, k, $K_a°$, and the stoichiometry at surface saturation, $^3/_2N/n$. Two of these, m and $^3/_2N/n$, can be constrained on the basis of experiment. Thus, for the experimental data presented, $m \geq 3$ and $^3/_2N/n = 69$ (from the minimum values attained in Table I). Values of m = 1–20 were tried to compare the domain binding model (m = 20) to a model consistent with our results (Figure 3, m = 3 or 4). By use of a simple grid-search algorithm, the other two parameters, k and $K_a°$, were assigned values that resulted in minimization of the residual between experimental and calculated semilogarithmic binding isotherms (e.g., see Figure 2) for all values of x examined for a given acidic phospholipid and protein.

Table III lists the residuals obtained in this way for various fixed values of m. A residual is given for the fit obtained to all four data sets (i.e., both proteins, both acidic lipids) and for the fits obtained to the two protein data sets individually. It is evident that the best overall fit was obtained for m = 3 or 4, with m = 4 giving a slightly better fit for prothrombin and m = 3 being preferred for factor X/X_a. It is also clear that M = 20, which depicts the acidic lipid domain model of Dombrose et al. (1979) or Mayer and Nelsestuen (1981), gave a significantly (>99% confidence limit by Student's t-test) poorer fit to the data.

Table IV summarizes the binding constants giving the best fits to each of the four data sets for m fixed at 4. Considering the simplicity of the model and the limited parameterization required, the fit to experiment was quite good for all four data sets (see reduced χ^2 values in Table IV). Only the data set

Table IV: Parameter Values Used To Obtain a Best Fit of Simulations to Semilogarithmic Binding Isotherms

protein	lipid	m[a]	$^3/_2N/n$[b]	k[c]	$K_a°$	s^2,[d] χ^2[e]
prothrombin	PG/PC	4	69	80–100	1	5.4, 0.379
prothrombin	PS/PC	4	69	190–210	1	2.4, 0.480
factor X/X_a	PG/PC	4	69	15–45	1	13, 2.38
factor X/X_a	PS/PC	4	69	260–320	1	8.2, 0.169

[a] Fixed according to Table III. [b] Fixed according to minimum values in the experimental stoichiometries in Table I. [c] The range given for the specific binding site constants, k, was defined by k values that resulted in an increase in s^2 of a factor of 2 relative to the minimized s^2. The value of k at the minimum was roughly the median of this range. [d] The variance of the fit (s^2) is calculated as the mean square deviation of the calculated from the observed concentration of bound protein divided by the number of degrees of freedom for a data set and presented $\times 10^{17}$. [e] The reduced "chi-square" of the fit (χ^2) is defined as $s^2/\langle\sigma^2\rangle$, where $\langle\sigma^2\rangle$ is the mean square standard deviation of all points in a data set. The reduced χ^2 is a measure of goodness of fit; a value near 1 indicates that the model provides a good description of the data (Bevington, 1969).

for the binding of factor X/X_a to DOPG/POPC membranes showed a reduced χ^2 greater than 1. This is not surprising, since this binding was so weak at low DOPG content that the signal was difficult to detect above the background.

Discussion

New View of Extrinsic Protein Binding. In this paper, we have suggested a new and, we believe, physically reasonable view of the specific binding of extrinsic proteins to membranes containing acidic (or other specific) phospholipids. Commonly, the protein ligand has been viewed as binding to ill-defined sites on the macromolecular membrane. We suggest instead that specific anionic lipid binding sites exist on certain extrinsic membrane proteins and that these sites are occupied according to a dynamic two-dimensional equilibrium obeying the laws of mass action (see Figure 1 of the Appendix). Our treatment is formally identical with the treatment of ligand binding to multiple sites on a macromolecule in a three-dimensional solution (Cantor & Schimmel, 1980). The existence of specific phospholipid binding sites on the protein can account for the variation in binding behavior observed with varying surface concentrations of acidic lipid: a greater availability of acidic phospholipids leads to a greater probability of occupying sites on proteins and, thus, to a greater probability of protein binding to a membrane, i.e., to a lower K_d and a decreased stoichiometry.

