Investigating the Interfacial Binding of Bacterial Phosphatidylinositol-Specific Phospholipase C†

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ABSTRACT: The interactions of PI-PLC with nonsubstrate zwitterionic [phosphatidylcholine (PC)] and anionic [phosphatidylmethanol (PMe), phosphatidylserine, phosphatidylglycerol, and phosphatidic acid] interfaces that affect the catalytic activity of PI-PLC have been examined. PI-PLC binding is strongly coupled to vesicle curvature and is tighter at acidic pH for all of the phospholipids examined. PI-PLC binds to small unilamellar vesicles (SUVs) of anionic lipids with much higher affinity (K_d is 0.01−0.07 μM for a site consisting of $n = 100 \pm 25$ lipids when analyzed with a Langmuir adsorption isotherm) than to zwitterionic PC SUVs (K_d is 5−20 μM and $n = 8 \pm 3$). The binding to PC surfaces is dominated by hydrophobic interactions, while binding to anionic surfaces is dominated by electrostatic interactions. The contributions of specific cationic side chains and hydrophobic groups at the rim of the $\alpha\beta$-barrel to zwitterionic and anionic vesicle binding have been assessed with mutagenesis. The results are used to explain how PC activates the enzyme for both phosphotransferase and cyclic phosphodiesterase activities.

Phospholipases are peripheral membrane proteins that catalyze the hydrolysis of phospholipids, generating several products critical in signal transduction of mammalian cells (*1*). Interfacial binding of these enzymes is an important aspect of their function since their substrates are organized in a two-dimensional matrix. Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC)[1] is a relatively simple phospholipase with specificity for phosphatidylinositol (PI). PI-PLC follows a general acid/general base mechanism where PI is first converted to diacylglycerol and *myo*-inositol 1,2-(cyclic) phosphate (cIP) by PI-PLC (phosphotransferase activity), followed by hydrolysis of cIP to *myo*-inositol 1-phosphate (I-1-P) (cyclic phosphodiesterase activity). The *Bacillus cereus* PI-PLC structure is a distorted $\alpha\beta$-barrel, with a solvent-accessible active site (*2*). There are two triads critical for catalysis: H32−D274−inositol 2-hydroxyl group as the general base complex (*3*, *4*) and H82−D33−R69 as the general acid complex (*5*). The top of the rim has several hydrophobic residues that are fully exposed to solvent in the crystal structure (*2*). It is thought that these residues might mediate binding of the enzyme to interfaces.

The sequence of PI-PLC from *Bacillus thuringiensis* is nearly identical to the enzyme from *B. cereus*. This phospholipase, like other phospholipases, exhibits the property of interfacial activation, where enhanced activity is observed when the substrate PI is present in an interface compared to monomeric substrate (*6*, *7*). However, it was observed that other nonsubstrate lipids such as phosphatidylcholine (PC), phosphatidic acid (PA), and other anionic lipids have an effect on the activity of PI-PLC toward both substrates PI and water-soluble cIP (*8*−*12*). In particular, the presence of PC enhanced the catalytic activity of the enzyme toward cIP. The activation was more pronounced when PC micelles (or vesicles) were present as opposed to PC monomers (*9*). This is a different type of interfacial activation where a nonsubstrate phospholipid surface alters the kinetic behavior of the enzyme and enhances catalysis. Low submicellar concentrations of anionic phospholipids could also activate the enzyme toward cIP but not as effectively as PC (*12*). Upon micellization (or with vesicles), anionic phospholipids such as PA, PMe, or PS effectively inhibited both phosphotransferase and cyclic phosphodiesterase activities (*8*, *12*). Determining the mode of binding of PI-PLC to lipid surfaces is critical to understanding how nonsubstrate interfaces affect PI-PLC catalytic activity and might shed light on how this bacterial PI-PLC is regulated.

Previous studies have monitored the binding of PI-PLC to interfaces (*3*, *8*, *10*−*13*), but results were not used to understand what is different in PI-PLC binding to activating (e.g., PC) versus other (e.g., PS or PMe) interfaces. In this work, we used a simple vesicle binding assay [previously

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[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol; cIP, D-*myo*-inositol 1,2-cyclic phosphate; I-1-P, D-*myo*-inositol 1-phosphate; CMC, critical micelle concentration; PC, phosphatidylcholine; PA, phosphatidic acid; diC_nL, 1,2-diacylphospholipid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPMe, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphomethanol; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; TX-100, Triton X-100; WT, wild-type PI-PLC.

