Interfacial Binding of Bee Venom Secreted Phospholipase A_2 to Membranes Occurs Predominantly by a Nonelectrostatic Mechanism†

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ABSTRACT: The secreted phospholipase A_2 from bee venom (bvPLA$_2$) contains a membrane binding surface composed mainly of hydrophobic residues and two basic residues that come in close contact with the membrane. Previous studies have shown that the mutant in which these two basic residues (K14 and R23) as well as three other nearby basic residues were collectively changed to glutamate (charge reversal), like wild-type enzyme, binds with high affinity to anionic phospholipid vesicles. In the present study, we have measured the equilibrium constants for the interaction of wild-type bvPLA$_2$, the charge-reversal mutant (bvPLA$_2$-E5), and the mutant in which the five basic residues were changed to neutral glutamine (bvPLA$_2$-Q5) with phosphatidylcholine (PC) vesicles containing various amounts of the anionic phosphatidylserine (PS). Remarkably, bvPLA$_2$-E5 with an anionic membrane binding surface binds more tightly to vesicles as the mole percent of PS is increased. Computational studies predict that this is due to a significant upward shift in the pK_a of E14 (and to some extent E23) when the enzyme binds to PC/PS vesicles such that the carboxylate of the glutamate side chain near the membrane surface undergoes protonation. The experimental pH dependence of vesicle binding supports this prediction. bvPLA$_2$-E5 binds more weakly to PS/PC vesicles than does wild-type enzyme due to electrostatic protein−vesicle repulsion coupled with the similar energetics of desolvation of basic residues and glutamates that accompanies enzyme−vesicle contact. Studies with bvPLA$_2$-Q5 show that only a small fraction of the total bvPLA$_2$ interfacial binding energy (∼10%) is due to electrostatics.

Secreted phospholipases A_2 (sPLA$_2$)[1] are 14−18 kDa, calcium-dependent, disulfide-rich enzymes that liberate free fatty acids from the *sn*-2 position of glycero phospholipids (*1*, *2*). These proteins are studied as a paradigm for enzymes that act at the lipid−water interface (interfacial enzymes) (*3*, *4*). sPLA$_2$'s are water-soluble but must absorb to the membrane surface to gain access to their water-insoluble phospholipid substrates. Binding of the enzyme to the membrane interface is distinct from the binding of a single phospholipid molecule in the active site (*5*, *6*). With suitable constraints on the exchange of enzyme, substrate, and products between substrate aggregates, it is possible to extract the primary rate and equilibrium constants that describe the interfacial reaction cycle (*3*, *4*).

Although binding of sPLA$_2$ to phospholipid−detergent mixed micelles had earlier been attributed to the interaction of the protein with a single phospholipid (*7*), it is now generally accepted that the interfacial binding surface of sPLA$_2$'s involves ∼10−20 amino acid residues that make contact with tens of phospholipids. sPLA$_2$'s sit on the vesicle surface, and hydrophobic amino acid side chains are thought to penetrate into the bilayer just below the polar headgroup region (*8*−*12*). Among hydrophobics, the side chain of aromatic amino acids, especially the indole of tryptophan, makes the largest contribution to interfacial binding of sPLA$_2$'s (*9*−*11*, *13*, *14*). Tryptophan often occurs on transmembrane helices near the lipid−water interface, for example, as observed for the potassium channel from *Aeropyrum pernix* (*15*), and ranks as the top amino acid in promoting the interfacial binding of peptides to membranes (*16*). Evidence for the propensity of tryptophan to exist near the phospholipid carbonyl groups near the lipid−water interface is particularly evident from the study of short transmembrane α-helical peptides (*17*) and from the study of the structure of the gramacidin transmembrane ion channel (*18*).

Electrostatic interaction of cationic lysine and arginine residues with anionic phospholipids including phosphati-

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[1] Abbreviations: bvPLA$_2$, group III phospholipase A_2 from bee venom (*Apis mellifera*); bvPLA$_2$-E5, the mutant of bvPLA$_2$ in which Lys-14, Arg-23, Lys-85, Lys-94, and Lys-133 were changed to glutamates; bvPLA$_2$-Q5, same as bvPLA$_2$-E5 but basic residues changed to glutamine; CH, cholesterol; DOPC and DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine or -phosphoserine, respectively; DO_{et}PC and DO_{et}PS, 1,2-dioleyl-*sn*-glycero-3-phosphocholine or -phosphoserine, respectively; EPR, electron paramagnetic resonance; PC, phosphatidylcholine; FDPB, finite difference Poisson−Boltzmann; PS, phosphatidylserine; SM, sphingomyelin; sPLA$_2$, secreted phospholipase A_2.

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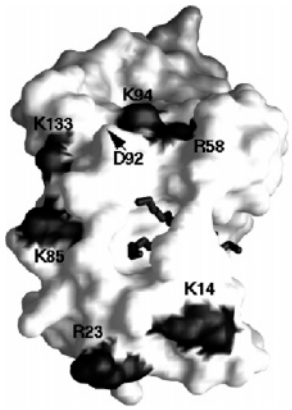

FIGURE 1: X-ray crystal structure of bvPLA$_2$ with a bound short-chain phospholipid in the active site slot. The collar of the enzyme around the active site slot opening contains six basic residues (K14, R23, R58, K85, K85, and K133, five of which have been mutated in this study and are shown in black) and one acidic residue (D92), and the remainder are hydrophobic residues. The figure was generated from the X-ray structure (*36*) using the GRASP program (*48*).

dylserine (PS) has been proposed to play an important role in the binding of specific sPLA$_2$'s to membranes (*10*, *19*, *20*). The idea that such electrostatic interactions dominate the binding of sPLA$_2$'s to membranes (*19*) has recently been challenged (*10*, *12*, *21*), although it is likely that basic residues play an important role in specificity of binding of sPLA$_2$'s to anionic phospholipid vesicles in preference to zwitterionic ones. In addition, it is expected that electrostatic interactions are important for membrane association in proportion to the density of basic residues on the interfacial binding surface of sPLA$_2$'s, for example, compare bee venom sPLA$_2$, which has a net charge of +2 and the membrane interaction of which is not highly dependent on electrostatic interactions (see below), and human group IIA sPLA$_2$, which has a net charge of +15 and binds significantly more strongly to PS membranes than membranes composed of electrically neutral lipids (*20*).

In this context, we have studied the interfacial kinetic action of the sPLA$_2$ from honey bee venom (bvPLA$_2$) on anionic phospholipid vesicles (*21*). The X-ray structure of this enzyme reveals the typical pattern for sPLA$_2$'s, namely, a ~15 Å deep active site slot, where a single phospholipid binds in proximity to the catalytic residues, which is surrounded by the interfacial binding surface (Figure 1). This surface is mainly composed of hydrophobic residues. The face of the enzyme that includes the active site slot also contains six basic residues (K14, R23, R58, K85, K94, and K133) and one anionic residue (D92). A reasonable working hypothesis is that these cationic residues support the higher affinity binding of bvPLA$_2$ to anionic versus zwitterionic membranes. However, the mutant in which five of these six basic residues were changed to the anionic residue glutamate, bvPLA$_2$-E5 (K14E/R23E/K85E/K94E/K133E mutant), like the wild-type enzyme, bound essentially irreversibly to anionic dimyristoyl phosphatidylmethanol vesicles (*21*). Tight binding of bvPLA$_2$-E5 to anionic vesicles was established by kinetic analysis, by the fact that the enzyme hydrolyses all of the phospholipids in the outer monolayer of the vesicles without desorption into the aqueous phase (scooting kinetics), and by fluorimetric studies that directly measure protein−vesicle binding (*21*).

Previous attempts to quantify the binding of bvPLA$_2$'s to vesicles of anionic phospholipids have been hampered by the high affinity of these enzymes for anionic vesicles. Thus, only upper limit values for the equilibrium constant for dissociation of the enzyme from the vesicle into the aqueous phase, K_d, have been obtained (for example, ref *21*). In the present study, we have examined the binding of bvPLA$_2$ and its interfacial binding site mutants to phosphatidylcholine (PC)/PS mixed vesicles with compositions reflecting those of physiological membranes under conditions where accurate values of K_d could be obtained. Binding studies as a function of pH together with finite difference Poisson−Boltzmann (FDPB) calculations of the electrostatic interactions of wild-type and mutant enzymes with PC/PS bilayers provide insight into the high-affinity binding of these proteins to anionic phospholipid vesicles. The results show that electrostatic interactions between lysine and arginine protein residues and anionic PS molecules in the membrane contribute minimally to the overall interfacial binding energetics of bv-PLA$_2$.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant bvPLA$_2$ Proteins. Recombinant bvPLA$_2$ and bvPLA$_2$-E5 were prepared as described previously (*21*, *22*). The plasmid encoding the mutant in which the same five basic residues were changed to glutamine, denoted bvPLA$_2$-Q5, was prepared from the same expression plasmid used for bvPLA$_2$ using the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's directions. The triple-site mutation R23Q/K85Q/K133Q was introduced first in one step, the entire bvPLA$_2$ coding region was confirmed by DNA sequencing, the double-site mutation K14Q/K94Q was then introduced in one step, and the entire bvPLA$_2$ coding region was resequenced to verify that no additional mutations were introduced.