Similar models have been described by Reynolds (1979) for the binding of antibodies to cells, by Tamm and Bartoldus (1988) for antibody binding to lipid model membranes, and by Dwyer and Bloomfield (1981) for the binding of any multivalent ligand to mobile membrane receptors. The papers by Reynolds and by Tamm and Bartoldus address problems of cellular immunology and not the issues dealt with here. In particular, the paper by Tamm and Bartoldus describes a very tight binding phenomenon and assumes that both sites on the bivalent ligand (antibody) are always occupied. Our treatment is distinguished by making no assumptions about site affinity and thus allowing for partial occupancy of multiple sites. Dwyer and Bloomfield, in a totally theoretical paper, conclude that bound multivalent ligands should always be saturated with mobile receptor, as was assumed by Tamm and Bartoldus. This conclusion results from the assumption that bound ligands lose completely one translational and two rotational degrees of freedom relative to free ligands. We believe this assumption to be an oversimplification, as rotational and translational degrees of freedom are not completely lost upon binding but rather are converted to vibrational degrees of freedom. Indeed,

treatment of our data demonstrates that partial occupancy of protein sites does occur for binding of coagulation proteins (Figure 3 and Table II).

Not all extrinsically bound membrane proteins are expected to associate with membranes by the mechanism suggested here. This mechanism assumes specific interaction of a particular membrane component with well-defined sites on a protein. However, other proteins or polypeptides might be expected to associate with charged membranes by other mechanisms such as nonspecific electrostatic absorption [e.g., polylysine (Hartmann & Galla, 1978)] or partial hydrophobic penetration [e.g., melittin (Vogel & Jähnig, 1986); α-lactalbumin (Berliner & Koga, 1987)]. All three types of mechanisms probably contribute to some extent to the binding of any extrinsic membrane protein. However, when one mechanism dominates, the dependence of apparent binding constant on mole fraction acidic phospholipid, as described here, should identify the dominant mechanism. Thus, nonspecific electrostatic absorption is expected to produce a dramatic dependence of association constant on mole fraction acidic lipid [e.g., see Chung et al. (1985)], while hydrophobic association should not be nearly as sensitive to the amount of acidic lipid as is the specific protein–lipid mechanism that we propose.

Application of Our Model to Prothrombin and Factor X/X_a Binding Data. The commonly accepted model for the binding of extrinsic membrane proteins involves the formation of domains or pools of negatively charged lipids under the surface-associated, positively charged protein (Birrell & Griffth, 1976; Hartmann et al., 1977; Barenholz et al., 1977; Wiener et al., 1985). In the case of γ-carboxyglutmic acid containing proteins such as prothrombin, binding to negatively charged lipids depends on Ca^{2+} and is thought to involve "Ca^{2+}-bridging" of negative γ-carboxyglutamic acid residues to the pool of acidic lipids (DiScipio et al., 1977; Lim et al., 1977; Dombrose et al., 1979; Mayer & Nelsestuen, 1981, 1983). [It should be noted that the bridging hypothesis also is not universally accepted (Rhee et al., 1982) but that we do not deal with the issue of bridging in this paper.] In recent years, we and others have questioned this negative lipid domain model both as it applies to coagulation proteins and as it applies to extrinsic proteins in general (Lentz et al., 1985; Jones & Lentz, 1986; Tendian & Lentz, 1989; Prigent-Dachary et al., 1986; Devaux et al., 1986). In particular, our work has shown that a domain induced by the binding of the fragment 1 region of prothrombin cannot contain more than 10 PG molecules (Lentz et al., 1985; Jones & Lentz, 1986).