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used to study the binding of phospholipase D to phospholipid interfaces (*14*)] to monitor the partitioning of PI-PLC to vesicles under a wide variety of conditions (pH, salt, phospholipid composition, vesicle size, etc). Binding to interfaces was also monitored by monolayer surface pressure techniques and CD spectroscopy. The data show a clear distinction between the binding of PI-PLC to zwitterionic and anionic surfaces, with hydrophobic interactions dominating the former and electrostatic interactions dominating the latter. Mutagenesis studies suggest that activating interfaces enhance partial insertion of the PI-PLC rim (notably Trp47 and Trp242 residues) into the bilayer while anionic interfaces anchor the PI-PLC electrostatically with far less hydrophobic contribution to binding. It is proposed that the partial insertion of rim residues leads to a conformational state of the enzyme with optimized activity.

MATERIALS AND METHODS

Materials. POPC, DMPC, POPMe, POPG, POPS, diC_6-PC, diC_6PS, diC_6PA, and cholesterol were purchased from Avanti and used without any further purification. Crude PI used in cIP preparation was purchased from Sigma. cIP was prepared as described previously (*9*). DiC_7PA wase prepared from the corresponding short-chain PCs using PLD (*12*) isolated from *Streptomyces chromofuscus* growth media (*14*). All resins used in PI-PLC purification were purchased from Amersham Pharmacia Biotech.

Overexpression of Bacterial PI-PLC and Construction of Mutants. The recombinant *Escherichia coli* cell strain MM294 transfected with the *B. thuringiensis* PI-PLC gene (obtained from Dr. Ming-Daw Tsai, The Ohio State University) was transformed into *E. coli* BL21 cells [BL21-Codonplus(DE3)-RIL from Stratagene]. The enzyme was isolated from culture supernatants and purified on a Q-Sepharose fast-flow column equilibrated with 20 mM Tris-HCl, pH 8.9. The protein was eluted using a gradient of 0−0.6 M NaCl in 20 mM Tris-HCl, pH 8.0. The protein was further purified using a phenyl-Sepharose column. The enzyme solution was applied to the column which had been equilibrated with 1 M NaCl in 20 mM Tris-HCl, pH 8.0, and eluted with a decreasing NaCl gradient ranging from 0.6 to 0 M. Enzyme purity, as checked by SDS−PAGE, was >90%. Millipore Centriplus 10 filters were used to concentrate the protein. PI-PLC concentration was measured by absorbance at 280 nm and the calculated extinction coefficient based on the sequence.

The K44A PI-PLC mutant was constructed using QuikChange methodology (*15*) with a site-directed mutagenesis kit from Stratagene. Two mutagenic primers containing the desired mutation (the codon for the desired mutation is indicated in boldface type) purchased from Operon, CAAGTTGCAAAATCCGATT**GCG**CAAGTGTGGGGAATGACGC and its complement, were annealed to the plasmid. Overexpression and purification of the K44A mutant was carried out as described above for wild type. PI-PLC activity was assayed by ^{31}P NMR spectroscopy as described previously (*9*). R69D was also constructed using QuikChange methodology and the primer CGCATTTTTGATATA**GAC**GGACGTTTAACAGATG and its complement. All mutations were confirmed by DNA sequencing carried out by the Beth Israel Deaconess Medical Center Sequencing Facility. W47A, W242A, and the double mutant W47A/W242A were constructed as described elsewhere (*16*).

Preparation of Vesicles. Appropriate aliquots of lipid (originally supplied in chloroform from Avanti) were placed in glass scintillation vials; the chloroform was removed by a stream of N_2. The lipid film was lyophilized overnight and then rehydrated with 10 mM Tris-HCl, pH 7.5, unless otherwise indicated. SUVs of different phospholipids were prepared by sonication on ice using a Branson sonifier cell disrupter and a microtip probe until maximum clarity was achieved (typically 5−10 min). LUVs of the different lipids were prepared by multiple passages of the aqueous lipid solutions through polycarbonate membranes (100 nm pore diameter) using a Lipofast extruder from Avestin. Incubation of PI-PLC with the different vesicles caused no change in the light scattering of the solution over several hours, a time considerably longer than that used in the binding assays.

CD Spectroscopy. The effects of phospholipid SUVs (1 and 2 mM) and short-chain phosphatidylcholine micelles (5 and 20 mM diC_6PC and 1 and 2 mM diC_7PC) on the secondary structure of WT PI-PLC were measured by CD spectroscopy using an AVIV 202 spectrophotometer. A stock PI-PLC protein solution (3.8 mg/mL) was dialyzed against 10 mM borate buffer, pH 8.0. Samples (typically 0.2−0.4 mg/mL in a 0.1 cm cell) were scanned from 300 to 185 nm at 25 °C. Estimation of secondary structure content was done with CDNN using ellipticity in the 195−300 nm range (*17*−*19*). Rather than reflect absolute amounts of secondary structure, the deconvolutions of PI-PLC spectra are used to highlight any changes induced by different phospholipid surfaces.