The plasmid encoding bvPLA$_2$-Q5 was used to transform *E. coli* M15/pRep4 for production of protein as described previously (*22*) except that the growth medium was 2×YT. Fusion protein was isolated from a 4 L bacterial culture and submitted to sulfonation and in vitro refolding as described (*22*). After refolding, precipitated protein was removed by centrifugation, and one-third of the protein solution was loaded directly onto a C4 reverse-phase column (Vydac 214TP1010) equilibrated with water with 0.06% trifluoroacetic acid (rather than dialyzed against SP buffer, (*22*)). The column was developed with a gradient of 0−100% acetonitrile/0.06% trifluoroacetic acid over 40 min at a flow rate of 2 mL/min. Several protein peaks were observed, and the desired peak (retention time ~21 min) was located using the fluorimetric sPLA$_2$ assay with 1-palmitoyl-2-pyrenedecanoyl phosphatidylmethanol (*22*). The desired fractions from three HPLC runs were combined, and water was added to bring the acetonitrile concentration to 10%. The solution was pumped directly onto a C18 reverse-phase column (Vydac 218TP1010) equilibrated with water with 0.06% trifluoroacetic acid at a flow rate of 2 mL/min. The column was developed with a gradient of 0−100% acetonitrile/0.06% trifluoroacetic acid over 40 min at a flow rate of 2 mL/min. The desired protein-containing fraction (located with the fluorimetric assay) eluted at ~21 min. The ~5 mL sample was concentrated to 0.5 mL in a Speed-Vac centrifugal vacuum concentrator (Savant Instruments) at room temper-

ature. A trace amount of solid, if present, was removed by centrifugation. SDS−PAGE analysis of bvPLA2-Q5 indicated that it was ~80% pure. The specific activity was estimated to be 120% of that of the wild-type enzyme based on the fluorimetric assay. The yield of bvPLA2-Q5 is ~50 μg per 4 L of bacterial culture, considerably lower than that for wild-type enzyme (*22*).

All $bvPLA_2$ proteins used in this study contain the N-terminal fusion peptide, RGSHHHHHHSPFR, fused to Ile-1, which is the N-terminus of native $bvPLA_2$. Although this fusion peptide has a kallikrein protease recognition site, SPFR, the fusion peptide was not removed because it was shown earlier that the specific activity of $bvPLA_2$ measured with the fluorimetric assay (described above) does not change after removal of the fusion peptide (*21*, *22*).

Interfacial Binding Studies. $DO_{et}PS$ was prepared as described (*10*). DOPC, DOPS, $DO_{et}PC$, cholesterol (CH), and egg sphingomyelin (SM) are from Avanti Polar Lipids Inc. The concentration of phospholipids in chloroform stock solutions was determined by inorganic phosphate assay after degradation with perchloric acid using the standard procedure. Interfacial binding studies using sucrose-loaded, 0.1 μm liposomes were carried out as described (*10*). For studies in the presence of calcium, vesicles were extruded in 5 mM MOPS, pH 7.4, 176 mM sucrose, and the binding buffer was 5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM $CaCl_2$, 0.5 mg/mL bovine serum albumin. Studies at pH 6.0 were carried out with BisTris replacing MOPS at pH 7.0 and at pH 8.0 with HEPES replacing MOPS. For studies in the absence of calcium, vesicles were extruded in 5 mM MOPS, pH 7.4, 2 mM EGTA, 176 mM sucrose, and the binding buffer was 5 mM MOPS, pH 7.4, 0.1 M KCl, 2 mM EGTA, 0.5 mg/mL bovine serum albumin. Binding reactions, 100 μL, contained 10−500 ng of $bvPLA_2$, depending on the total lipid concentration (given in the figures) so that the lipid-to-protein ratio was at least 200 (to avoid crowding of enzyme on vesicles). The amount of $bvPLA_2$ in the supernatant after vesicles were pelleted in the ultracentrifuge was measured using the fluorimetric assay with 1-palmitoyl-2-pyrenedecanoyl phosphatidylmethanol (*10*, *22*). Studies with radiolabeled CH and SM showed that these lipids and phospholipids present in the covesicles are recovered with similar yield after extrusion (*10*).

The percent of $bvPLA_2$ remaining in the supernatant, based on enzymatic activity (100% is the amount of $bvPLA_2$ activity in the supernatant of a sample processed as above but in the absence of vesicles), was plotted versus the total phospholipid present in the binding reaction. The curve was fit to the standard equation for equilibrium dissociation: $100\text{-}([E]/[E]_T) = K_d/([L]_T + K_d)$, where [E] is the concentration of $bvPLA_2$ in the aqueous phase, $[E]_T$ is the concentration of total enzyme (free and vesicle bound) in the binding reaction, $[L]_T$ is the total phospholipid concentration in the binding reaction (expressed as total moles of phospholipid, PS + PC, divided by the volume of binding reaction sample), and K_d is the equilibrium constant for the dissociation of vesicle-bound enzyme into the aqueous layer. The above equation applies to the present case where the total phospholipid concentration is in large excess over the total enzyme concentration so that depletion of vesicle binding sites due to enzyme binding can be ignored. The recovery of all $bvPLA_2$'s used in the study from centrifuged samples that lacked vesicles was >80%. Thus, no correction was needed for loss of enzyme to the walls of the polyallomar tubes.

Electrostatic Calculations. The electrostatic free energy component of the membrane interaction of $bvPLA_2$, $bvPLA_2$-Q5, $bvPLA_2$-E5, and various protonated forms of $bvPLA_2$-E5 was obtained from a modified version of the DelPhi program (*23*) that solves the nonlinear Poisson−Boltzmann equation for a protein/membrane system (*24*). DelPhi produces finite difference solutions to the Poisson−Boltzmann equation (the FDPB method) for a system where the solvent is described in terms of a bulk dielectric constant and concentrations of mobile ions, while solutes (here, enzyme and phospholipid membranes) are described in terms of the coordinates of the individual atoms, as well as their atomic radii and partial charges. The FDPB method has previously been shown to yield excellent agreement with experimental measurements of the binding of peptides and proteins to charged membranes (e.g., refs *24*−*28*).

In the calculations described in this work, the proteins and phospholipid bilayers are represented in atomic detail, while the solvent is modeled as a homogeneous dielectric medium. Each atom of a protein/bilayer system is assigned a radius and partial charge that is located at its nucleus. The protein/membrane model is then mapped onto a three-dimensional lattice of l^3 points, each of which represents a small region of the protein, membrane, or solvent. The charges and radii used for the amino acids were taken from the CHARMM22 parameter set (*29*), and those used for the lipids are the ones described by Peitzsch et al. (*30*) and used in previous studies (*24*, *26*). Regions inside the molecular surfaces of the protein and membrane are assigned a dielectric constant of 2 to account for electronic polarizability, and those outside are assigned a dielectric constant of 80 (*31*). An ion exclusion layer is added to the solutes and extends 2 Å beyond the molecular surfaces (*24*). The nonlinear Poisson−Boltzmann equation is solved in the finite difference approximation, and the numerical calculation of the potential is iterated to convergence, which is defined as the point at which the potential changes less than 10^{-4} kT/e between successive iterations. Electrostatic free energies are obtained from the calculated potentials (*32*), and the electrostatic free energy of interaction is determined as the difference between the electrostatic free energy of a protein in a specific orientation with respect to the membrane surface, $G_{el}(P\cdot M)$ and the electrostatic free energies of the protein, $G_{el}(P)$, and membrane, $G_{el}(M)$, infinitely far apart, that is, taken separately:

$$\Delta G_{el} = G_{el}(P\cdot M) - [G_{el}(P) + G_{el}(M)] \qquad (1)$$

Misra and Honig (*33*) used solutions to the nonlinear Poisson−Boltzmann equation from the FDPB method to calculate the pK_a shift of a titratable group on an antibiotic upon binding to DNA. Their results are in excellent agreement with experimental observations. We applied this approach to calculate the pK_a shift of each of the glutamic acid substitutions in $bvPLA_2$-E5 upon interaction of the protein with a membrane surface:

$$\Delta pK_a = 1/(2.3kT)[\Delta G_{el}^{-} - \Delta G_{el}^{\circ}] \qquad (2)$$

where ΔG_{el}^{-} and ΔG_{el}° are calculated as described for eq 1 and ΔG_{el}^{-} represents the electrostatic free energy of membrane interaction of bvPLA$_2$-E5 with all five glutamates unprotonated while ΔG_{el}° represents the electrostatic free energy of membrane interaction of bvPLA$_2$-E5 with one of the glutamic acids (E14, E23, E85, E94, or E133) in its protonated form.