The new view of extrinsic protein binding presented here makes no a priori assumptions about the number of acidic lipids associated with a membrane-bound protein. For this reason, it offers the opportunity to test the ability of the domain model to account for the dependence of observed binding isotherms on the surface concentration of acidic phospholipids. This test was made in two ways. First, the quantitative treatment of our model given in the Appendix shows how to derive directly from the dependence of binding on the surface concentration of acidic lipid an estimate that only 2–3 acidic lipids are associated with sites on bound prothrombin or factor X/X_a molecules (Figure 3). Second, when a simplified version of our model was used to simulate our binding isotherms, it was very clear that the cooperative formation of relatively large acidic lipid domains (ca. 20 acidic lipids) provided a statistically worse description (Table III) of the binding data than did a model involving binding of very few acidic lipids to a limited number of specific sights on prothrombin or factor X/X_a.

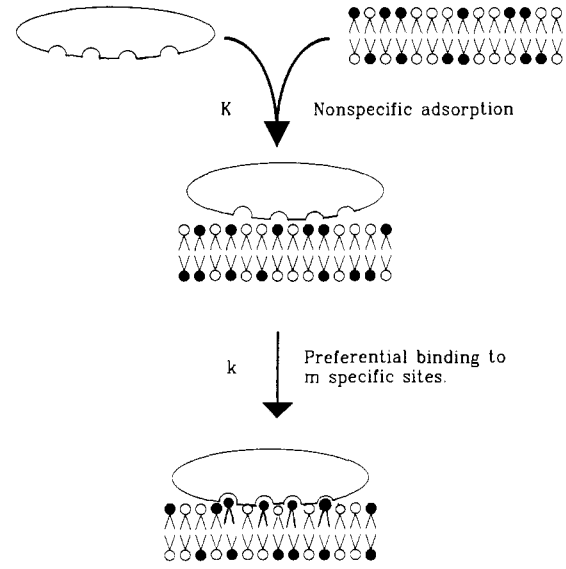

FIGURE A1: Model for the recruitment of negative phospholipids during the binding of prothrombin and factor X/X_a to a charged lipid membrane. The model stresses a two-step binding process in which the first step is nonspecific adsorption to the membrane surface. The second step is a specific two-dimensional dynamic binding equilibrium in which sites on the protein are occupied depending on the surface concentration of negative phospholipids in the bilayer. Quantitative treatment of this model indicates that both proteins display at least three sites but that at least some of these sites have different affinities for PS as compared to PG.

The results of the simulation of our experimental data by the negative phospholipid binding model suggest that the affinity of the acidic sites on factor X/X_a have approximately $^1/_{10}$ the affinity for PG as for PS (see Table IV), even though factor X/X_a appears to bind with comparable (and sometimes even lower) dissociation constants to PG- as compared to PS-containing membranes (see Table I). This apparent paradox is resolved in terms of our model by the prediction that larger ratios of PG to protein on the surface are needed to maintain occupancy of acidic lipid sites on factor X/X_a compared to that required to maintain occupancy by PS or to occupy sites on prothrombin. This explains why binding to low-PG-containing membranes occurs at a very low apparent surface saturation (see large stoichiometries in Table I) and why binding of factor X/X_a could not be observed to membranes of less than 25% PG content.

The model presented here contains a parameter, $^3/_2N/n$, that accounts for saturation of binding when the amount of protein bound per membrane vesicle becomes large. This saturation stoichiometry is obtained experimentally and presumably is set by crowding of the protein on the membrane surface. However, at very low concentrations of negatively charged phospholipids, binding is so weak that this limit is never reached, at least at experimentally obtainable protein concentrations. At such low concentrations of negatively charged phospholipid and when $K_a°$ and k are very different, our model can produce apparent saturation at a higher apparent lipid to bound protein stoichiometry (low surface occupancy), with ultimate saturation at $^3/_2N/n$ only at very high protein concentrations.

Finally, our model offers an explanation for the slight negative cooperativity reported by Dombrose et al. (1979) for the binding of prothrombin fragment 1 to DOPG/DOPC membranes. Dwyer and Bloomfield (1981) point out how the model presented here can produce negative cooperativity and thereby an incorrect estimate for the stoichiometry. As shown in eq A12 of the Appendix, the apparent equilibrium constant

for binding of protein to a membrane should be greater at low surface occupancy (high surface concentration of unbound acidic lipid, x_f) than near saturation (low x_f). It must be stressed that this does not reflect the "statistical crowding" phenomenon previously reported for binding of multivalent ligands to DNA (McGhee & von Hippel, 1974) and treated for fluid lipid bilayers by Stankowski (1984). Statistical crowding is not considered at this stage in our treatment but is expected to lead to even greater negative cooperativity in the observed binding isotherms.