PI-PLC Phosphotransferase and Phosphodiesterase Activity. PI-PLC activity toward PI or cIP was measured between pH 5.5 and pH 8.2 using ^{31}P NMR spectroscopy. MES (50 mM) was used in assaying PI-PLC activities between pH 5.0 and pH 6.5, and Tris-HCl (50 mM) was used from pH 7.0 to pH 8.2. With PI as the substrate, mixed micelles of PI/diC_7PC (1:4) were used.

Vesicle Binding Assay. Solutions of PI-PLC incubated with phospholipid vesicles were centrifuged through a filter to separate free from vesicle-bound enzyme. This assay method was previously used to monitor a bacterial phospholipase D binding to vesicles (*14*). Solutions of PI-PLC incubated with phospholipid vesicles were centrifuged through a filter to separate free from vesicle-bound enzyme. Typically, 25 μg of PI-PLC (E_T) was added to an aliquot of the vesicle solution (2 mL) in 10 mM Tris-HCl, pH 7.5. Vesicle-bound enzyme (E_B) was separated from free enzyme (E_F) using an Amicon centricon-100 filter (100 kDa molecular mass cutoff). A control containing the same amount of enzyme in Tris-HCl, pH 7.5, with no lipid vesicles present was used to measure the efficiency of recovering free enzyme from the centrifugation step. With total enzyme usually 0.34−0.36 μM, less than 5% of E_T was retained on the filters in the control. Filtrates were lyophilized and analyzed by SDS−PAGE. Band intensities were quantified by NIH Image 1.61 software and used to calculate the concentration of E_F by comparing intensities from vesicle-containing samples to the E_T value of the control. The concentration of enzyme bound, E_B, was then evaluated as $E_T - E_F$. Binding was analyzed using a Langmuir adsorption isotherm (*20*) as shown in the equation:

$$E_B = ([L_0]/n)/(1 + K_d/E_F) \quad (1)$$

where n is the number of lipid molecules in the surface that each enzyme molecule requires (n can reflect direct and specific binding interactions of lipids with the enzyme, the number of lipids occluded by the enzyme when it is bound to the surface, and/or the number of lipids needed to generate an appropriate electric field that ties the enzyme to the surface), K_d is the dissociation constant for the binding site represented by n lipids, and $[L_0]$, E_F, and E_B are the bulk lipid and free and vesicle-bound enzyme concentrations, respectively. $[L_0]$ was varied between 0 and 0.2 mM for SUVs and between 0 and 2 mM for LUVs. To extract n and K_d over a wide total lipid concentration range, the data were fit to the following equation using a nonlinear least-squares analysis:

$$E_B = \{E_T + K_d + [L_0]/n - \sqrt{(E_T + K_d + [L_0]/n)^2 - 4E_T[L_0]/n}\}/2 \quad (2)$$

Most binding assays (except for mutants) were repeated at least twice; K_d and n values reported are averages of both data sets. nK_d is the apparent dissociation constant in terms of bulk lipid concentration; it was estimated as the average from multiple analyses for a given phospholipid vesicle. nK_d is similar to the partition coefficient from a simple bulk binding isotherm $[E_B/E_T = [L_0]/([L_0] + K_d)]$ when the concentration of lipid sites $([L_0]/n)$ is significantly greater than E_T. In these limits, the partition constant, K_d, is the same as nK_d from the Langmuir analysis. This latter analysis was used for vesicles to which PI-PLC bound weakly.

Monolayer Measurements. Surface pressure (π) of solution in a circular Teflon trough was measured using a Wilhelmy plate attached to a computer-controlled tensiometer (*20*). The trough (4 cm diameter × 1 cm depth) has a 0.5 cm deep well for a magnetic stir bar and a small hole drilled at an angle through the wall to allow an addition of protein solution. Five to ten microliters of phospholipid solution in ethanol/hexane [1:9 (v/v)] was spread onto 10 mL of subphase (20 mM Tris-HCl for pH 7.5 and 20 mM MES for pH 5.5, each containing 0.1 M KCl) to form a monolayer with a given initial surface pressure, π_o (mN/m). The subphase was continuously stirred at 60 rpm with a magnetic stir bar. Once the surface pressure had been stabilized (after ~5 min), the protein solution (typically 50 μL) was injected into the subphase through the hole, and the change in surface pressure ($\Delta\pi$) was measured as a function of time. Typically, the $\Delta\pi$ value reached a maximum after 20 min. The maximal $\Delta\pi$ depended on the protein concentration at the low concentration range and reached a saturation when the protein concentration was higher than 3 μg/mL. Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed $\Delta\pi$ represented a maximal value. Extrapolation of the surface pressure with enzyme to $\pi_o = 0$ provides the maximum $\Delta\pi$ obtainable; extrapolation to $\Delta\pi = 0$, provides π_c, the pressure above which there is no penetration of the enzyme into the phospholipid monolayer.