In all FDPB calculations, a sequence of focusing runs (*34*) of increasing resolution was employed to calculate the electrostatic potentials (e.g., 0.5, 1.0, 2.0, and 4.0 grids/Å). In the initial calculation, the protein/membrane model encompasses a small percentage of the lattice (~20%) and the potentials at the boundary points of the lattice are approximately zero; this procedure ensures that the system is electroneutral. Lattice sizes of 289^3 and 385^3 were used, and the calculations were performed to final resolutions of 3 and 4 grids/Å, respectively. The precision in the electrostatic free energies of interaction, determined as the difference between the results obtained at the two highest resolution scales, is ≤0.3 kcal/mol for all calculations. The precision in the calculated pK_a shifts is ≤0.2 units. The pK_a's of each of the five glutamic acids on the isolated protein, that is, in solution and far from the membrane, were calculated using the multiconformation continuum electrostatics (MCCE) method (*35*); we obtained values of 4.4, 4.3, 4.2, 5.4, and 4.1 for E14, E23, E85, E94 and E133, respectively. Only E94 was significantly shifted from its intrinsic value; this may be because this residue is less solvent exposed than the other glutamates (see Figure 5A).

Phospholipid bilayers were built as described previously (*24*, *30*). In this study, we considered membranes with the following compositions: 0:1, 3:1, 2:1, 5:1, 8;1 and 1:0 PC/PS. bvPLA$_2$ was represented by the coordinates of its crystal structure (*36*, PDB identifier 1POC). Hydrogen atoms were added to the heavy atoms, and the mutant forms of the protein were constructed with the program CHARMM (*29*). We assumed that all forms of the enzyme are oriented at the surface of a membrane, regardless of composition, as suggested by EPR spectroscopy measurements of wild-type bvPLA$_2$, site-selectively spin-labeled, at the surface of phosphatidylmethanol membranes (*8*) (see below). The calcium ion bound in the active site was included in the calculations in correspondence with the experiments with which we were comparing our predictions. Furthermore, in our calculations, we assumed that the bilayer lipids change neither their structure nor their position upon interaction with the protein. These approximations are not unreasonable for bvPLA$_2$, which does not penetrate the membrane interface to a large degree (*8*).

RESULTS

Approach To Study Interfacial Binding of bvPLA$_2$'s. Interfacial binding of bvPLA$_2$ to 0.1 μm unilamellar vesicles, prepared by extrusion, of varying phospholipid composition was quantified by sedimenting vesicles and measuring the fraction of enzyme remaining in the supernatant. Vesicles are loaded with sucrose so that they can be sedimented in an ultracentrifuge (*10*, *37*). Sucrose-loaded vesicles are not under osmotic stress because of the presence of 0.1 M KCl in the binding buffer outside of the vesicles (*37*). Also, the addition of 0.1 M KCl to the binding buffer brings the ionic strength to a value close to the physiological one.

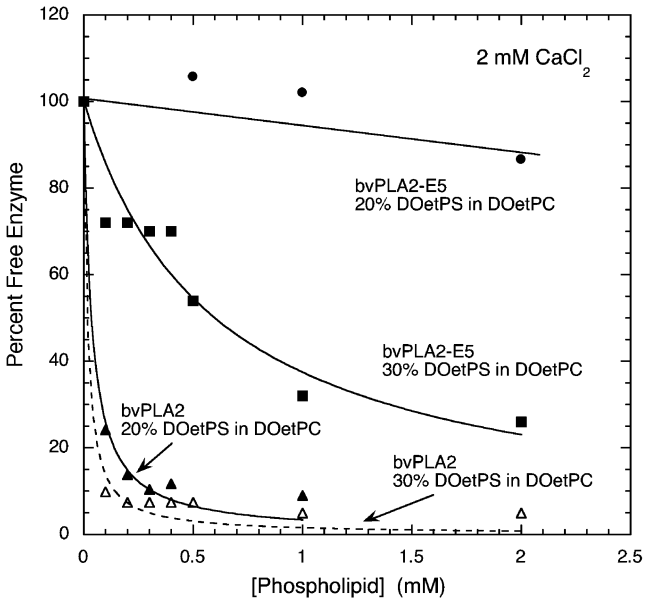

FIGURE 2: Binding of bvPLA$_2$ and bvPLA2-E5 to DO$_{et}$PS/DO$_{et}$PC vesicles. Binding buffer at pH 7.4 contained 2 mM $CaCl_2$ (see Experimental Procedures for additional details). The solid lines are the fit of the data to the binding equation given in Experimental Procedures.

It is important to use a sufficiently high lipid-to-protein ratio to ensure that enzyme is not crowded on vesicles. Otherwise interactions among proteins bound to the vesicle may complicate the meaning of K_d, and the amount of enzyme in the aqueous phase may be an overestimate due to insufficient vesicle surface available for enzyme binding. Since the binding of a typical sPLA$_2$ to vesicles prevents about 40−50 phospholipids from interacting with other sPLA$_2$'s bound on the same vesicles (*6*) and because ~50% of the total phospholipid is on the inner vesicle monolayer and not accessible to enzyme, a lipid-to-protein ratio of at least ~200 is required to prevent crowding of enzyme on vesicles. In the present study, we used total lipid concentrations in binding mixtures down to 1 μM. In this case, the maximum amount of bvPLA$_2$ was 10 ng to give a lipid-to-protein ratio of 200. It was found that inclusion of 0.5 mg/mL bovine serum albumin in the binding buffer was necessary to prevent absorption of bvPLA$_2$ to the walls of the polyallomar centrifuge tubes.

Binding of Wild-Type and Mutant bvPLA$_2$'s to Vesicles. Figure 2 shows initial studies of the binding of bvPLA$_2$ and bvPLA$_2$-E5 to DO$_{et}$PS/DO$_{et}$PC vesicles. In these experiments, saturating calcium is present in the binding buffer, and thus it is necessary to use diether phospholipid vesicles to prevent phospholipid hydrolysis by vesicle-bound enzyme. The binding of bvPLA$_2$ to vesicles containing 20 or 30 mol % DO$_{et}$PS in DO$_{et}$PC is too tight to determine the K_d accurately (K_d < 0.05 mM). Additional studies using lower lipid concentrations were carried out as described below. For bvPLA$_2$-E5, very little binding to vesicles containing 20 mol % DO$_{et}$PS in DO$_{et}$PC is seen with phospholipid concentrations up to 1 mM (K_d > 1 mM), and binding increases significantly when the amount of anionic phospholipid is increased to 30 mol % ($K_d = 0.3 \pm 0.04$ mM). Note, values of K_d are expressed in terms of the concentration of lipid in the outer monolayer of vesicles, taken as half of the total lipid concentration. Given that the interfacial binding surface

of bvPLA$_2$-E5 has a high anionic character (due to the presence of five glutamates and only a single cationic residue, R58), it is remarkable that this mutant shows enhanced interfacial binding as the amount of anionic DO$_{et}$PS in DO$_{et}$PC vesicles is increased. These results are consistent with our earlier studies showing essentially irreversible binding of bvPLA$_2$-E5 to vesicles composed of 100 mol % of the anionic phospholipid dimyristoyl phosphatidylmethanol (*21*). These initial studies show that the bvPLA$_2$ binds at least 20-fold more tightly than bvPLA$_2$-E5 to these PS/PC vesicles and that both proteins show enhanced binding to vesicles as the mole percent of PS is increased.