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Appendix: A New Model To Describe Extrinsic Protein Binding to Phospholipid Membranes of Varying Composition: Quantitative Development

Abstract

A new model is presented and quantitatively treated to explain the surface binding of certain extrinsic membrane proteins. This simple model allows for treatment of extrinsic protein binding data obtained over a wide range of conditions in such a way as to permit maximal extraction of detailed molecular interpretations. The model presumes that there exist on such protein molecules a limited number of binding sites specific for a subclass of special lipids. Macroscopic binding affinity is determined by two processes: nonspecific adsorption of protein to the membrane surface and association of a subclass of membrane lipids with the specific sites on the bound protein. Analysis of binding data is presented on two levels. First, analysis of semilogarithmic binding isotherms at low surface occupancy reveals the number of sites occupied. Second, the model was treated exactly for the simple case of equivalent, noninteracting, protein-associated binding sites in such a way as to allow estimation of the specific site binding constant as well as the nonspecific adsorption constant.

(I) Traditional Analysis of Membrane Binding

The macroscopic binding of surface proteins to membrane vesicles can be described in terms of an independent-site binding model (Klotz & Hunston, 1975; Nelsestuen & Lim, 1977) with n equivalent binding sites

$$P + S \rightleftharpoons P{\cdot}S$$

$$K_{a,obs} = [P{\cdot}S]/[S][P_f] \qquad \nu = n[P_f]K_a/(1 + K_a[P_f]) \quad \text{(A1)}$$

where P is the protein and S is an individual binding site on the membrane, ν/n is the fraction of all sites on a vesicle that are occupied, and $K_{a,obs}$[1] is the experimentally determined site binding constant. [P·S] is the concentration of bound protein, $[P_b]$, while $[P_f]$ is the concentration of free protein. The concentration of binding sites, [S], can be related, in the absence of binding, to the vesicle concentration, [V], or to the concentration of phospholipids, [PL], by the number of binding sites per vesicle, n, and by the number of lipids per vesicle, $^3/_2\,N$ (N being the number of lipids in the outer leaflet of a small, unilamellar vesicle; Barenholz et al., 1977):

$$[V] = [S]/n \qquad [PL] = [S]^3/_2\,N/n \tag{A2}$$

Measured site binding constants are often reported in units of (molar lipid)$^{-1}$, a quantity equal to the inverse of $K_{a,obs}\cdot {}^3/_2 N/n$, with stoichiometries usually reported as $^3/_2 N/n$.

(II) Quantitative Development of the New Model

Our model to describe extrinsic protein binding does not make any assumptions about the existence or nature of a discrete binding site on the membrane. Rather, we presume that, as in other biological systems, specific binding properties should be assigned to proteins rather than to pools of lipids. For proteins that bind via specific interactions rather than by electrostatic double-layer effects, macroscopic binding affinity is viewed as being determined by two simple, equilibrium processes: nonspecific adsorption of protein to the membrane surface and association of a subclass of membrane lipids with specific sites on the bound protein (see Figure A1). This simple formulation minimizes the need for assumptions and extracts the maximum information from measurements.

The *first* process is a nonspecific binding of the protein to a neutral phospholipid membrane. The affinity of a protein such as prothrombin for neutral phospholipid membranes is, of course, very low, and binding is sufficiently weak that it is in practice often negligible. Nonetheless, the binding interaction must be included for the sake of completeness. Following a traditional treatment of binding to a membrane, we describe this in terms of binding to multiple, independent sites. Writing P_f and P_m for the concentrations of protein free in solution and membrane bound, respectively, one can write

$$P_f + M \rightleftharpoons P_m$$
$$K_a^\circ = [P_m]/[P_f][PL_f] \qquad K_a^\circ << [P_f] \tag{A3}$$

with $[PL_f]$ the concentration of unbound phospholipid, i.e., the amount not carrying bound protein. This is formally equivalent to describing the binding equilibrium with a Langmuir isotherm. The dissociation constant is much larger than the protein concentration in most instances, and, therefore, the fraction of all binding sites that are occupied is small.

The *second* equilibrium that we consider occurs when the phospholipid membrane contains some specific phospholipid, e.g., negatively charged phospholipid, L^-, as some (small) mole fraction, x, of the total phospholipid. The presence of this specific phospholipid greatly increases the affinity of protein for the membrane and is interpreted to indicate that, in the protein–membrane complex, specific phospholipid molecules are preferentially associated with the protein. We incorporate this two-dimensional binding equilibrium into the model by writing n successive association equilibria between membrane-bound protein and membrane-associated specific phospholipid, using a stoichiometric binding model (Klotz & Hunston, 1975):

$$P_m + L^- \rightleftharpoons P_m{\cdot}L^-;\ K_1 = [P_m{\cdot}L^-]/[P_m]x_f$$
$$\vdots$$
$$P_m{\cdot}L^-_{n-1} + L^- \rightleftharpoons P_m{\cdot}PL^-_n;\ K_n = [P_m{\cdot}L^-_n]/[P_m{\cdot}L^-_{n-1}]x_f \tag{A4}$$

Note that the K_i are two-dimensional binding equilibrium constants, with the units of specific lipid concentration being surface mole fraction of unbound specific lipid, x_f. The units of $[P_m{\cdot}L^-]$ and $[P_m]$ are taken as bulk concentrations, since division of both by the bulk total lipid concentration will convert these bulk concentrations to surface concentrations. The total concentration of bound protein, $[P_b]$, is the sum of the concentrations of all these species

$$[P_b] = \sum_{i=0}^{n}[P_m{\cdot}L^-_i] \tag{A5}$$

In addition, we may write the concentration of the ith species as

$$[P_m{\cdot}L^-_i] = \kappa_i[P_m](x_f)^i;\ \kappa_i = K_1K_2...K_i \tag{A6}$$

This result and the preceding equations here and in Section I are valid under conditions of "ideality", which must apply both in the solution and on the membrane surface. The solution concentrations of total phospholipid and of protein, in most experiments and in vivo, are sufficiently low to be considered ideal solutions for the purposes of describing the equilibrium A1. Ideality of the membrane required for application of eq A4–A6 means that the mole fraction of specific lipid, x, must be low, that the lipids mix well in the plane of the membrane, and that the surface concentration of protein is sufficiently low that steric crowding and the interactions between bound protein molecules do not interfere with binding. Experience indicates that most phospholipids with similar acyl chains mix close to ideally in the fluid phase. Protein–protein interactions can be minimized by confining our attention to conditions of low surface occupancy. It is also important to notice that the concentrations of protein, $[P_f]$ (eq A1), and of specific phospholipid, as expressed by the mole fraction, x_f, refer to concentrations of protein molecules free in solution and of specific lipids *not* associated with protein. As binding occurs, these will obviously be lower than the total concentrations and appropriate corrections will be made.

We can rewrite eq A1 using eq A5 and A6 for the total concentration of membrane-bound protein:

$$K_{a,obs} = [P_b]/[P_f][PL_f] = [P_m]\sum_{i=0}^{n}\kappa_i x_f^i/[P_f][PL_f] \tag{A7}$$

From eq A3, $[P_m] = K_a^\circ[P_f][PL_f]$, giving

$$K_{a,obs} = K_a^\circ\sum_{i=0}^{n}\kappa_i x_f^i \tag{A8}$$

---

[1] Some symbol definitions: N/n, stoichiometry of binding in terms of the ratio of outer leaflet phospholipids to protein binding sites on the outer leaflet of a membrane; $K_{a,obs}$, apparent equilibrium constant observed for binding of protein to sites on a membrane surface; K_a°, nonspecific equilibrium constant for binding of protein to an unchanged membrane surface; k, 2D site binding constant for binding of specific lipid molecules to independent and equivalent sites on membrane-associated protein; m, number of independent and equivalent specific lipid binding sites on a membrane-associated protein; x (x_f), mole fraction of total (free) specific lipid in the target membrane; Γ, partition coefficient between bound (P_b) and free (P_f) protein; [PL°] ([PL_f]), total (free or "unbound") phospholipid concentration.