To measure accurate values for K_d for bvPLA$_2$, we used 25% DO$_{et}$PS in DO$_{et}$PC vesicles down to a lipid concentration of 1 μM. We also determined values of K_d at pH 6.0, 7.0, and 8.0, all in the presence of 2 mM CaCl$_2$. Binding curves for bvPLA$_2$, bvPLA$_2$-E5, and bvPLA$_2$-Q5 are shown in Figure 3, panels A, B, and C, respectively, and values of K_d are listed in Table 1. bvPLA$_2$ showed a 2-fold increase in affinity for vesicles as the pH was lowered from 8.0 to 7.0 and a 4-fold increase in affinity when the pH was lowered from 7.0 to 6.0. In contrast, the membrane association of bvPLA2-E5 was significantly affected by pH. This protein did not bind to vesicles at pH 8.0 up to the maximum lipid concentration tested (2.5 mM), and binding increased 30-fold when the pH was decreased from 7.0 to 6.0. At pH 7.0, bvPLA$_2$ bound 3400-fold more tightly than bvPLA$_2$-E5 to the 25% DO$_{et}$PS/75% DO$_{et}$PC vesicles, and at pH 6.0, the difference in affinity is reduced to 520-fold. bvPLA$_2$-Q5 showed a pH dependence of vesicle binding comparable to that for wild-type enzyme; interfacial binding affinity increased 2-fold in going from pH 8.0 to 7.0 and 1.5-fold increase in going from pH 7.0 to 6.0. At pH 8.0, bvPLA$_2$ binds 4-fold more tightly to vesicles than does bvPLA$_2$-Q5, at pH 7.0 the difference is 3-fold, and at pH 6.0 the difference is 10-fold.

We studied the binding of bvPLA$_2$ and bvPLA$_2$-Q5 to PC vesicles containing various amounts of PS. Studies were carried out at a constant pH of 7.4. Binding was studied in the presence of CaCl$_2$ using diether phospholipids or in the absence of calcium (2 mM EGTA) using diester phospholipids. Since sucrose-loaded pure DO$_{et}$PC vesicles do not sediment well (presumably due to leakage of sucrose), we included 30% CH and 30% SM in the vesicles. Additionally, these levels are similar to those found in mammalian cell membranes, and previous studies have shown that sucrose-loaded DO$_{et}$PC/CH/SM vesicles readily pellet after ultracentrifugation (*10*). In the presence of CaCl$_2$, vesicles contained 0, 5, or 10 mol % DO$_{et}$PS (Table 2). Since bvPLA$_2$'s bound less tightly to vesicles in the absence of calcium, we used 10, 20, and 30 mol % DOPS in DOPC/CH/SM vesicles for experiments in the absence of calcium (Table 2). Representative binding curves are shown in Figure 4A,B, and all of the K_d values are listed in Table 2. bvPLA$_2$ binds 70-fold more tightly to diether phospholipid vesicles containing 10% PS (10% DO$_{et}$PS/30% DO$_{et}$PC/30% CH/30% SM) in the presence of saturating CaCl$_2$ than in the absence of CaCl$_2$, whereas bvPLA$_2$-Q5 shows a 30-fold increase in vesicle affinity in the presence of CaCl$_2$. The phospholipid ester carbonyl groups are not important for bvPLA$_2$ binding; in the absence of CaCl$_2$, bvPLA$_2$ and bvPLA$_2$-Q5 bind to diether vesicles with 10% PS only 1.5-fold more tightly than to diester vesicles containing 10% PS (Table 2). In the presence of CaCl$_2$, bvPLA$_2$ shows a 15-fold increase in affinity to diether phospholipid vesicles as the mole percent PS is increased from 0 to 10%, whereas bvPLA$_2$-Q5 shows a 10-fold increase. In the absence of CaCl$_2$, bvPLA$_2$ shows a 130-fold increase in affinity to diester phospholipid vesicles as the mole percent PS is increased from 10 to 30%, whereas bvPLA$_2$-Q5 shows a 40-fold increase.

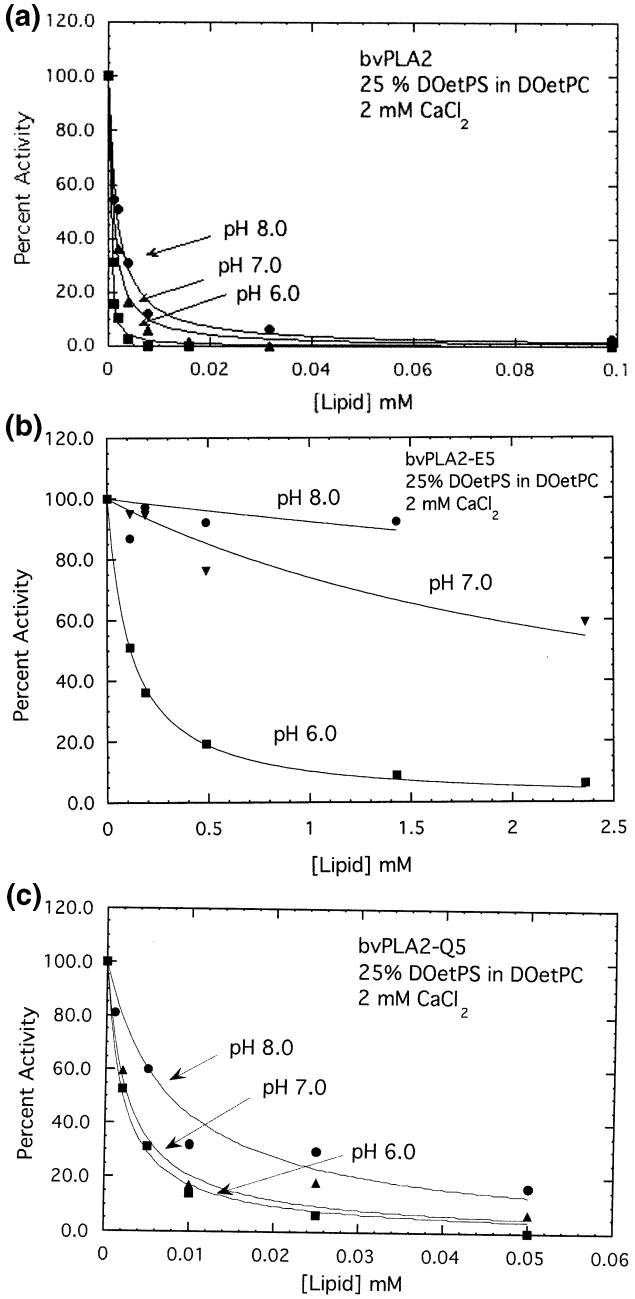

FIGURE 3: Binding of bvPLA$_2$'s to 25% DO$_{et}$PS/75% DO$_{et}$PC vesicles versus pH: (A) bvPLA$_2$; (B) bv-PLA$_2$-E5; (C) bvPLA$_2$-Q5. Binding buffer contained 2 mM CaCl$_2$ (see Experimental Procedures for additional details). The solid lines are the fit of the data to the binding equation given in Experimental Procedures.

Glutamates on the Interfacial Binding Site of bvPLA$_2$-E5 Are Predicted To Be Protonated upon Association of the Enzyme with PS-Containing Vesicles. In the calculations presented here, bvPLA$_2$ was docked at the membrane surface as suggested by EPR spectroscopy measurements (*8*). In this

Table 1: Binding of bvPLA2 to Vesicles—pH Dependence[a]

vesicle composition	pH	[Ca^{2+}] (mM)	K_d (μM) bvPLA$_2$	K_d (μM) bvPLA$_2$-E5	K_d (μM) bvPLA$_2$-Q5
25% $DO_{et}PS$ 75% $DO_{et}PC$	8.0	2	0.8 ± 0.1 0.9 ± 0.1	*b*	3.8 ± 1 3.8 ± 1
25% $DO_{et}PS$ 75% $DO_{et}PC$	7.0	2	0.46 ± 0.05 0.49 ± 0.05	1500 ± 300 1700 ± 400	1.4 ± 0.2 1.8 ± 0.3
25% $DO_{et}PS$ 75% $DO_{et}PC$	6.0	2	0.10 ± 0.01 0.11 ± 0.02	50 ± 5 60 ± 2	1.0 ± 0.1 1.1 ± 0.1

[a] Shown are the estimated errors from the nonlinear regression fit of the data to the binding equation. Two independent binding analyses were carried out per condition, and values of K_d for each are listed. [b] No binding up to 2.5 mM phospholipid.