[2] The requirement of constant total lipid concentration should not present any experimental constraint, since data obtained at different lipid concentrations but at constant free protein concentration can be shown rigorously to normalize as

$$([P_b]/[P_f])_1/([P_b]/[P_f])_2 = [PL^\circ]_1/[PL^\circ]_2$$

If we consider the partition coefficient, Γ, at constant total lipid concentration,[2] of protein between the membrane surface and the solution phase

$$\Gamma = [P_b]/[P_f] = \sum_{i=0}^{n}[P_m \cdot L_i^-]/[P_f] \quad (A9)$$

It is easy to see that for low surface occupancy

$$\langle i \rangle = \left(\frac{d \log \Gamma}{d \log x}\right)_{[P_f]} \quad (A10)$$

The function Γ is obtained by plotting the data in semilogarithmic form as $[P_b]$ versus log $[P_f]$ (see Figure 2) for several values of x, drawing smooth curves through each set of data and then reading $[P_b]$ from these curves for fixed values of $[P_f]$. By use of this method, the condition of low surface occupancy can be rigorously met independent of whether binding saturates the surface at a particular specific lipid content.

(III) Behavior of a Simplified Model

A simplified form of our model was used to describe the binding data by assuming that the protein has *m independent and equivalent sites*, each with a site binding constant, k, for association with specific phospholipid. For this situation, a simple expression holds for the concentration of bound protein (i.e., the sum on the right side of eq A5)

$$\sum_{i=0}^{n}[P_m \cdot L_i^-] = P_m(1 + kx_f)^m \quad (A11)$$

where the surface concentration of free, specific phospholipid, x_f, is expressed as a mole fraction. Combining eq A3, A7, and A11, it is easy to show that the observed binding constant for binding of protein to a membrane, $K_{a,obs}$, can be written

$$K_{a,obs} = K_a^\circ(1 + kx_f)^m \quad (A12)$$

where K_a° is the binding constant in the absence of specific phospholipids as described by eq A3.

Using eq A1, A2, and A11 and recognizing that the total lipid concentration, [PL°], is related to the sum of occupied and unoccupied membrane surface binding sites, we obtain for the concentration of bound protein

$$[P_b] = K_{a,obs}[P_f][PL^\circ]/(1 + {}^3/_2N/n[P_f]) \quad (A13)$$

where the surface stoichiometry (moles of total phospholipid/moles of surface binding sites) is ${}^3/_2N/n$ (see eq A2). Further, the mole fraction of specific phospholipid associated with sites on the protein can be shown to be

$$x_b = mkx_f[P_b]/\{[PL^\circ](1 + kx_f)\} \quad (A14)$$

We have used an iterative calculation for obtaining the concentration of bound protein, $[P_b]$, and the mole fraction of specific phospholipid not associated with protein binding sites, x_f, in terms of observable quantities: the concentration of unbound protein, $[P_f]$, the total phospholipid concentration, [PL°], and the total mole fraction of specific phospholipid, x. Initially, we have taken $x_f = x$ and used eq A12–A14 to obtain X_b. Thereupon, a new estimate of x_f was made

$$x_f = x - x_b \quad (A15)$$

and used in eq A12 to begin a new iteration. When the change in x_f through an interation became less than a prescribed amount (1 ppm has worked well in practice), the iteration was terminated.

This simple treatment of our model contains four parameters: m, k, K_a°, and the macroscopic stoichiometry ${}^3/_2N/n$. Two of these, m and ${}^3/_2N/n$, can be constrained on the basis of experiment. By use of a simple grid-search algorithm, the other two parameters, k and K_a°, may be assigned values that result in minimization of the residual between experimental and calculated logarithmic binding isotherms.

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