Table 2: Binding of bvPLA2 to Vesicles at pH 7.4—PS and Calcium Dependence[a]

vesicle composition	[Ca^{2+}] (mM)	K_d (μM) bvPLA$_2$	K_d (μM) bvPLA$_2$-Q5
0% $DO_{et}PS$ 40% $DO_{et}PC$ 30% CH 30% SM	2	14 ± 2 14 ± 1	60 ± 7 50 ± 4
5% $DO_{et}PS$ 35% $DO_{et}PC$ 30% CH 30% SM	2	8 ± 2 8 ± 2	12 ± 2 17 ± 3
10% $DO_{et}PS$ 30% $DO_{et}PC$ 30% CH 30% SM	2	0.9 ± 0.2 1.2 ± 0.2	5 ± 0.3 7 ± 0.1
10% $DO_{et}PS$ 30% $DO_{et}PC$ 30% CH 30% SM	0	79 ± 8 55 ± 10	190 ± 20 190 ± 20
10% DOPS 30% DOPC 30% CH 30% SM	0	110 ± 10 130 ± 20	270 ± 50 280 ± 50
20% DOPS 20% DOPC 30% CH 30% SM	0	5 ± 1 6 ± 1	21 ± 5 23 ± 5
30% DOPS 10% DOPC 30% CH 30% SM	0	0.8 ± 0.1 1.0 ± 0.1	8 ± 2 7 ± 1

[a] Shown are the estimated errors from the nonlinear regression fit of the data to the binding equation. Two independent binding analyses were carried out per condition, and values of K_d for each are listed.

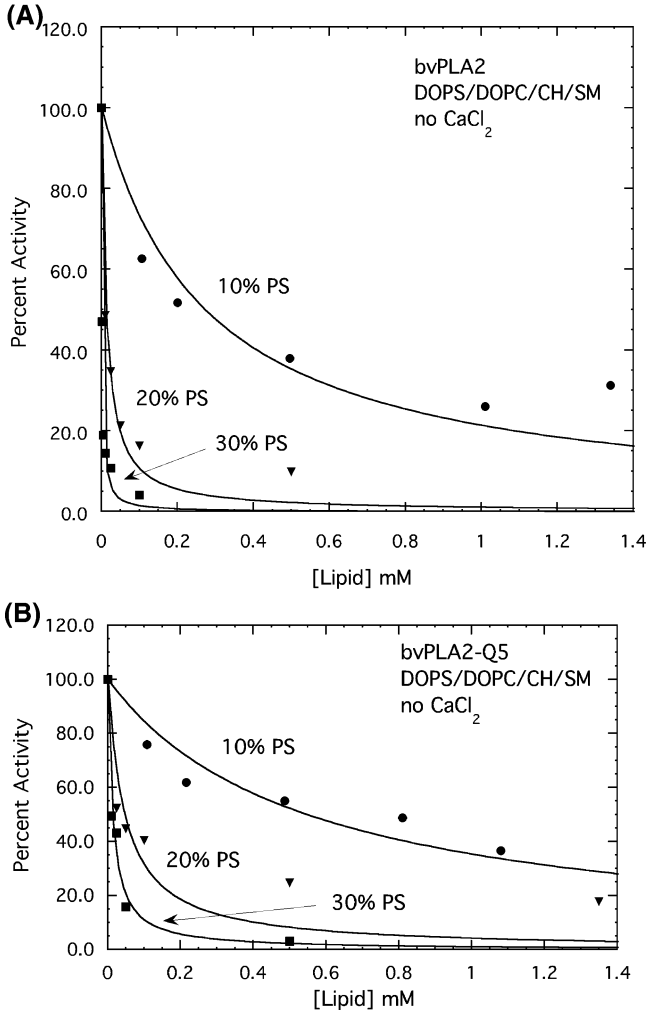

FIGURE 4: Binding of bvPLA$_2$'s to DOPS/DOPC/CH/SM vesicles versus the amount of DOPS: (A) bvPLA$_2$; (B) bv-PLA$_2$-Q5. Binding buffer at pH 7.4 lacked $CaCl_2$ (see Experimental Procedures for additional details). The solid lines are the fit of the data to the binding equation given in Experimental Procedures.

orientation (Figure 5A), basic and hydrophobic residues on the interfacial binding site (Figure 1) can interact with the membrane surface, and the active site is well positioned to bind a lipid substrate. In our treatment, we considered changes only in the electrostatic component of the membrane interaction. Hence, we calculated the relative binding, rather than the absolute binding, of bvPLA$_2$ to membranes. Previous work has shown that consideration of a single orientation of a protein with respect to the membrane is sufficient for predicting how membrane association changes as a function of various properties of the system, such as the mole percent acidic lipid in the membrane and mutations of amino acids on the protein (*24*−*26*). In the calculations presented here, the PS lipids have a net charge of −1 and the PC lipids have a net charge of 0; we do not consider changes in the protonation states of groups on the lipid heads. We assume that bvPLA$_2$ and the bvPLA$_2$-E5 and bvPLA$_2$-Q5 mutants adopt the same orientation with respect to the membrane surface and that the mutations do not significantly alter the conformation of the enzyme. These assumptions are consistent with the fact that there are determinants in addition to electrostatic interactions that mediate the membrane association. For example, as shown in Figure 1 and Figure 5A, the surface of the protein surrounding the active site and predicted by the EPR study to mediate membrane association is rich in hydrophobic residues. We further assume that the nonpolar contribution to membrane association is the same in all cases and that the only residues that may change ionization state upon membrane association are the five glutamic acid mutations in bvPLA$_2$-E5 (E14, E23, E85, E94, and E113). In order not to bias the results, no attempt was made to optimize the calculated pK_a shifts (eq 2) by altering the orientation of the protein from that suggested by the EPR study. However, calculations based on other orientations of the enzyme with respect to the membrane surface, that is, orientations different from that suggested by the EPR data (data not shown), support the conclusions reported here: that at least one site (E14) and possibly two (E14 and E23) on bvPLA$_2$-E5 are protonated

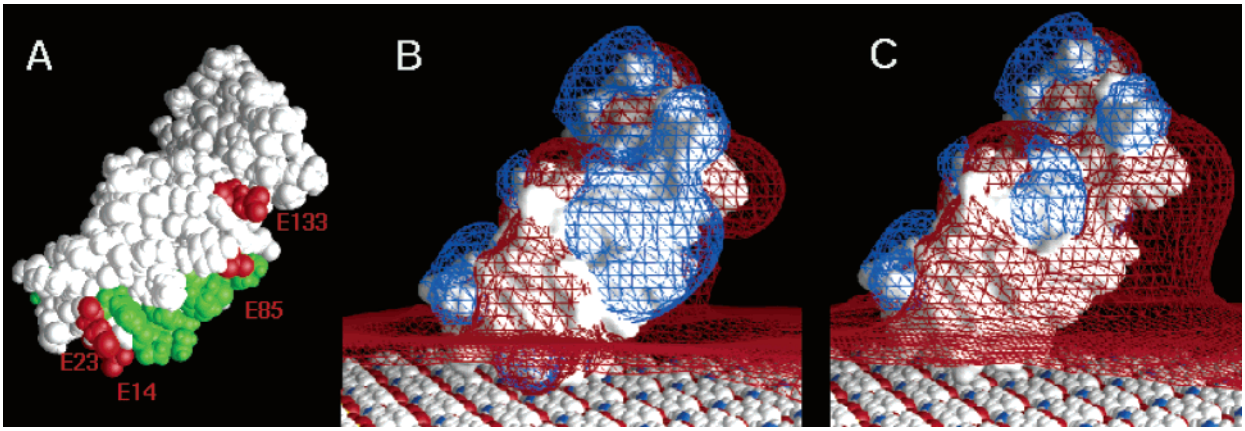

FIGURE 5: Models and electrostatic properties of membrane-adsorbed bvPLA$_2$'s. The proteins in all three panels are in the same orientation. Panel A presents a space-filling model for bv-PLA$_2$-E5. In our calculations, the membrane is located beneath the protein. The residues mutated to glutamates are colored red and labeled with the exception of E94, which is located on the back side of the protein. Hydrophobic residues on the interfacial binding site are colored green. Panels B and C present models for the membrane-associated forms of wild-type bvPLA$_2$ (B) and bvPLA$_2$-E5 (C) used in the FDPB calculations. The proteins are represented by their molecular surfaces (white). The membrane composition is 2:1 PC/PS; oxygen atoms are colored red and nitrogen atoms are colored blue. Red (blue) meshes represent the −1 kT/e (+1 kT/e) equipotential contours as calculated by the FDPB method (see Experimental Procedures) for 0.1 M KCl and visualized in GRASP (*48*).

Table 3: Calculated Dependence of the pK_a Shift, ΔpK_a, of E14 on bvPLA$_2$-E5 on the Mole Percent of PS in Vesicles[a]

% PS	ΔpK_a	pK_a(membr)	ΔG_{el}(E5/E14p) − ΔG_{el}(E5), kcal/mol
0	1.0	5.4	−1.4
17	1.3	5.7	−1.8
25	1.8	6.2	−2.4
33	2.1	6.5	−2.9
100	2.5	6.9	−3.5

[a] ΔpK_a was calculated with eq 2, as described in Experimental Procedures. The rest of the glutamic acids were assumed to be unprotonated. [KCl] = 0.1 M, and a calcium ion is bound in the active site of the enzyme. pK_a(membr) is the sum of ΔpK_a and the pK_a of E14 when the protein is in solution, far from the membrane. ΔG_{el}(E5/E14p) − ΔG_{el}(E5) is the predicted difference between the electrostatic free energies of the membrane interaction of bvPLA$_2$-E5 with E14 protonated and bvPLA$_2$-E5 with no glutamates protonated.

when the membrane contains a sufficient fraction of acidic phospholipid (see below).

Our calculations with bvPLA$_2$-E5 in the orientation depicted in Figure 5 predict that E14, which is the glutamate closest to the membrane surface, is the only glutamate that experiences a large shift in pK_a when the protein associates with the membrane. For the enzyme in solution far from the membrane, the calculated pK_a of E14 is 4.4. As determined by eq 2 (Experimental Procedures), the pK_a shift at the surface of pure PS membranes is predicted to be 2.5, so the pK_a of this residue when bvPLA$_2$-E5 is membrane-bound, as depicted in Figure 5, is 6.9 (Table 3, row 5). Hence, at pH 6, E14 is likely to change from a mostly deprotonated form to a mostly protonated form upon association with PS vesicles. E23 is predicted to experience a smaller pK_a shift upon binding to PS membranes (1.0 unit), while the other glutamic acids (E85, E94, and E113), which are located much farther from the membrane surface for this orientation of the enzyme (Figure 5A), are predicted to experience insignificant shifts from their solution pK_a values (Table 3).

Other FDPB calculations (not shown) predict that the electrostatic interaction of bvPLA$_2$ with pure PS vesicles is 5 kcal/mol more favorable than the interaction of bvPLA$_2$-E5 when all five glutamates are fully deprotonated for enzyme in solution and membrane bound, but only 1.5 kcal/mol more favorable than the interaction of bvPLA$_2$-E5 with E14 fully protonated. With both E14 and E23 fully protonated, the electrostatic interaction of bvPLA$_2$-E5 with pure PS membranes is predicted to be the same as for bvPLA$_2$.

When bvPLA$_2$ is docked at the membrane surface as suggested by the EPR studies and shown in Figure 5, there is significant desolvation of both the membrane contact surface of the protein and the lipids with which the protein associates. Therefore, close to the membrane surface, electrostatic attraction between basic residues on bvPLA$_2$ and acidic lipids is counterbalanced by these desolvation costs. Electrostatic charge−charge repulsion between E14 (and E23) and anionic PS is significantly decreased by protonation of the glutamates. Furthermore, desolvation of the glutamates has a lower penalty if they are protonated. The net effect is a similar electrostatic interaction predicted for the interaction of the E14/E23 diprotonated bvPLA$_2$-E5 and bvPLA$_2$ with pure PS vesicles.

As summarized in Table 3, the pK_a of E14 on bvPLA$_2$-E5 is predicted to increase significantly as a function of mole percent of PS in the membrane. Even at the surface of electrically neutral PC membranes, the pK_a of E14 is predicted to increase from its solution value of 4.4 to 5.4. This is due to the desolvation of E14 by the low dielectric membrane interface (see Figure 5A), which favors protonation. Introduction of acidic lipids into the membrane further increases the pK_a by providing a negatively charged environment: the pK_a of E14 is predicted to increase by 2.5 units to 6.9 as the mole percent acidic lipid increases from 0 to 100 (Table 3).

Comparison of the Predicted Electrostatic Component of the Membrane Interaction of Wild-Type and Mutant Forms of bvPLA$_2$. As depicted in Table 4, our computational model, which considers electrostatic interactions alone, provides, in most cases, an adequate qualitative description of the difference in the membrane association of the different forms of bvPLA$_2$ considered in this study. Our calculations predict that for protein bound to 25% PS in PC membranes, E14 is

Table 4: Comparisons of the Calculated Electrostatic Free Energies of Interaction of bvPLA$_2$, bvPLA$_2$-E5, and bvPLA$_2$-Q5 with PC/PS Vesicles[a]

pH	comparison	$\Delta\Delta G_{el}$, kcal/mol, theory	fold change in K_D, theory	fold change in K_D, experiment
7	ΔG_{el}(wt,25%) − ΔG_{el}(E5,25%)	−4.7	2800	3400
6	ΔG_{el}(wt,25%) − ΔG_{el}(E5/E14p,25%)	−2.2	40	520
6, 7	ΔG_{el}(E5/E14p,25%) − ΔG_{el}(E5,25%)	−2.4	60	30
7	ΔG_{el}(wt,25%) − ΔG_{el}(Q5,25%)	−0.7	3	3
7	ΔG_{el}(wt,10%) − ΔG_{el}(wt,0%)	−1.2	8	15
7	ΔG_{el}(Q5,33%) − ΔG_{el}(Q5,0%)	+0.5	0.4	12
7	ΔG_{el}(wt,10%) − ΔG_{el}(wt,10%,no Ca)	−0.7	3	70
7	ΔG_{el}(wt,33%) − ΔG_{el}(wt,33%,no Ca)	−1.8	22	*b*

[a] E5/E14p refers to bvPLA$_2$-E5 with E14 protonated. [KCl] = 0.1 M, and a calcium ion is bound in the active site of the enzyme except where noted. The composition of the membranes is denoted by the mole percent (%) PS given in the parentheses. ΔG_{el} is calculated using eq 1, as described in Experimental Procedures, and it is assumed that, at pH 7, E14 in bvPLA$_2$-E5 is unprotonated and that, at pH 6, E14 in bvPLA$_2$ is protonated (Table 3). $\Delta\Delta G_{el}$ corresponds to the difference in ΔG_{el}'s as depicted in the "comparison" column. [b] Not determined.

unprotonated at pH 7 and at least partially protonated at pH 6 (Table 3, row 3; pK_a 6.2). In qualitative agreement with experiment, we predict that, at pH 7, bvPLA$_2$ binds 2800-fold more strongly to these vesicles than bvPLA$_2$-E5, which is assumed to be unprotonated in the calculations (Table 4, row 1), and, at pH 6, only 40-fold more strongly than bvPLA$_2$-E5, which is assumed to be protonated at E14 (Table 4, row 2). However, at pH 6, it is observed experimentally that bvPLA$_2$ binds 520-fold more strongly than bvPLA$_2$-E5. This order of magnitude discrepancy between theory and experiment suggests that E14 of bvPLA$_2$-E5 is not fully protonated at the surface of a 25% PS membrane at pH 6, as indicated by the predicted pK_a of 6.2 (Table 3), or that there are differences in the membrane-associated orientations of bvPLA$_2$ and bvPLA$_2$-E5. However, as shown in row 3 of Table 4, the computational comparison of the electrostatic component of membrane binding of bvPLA2-E5 at (1) pH 7, where we assume E14 is unprotonated, and (2) pH 6, where we assume E14 is protonated, is in agreement with the observed 30-fold increase in membrane association of bvPLA$_2$-E5 when the pH is lowered from 7 to 6, suggesting that E14 is indeed mostly protonated at the surface of the membrane. Furthermore, our calculations correctly predict that the membrane association of bvPLA$_2$ increases as the mole percent PS in the membrane increases (Table 4, row 5). In addition, our calculations correctly predict that wild-type bvPLA$_2$ binds to 25% PS membranes slightly more strongly than bvPLA$_2$-Q5 (Table 4, row 4). However, we incorrectly predict that the membrane association of bvPLA$_2$-Q5 decreases as the mole percent PS increases, whereas the membrane association is observed to increase slightly (Table 4, row 6). Possible reasons for this discrepancy are given in the Discussion section.

The calculations allow us to examine the contribution of the active site calcium ion to the nonspecific electrostatic component of the membrane association of bvPLA$_2$ without the complication of a lipid being bound in the active site cleft in the presence of calcium, as is the case in the experiments with calcium in the buffer. We predict that calcium increases the nonspecific association of bvPLA$_2$ to 8:1 PC/PS and 2:1 PC/PS membranes by 3-fold and 20-fold, respectively (Table 4, rows 7 and 8). These values are lower than the differences observed experimentally because our calculations do not account for the interaction of a lipid with the catalytic cleft (see Discussion).

Overall, the qualitative agreement of the computational predictions with the experimental observations indicates that changes in the pK_a's of glutamic acid residues, specifically E14, significantly affect the membrane association of bvPLA$_2$-E5.

DISCUSSION

In our earlier study, we found that bvPLA$_2$ and bvPLA$_2$-E5 bound tightly to vesicles composed purely of the anionic phospholipid dimyristoyl phosphatidylmethanol (*21*). In the present study, we see that the use of PS/PC covesicles allows values of the enzyme−vesicle dissociation equilibrium constant K_d to increase into the measurable range. With this new data and the study of bvPLA$_2$-Q5 for the first time, we can summarize the interfacial binding trends as follows: (1) Electrostatic interactions between the five basic residues (K14, R23, K85, K94, and K133) on the face of the protein that includes the opening to the active site slot and acidic lipids in the membrane do not constitute a major determinant of binding. This is based on the observation that bvPLA2-Q5 binds only 3- to 10-fold more weakly than bvPLA$_2$ to PS/PC vesicles (Tables 1 and 2). This is consistent with the results from our previous EPR studies (*8*), which suggest that only two of the five basic residues are within a Debye length (~10 Å for 0.1 M KCl) of the membrane surface so that the electrostatic component to membrane association is expected to be weak. (2) Replacing the five basic residues with anionic glutamates (charge reversal) overestimates the contribution of the five basic residues to electrostatic binding to the anionic PS/PC vesicles. This is based on the fact that at pH 7, bvPLA2-E5 binds to PS/PC vesicles 3000-fold more weakly than bvPLA2 and yet mutation of basic residues to charge-neutral glutamines produces only a 3-fold reduction (Table 1). (3) The remarkable finding that the affinity of bvPLA$_2$-E5 to anionic PS/PC vesicles increases as the mole percent of anionic PS is increased is explained by the increase in the pK_a values of a subset of the introduced glutamates when these residues transfer from bulk solution to the surface of the anionic vesicles and that the increase in pK_a is larger as the amount of PS in the vesicles is increased (supported by pH-dependent interfacial binding studies (Table 1) and calculations of pK_a shifts (Table 3)). (4) The binding of calcium to the active site of bvPLA$_2$ increases the affinity of the enzyme for PS/PC vesicles by both a mass action effect (substrate binding in the active site is calcium-dependent) and an electrostatic attraction of calcium with the anionic vesicle interface. Both effects are not large. These points are discussed in more detail below.

We present a molecular model, based on numerical solutions of the nonlinear Poisson−Boltzmann equation (the FDPB method), to describe the membrane binding behavior

of bvPLA$_2$. Despite the complexity of the system that we are considering, our simple computational model is able to describe the membrane binding behavior of bvPLA$_2$, as well as the E5 mutant. As depicted by the agreement between theory and experiment, it is clear that there is a strong electrostatic effect on membrane association resulting from replacing basic residues on the interfacial binding site of bvPLA$_2$ with glutamates (Table 4, row 1). However as discussed further below, this result does not imply that the binding of bvPLA$_2$ to PS/PC vesicles is dominated by electrostatic interactions. The weaker binding of bvPLA$_2$-E5 versus bvPLA$_2$ to PS/PC is due to a combination of electrostatic repulsion of anionic glutamates by the anionic vesicle interface and of the fact that the both the anionic glutamates of bvPLA$_2$-E5 and the basic residues of bvPLA$_2$ have a high cost of desolvation that accompanies the enzyme−vesicle binding. Thus, it is clear that replacement of basic residues with anionic ones overestimates the contribution of basic residues to electrostatic binding to anionic vesicles.

Our model for protonation of glutamates on bvPLA$_2$-E5 describes the difference in membrane association between wild-type bvPLA$_2$ and bvPLA$_2$-E5 (Table 4, rows 1−3). The glutamate protonation model is strongly supported by the pH-dependence of protein binding to PS/PC vesicles (Table 1) showing that bvPLA$_2$-E5 binding to PS/PC vesicles increases much more than for bvPLA$_2$ as the pH is dropped from 8 to 6. This glutamate protonation model nicely explains the seemingly paradoxical result that the binding of the highly anionic bvPLA$_2$-E5 increases as the mole percent of anionic PS is increased in PC vesicles. Recently, Johnson et al. suggested that gutamates present on the membrane binding region of cytidylyltransferase conferred specificity for anionic membranes due to an increase in their pK_a values as the mole percent acidic lipid in the membrane increased (*38*). The calculation of pK_a shifts has previously been applied to describe pH effects on protein folding and protein/protein, protein/DNA, and DNA/ligand association (*39*).

Figure 5 illustrates how electrostatic forces may drive the protonation of glutamates on bvPLA$_2$-E5. Wild-type bvPLA$_2$ (Figure 5B) and bvPLA$_2$-E5 (Figure 5C) are shown adsorbed to the surface of a 2:1 PC/PS membrane ([KCl] = 0.1 M); these configurations are consistent with our EPR studies (*8*) and were used in our FDPB calculations. Red meshes represent the −25 mV or −1 kT/e equipotential contours, and blue meshes represent the +25 mV or +1 kT/e equipotential contours determined by solving the nonlinear Poisson−Boltzmann equation, as described in Experimental Procedures. Away from the proteins, the negative electrostatic potential contour of the membrane is flat and located about 10 Å beyond the membrane envelope (*40*). Wild-type bvPLA$_2$ experiences a favorable electrostatic interaction with the membrane as illustrated by the overall diminution of the negative potential of the membrane in the vicinity of the protein; this interaction is due mainly to K14 and R23, which are located near the membrane surface (see Figure 5A), though other basic residues on the interfacial binding site may contribute as well. The FDPB calculations are in agreement with the experimental observations that, although the binding of bvPLA2 increases as the negative charge density of the membrane increases (Table 4, row 5), the electrostatic contribution to binding is relatively small as shown qualitatively in Figure 5B. The E5 mutation introduces a large change in the net charge of the protein, from +4 to −6. The membrane adsorption of bvPLA$_2$-E5, thus, dramatically enhances the negative character of the membrane in this region, as illustrated by the extended red contour in Figure 5C. As a consequence, bvPLA$_2$-E5 experiences a strong electrostatic repulsion from the membrane surface due to the replacement of the interfacial binding site basic residues with glutamates. The placement of E14 in a highly negatively charged region as well the desolvation of the protein in the vicinity of E14 both contribute to the increased affinity of the side chain carboxylate for a proton. Protonation of this glutamic acid on the interfacial binding site decreases both the electrostatic repulsion with acidic lipids in the membrane and the desolvation penalty of the protein and, thus, results in more favorable membrane association, as indicated by the qualitative agreement of the computational results with the experimental observations (Table 4).

The effect of the active site calcium ion on interfacial binding cannot be accurately dissected from the present studies. This is because the binding of a single phospholipid molecule to the active site slot of membrane-bound sPLA$_2$'s requires calcium, as suggested by structural studies that reveal the substrate−calcium ligation (*41*) and as unequivocally demonstrated using kinetic studies (*42*, *43*). Thus, calcium-dependent substrate binding drives interfacial binding by mass action. The interfacial K_M for the dissociation of dimyristoyl phosphatidylmethanol from the bvPLA$_2$−substrate−calcium complex is about 0.4 mole fraction (*43*). If we assume that free enzyme and the enzyme−calcium complex bind to vesicles with the same value of K_d and if we assume that the interfacial K_M values for the PS and PC phospholipids used in this study are also 0.4 mole fraction, the presence of saturating calcium should increase the interfacial binding of bvPLA$_2$ by 3.5-fold (i.e., by a factor of $1 + X_S/K_M$, where X_S is the mole fraction of substrate, equal to unity). On the other hand, it is likely that the binding of bvPLA$_2$ to vesicles of anionic phospholipid is enhanced by the electrostatics of a calcium dication bound to the active site. Our FDPB calculations predict that the presence of bound calcium increases the binding of bvPLA$_2$ to 10% PS/PC vesicles and to 30% PS/PC vesicles by 3-fold and 20-fold, respectively (Table 4, rows 7 and 8). As shown in Table 2, the addition of saturating calcium increased the affinity of bvPLA2 for 10% DO$_{et}$PS/30% DO$_{et}$PC/30% CH/30% SM vesicles by 60-fold. This value differs from the predicted value of $3.5 \times 3 = 10$-fold (not surprisingly given the assumptions made), and the 6-fold discrepancy is small in energetic terms (∼1 kcal/mol).

Note also that calcium increases the interfacial affinity of bvPLA$_2$-E5 to about the same extent as for bvPLA$_2$ (Table 2). All together, the results show that protein-bound calcium enhances interfacial binding of bvPLA$_2$ to anionic vesicles by a mass action effect due to active site substrate binding and by an electrostatic effect. Both of these effects are relatively small compared to the total interfacial binding energy (see below).

It is possible that calcium acts as a bridging metal to link anionic headgroups of PS and the carboxylates of bvPLA$_2$-E5. However, this seems unlikely based on two reasons. First, McLaughlin and co-workers have shown that the affinity constant of calcium for PS in bilayers is 12 M^{-1} at an ionic

strength very similar to that of our samples (*44*). Thus, in our studies with 2 mM $CaCl_2$, the amount of PS bound to calcium in the absence of $bvPLA_2$ is only about 2% of total bilayer PS. Stronger evidence against this calcium-bridging model is the data in Table 1 showing that the interfacial binding of $bvPLA_2$-E5 to 25% $DO_{et}PS$/75% $DO_{et}PC$ vesicles in the presence of saturating calcium increases significantly as the pH is lowered from 8 to 6 (Table 1). Protonation of carboxylate side chains is expected to break any putative coordination of calcium, which should weaken interfacial binding, in contrast to what is observed.

The experimental and theoretical studies presented here show that the active site calcium and one or two lysine residues (K14 and R23) make an electrostatic contribution to the interfacial binding of $bvPLA_2$ to anionic PS/PC vesicles. It is clear however that electrostatic interactions are not the main force driving interfacial binding of $bvPLA_2$ to vesicles. This is most apparent from the observation that K_d values for $bvPLA_2$ and $bvPLA_2$-Q5 differ by 10-fold or less (Table 2). Thus, the five basic residues that lie on the face of $bvPLA_2$ that includes the opening to the active site slot cannot be the main anchoring mechanism for interfacial binding of this protein. This is also suggested by the relative positioning of these lysine residues in our EPR membrane-docked model (see Figure 5). Thus, it is concluded that the large number of hydrophobic residues near the active site slot on $bvPLA_2$ contribute significantly to interfacial binding.

In the case of $bvPLA_2$, what fraction of the total interfacial binding energy is due to electrostatics? This fraction cannot be obtained by comparing the difference in free energy of binding for $bvPLA_2$ versus $bvPLA_2$-E5 ($\Delta\Delta G°$) to the free energy for binding of $bvPLA_2$ to vesicles (as was done previously, *19*). One has to compare the difference in "intrinsic" binding free energy for $bvPLA_2$ versus $bvPLA_2$-Q5 to the intrinsic binding free energy for $bvPLA_2$ as discussed previously (*10*, *45*). The intrinsic binding free energy, $\Delta G_i°$, is the observed binding free energy minus the free energy associated with the loss of transitional and rotational entropy of $bvPLA_2$ in solution as it binds to vesicles (cratic entropy term, $\Delta G_S°$). An estimated value of this cratic term is 1.5 kcal/mol for binding of a solute in solution to a membrane interface (*46*). Thus, the intrinsic free energy for the binding of $bvPLA_2$ to 30% DOPS/10% DOPC/30% CH/30% SM vesicles is estimated to be -9.8 kca1/mol ($-1.5 + 0.59 \times \ln(10^{-6})$, Table 2), and the value for bvPLA2-Q5 is -8.5 kcal/mol. Thus, the difference in intrinsic binding free energy is estimated to be -1.3 kcal/mol, which is only 13% of the intrinsic binding free energy for $bvPLA_2$. This is consistent with the fact that only two of the basic residues on the interfacial binding surface model are in close proximity to the membrane surface, as shown by our EPR docking studies. Thus, electrostatic attraction of the five basic residues of $bvPLA_2$ with the anionic vesicle interface is not the major force driving interfacial binding.

In the case of human group IIA $sPLA_2$, we have recently shown that this highly basic protein (23 surface lysine and argine residues) binds much more tightly to anionic PS vesicles than to zwitterionic PC vesicles and that the interaction with PS vesicles results in a supramolecular aggregation involving several enzymes and vesicles (*20*). Thus high affinity of this $sPLA_2$ for anionic vesicles cannot be attributed to the electrostatic interaction of only a few lysine or arginine residues on the membrane binding surface with anionic vesicles; rather the combined effect of many basic residues distributed over the surface of the protein contributes to membrane association through electrostatic interactions. In an earlier study of the interfacial binding of venom $sPLA_2$ (*Agkistrodon piscivorus piscivorus*, D49 isoform) to anionic vesicles of polymerized phosphatidylglycerol, it was suggested that K7 and K10 on the interfacial binding site of this $sPLA_2$, together, account for nearly half of the total interfacial binding energy (*19*). Mutation of these two basic residues to glutamates (charge reversal) led to a 500-fold increase in the value of K_d. Based on the present study with $bvPLA_2$, it seems reasonable to suggest that this charge reversal mutagenesis overestimates the electrostatic contribution of the two basic residues to interfacial binding.

Gadd and Biltonen studied the binding of the same venom $sPLA_2$ to vesicles of dipalmitoyl PC containing various amounts of dipalmitoyl phosphatidylglycerol (*47*). A value of $K_d = 5\ \mu M$ was obtained for pure anionic vesicles, and a value of $K_d = 2800\ \mu M$ was obtained for pure zwitterionic vesicles (by extrapolation of data obtained from vesicles with 0.05−1 mole fraction phosphatidylglycerol). A value of $\Delta\Delta G° = 3.6$ kcal/mol is obtained for the difference in PC and phosphatidylglycerol interfacial binding energies. Since $\Delta G_i°$ for binding of the venom $sPLA_2$ to PC vesicles is larger than $\Delta G° = 3.5$ kcal/mol (calculated from $K_d = 2800\ \mu M$), again due to the positive value of $\Delta G_S°$, these authors correctly state, "thus the interaction to the zwitterionic surface is stronger than the enhancement achieved by replacing the them with anionic lipids. Although the binding does have an electrostatic component, another thermodynamic component, likely of a hydrophobic or van der Waals type, must be playing a major role in the interaction." These results are thus seen to be consistent with those of the present study indicating that electrostatics is not the major force driving interfacial binding of at least some $sPLA_2$'s.

We also examined the membrane association of $bvPLA_2$-Q5. The FDPB calculations correctly predict that $bvPLA_2$ binds to 3:1 PC/PS slightly more strongly than $bvPLA_2$-Q5 (Table 4, row 4). Interestingly, decreasing the net charge of the protein by 10 (as for $bvPLA_2$-E5) decreases the observed membrane association by 3 orders of magnitude, whereas decreasing the net charge by 5 (as for $bvPLA_2$-Q5) decreases the membrane association by only 3-fold. This exemplifies the complex interplay among charge/charge interactions and macromolecular desolvation in protein/membrane interactions. However, our FDPB calculations incorrectly predict that the membrane association of $bvPLA_2$-Q5 decreases as the mole percent PS increases, whereas the membrane association is observed to increase (Table 4, row 6). These results suggest that factors other than electrostatic interactions, for example, changes in the depth of penetration into the membrane interface or a shift in the membrane-associated orientation, may affect membrane binding as the composition of the membrane changes. In addition, interactions that depend on structural detail, such as hydrogen bonding between groups on the enzyme and lipid heads, are not accounted for in our computational model and may also play a role in stabilizing the membrane-associated state. For example, hydrogen bonding between the amide group of glutamine and the carboxyl group of PS may explain the increased membrane binding of $bvPLA_2$-Q5 as the mole

percent PS is increased. The polar but neutral glutamines are expected to experience a lower desolvation penalty at the membrane interface than either lysine/arginine or glutamate. The combined effect of hydrogen bonding, lower desolvation, and, perhaps, the consequently more efficient membrane penetration may combine to produce the relatively strong binding of bvPLA$_2$-Q5 to PC/PS vesicles.

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