RESULTS

Binding of PI-PLC to SUVs. The vesicle binding assay provides information on the partitioning of *B. thuringiensis* PI-PLC to vesicles as a function of bulk lipid concentration. Using Langmuir analysis, an estimate of n (the number of molecules of lipid bound per enzyme molecule) and K_d (the dissociation constant per binding site) can be determined. The product, nK_d, is an apparent binding constant that relates the partitioning of the enzyme under specific conditions to the bulk concentration of phospholipid. Previous NMR and fluorescence experiments have shown that PI-PLC binds to monomers of PC (diC_6PC and diC_7PC) with an apparent binding constant of 0.2−0.5 mM (*10*). Apparent binding constants to PC vesicles were even lower, 0.02−0.08 mM, as measured by fluorescence methods (*8*, *13*). Binding to anionic vesicle surfaces (substrate PI as well as nonsubstrates such as PMe, PG, PS, or PA) appeared to be significantly stronger (*8*, *13*). However, little is known about what controls the binding of the enzyme to these different surfaces.

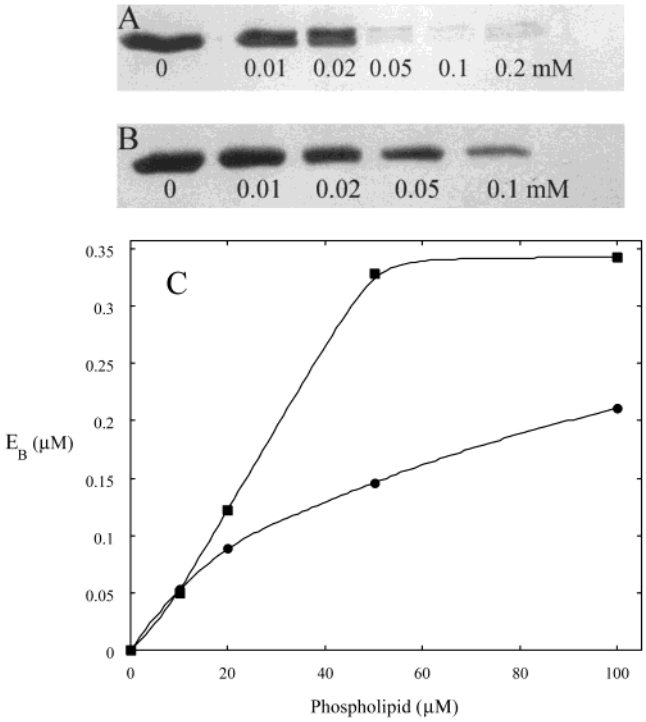

FIGURE 1: SDS−PAGE analysis of free PI-PLC separated from PI-PLC bound to SUVs of (A) POPG and (B) POPC as a function of bulk lipid concentration (indicated beneath each lane). The total concentration of PI-PLC used for each binding assay was 0.36 μM. (C) Binding isotherms constructed from data in (A) and (B) for PI-PLC binding to PG (■) and PC (●).

Under conditions of moderate ionic strength, PI-PLC bound tightly to PG SUVs (Figure 1A). At low PG concentrations, very little binding of PI-PLC occurred. Above a threshold concentration there was significant binding that did not increase further. Between the two extremes, the profile did not look like the typical hyperbolic Michaelis−Menten profile (see Figure 1C) but showed a nearly linear increase in the amount of enzyme bound to the vesicles. This type of behavior is consistent with PI-PLC binding to a "site" on the vesicle composed of many (n) phospholipid molecules and can be analyzed using the Langmuir adsorption isotherm. A fit to the data (Figure 1C) shows that n is ~120 with K_d ~0.01 μM to this site. The value of nK_d was 1.2 μM. Similar values were observed with other anionic phospholipid vesicles (Table 1). For assessing binding of PI-PLC to PA

Table 1: Apparent Dissociation Constants[a] for WT PI-PLC Binding to Unilamellar Phospholipid Vesicles[b]

phospho-lipid	vesicle size	n	K_d	nK_d (μM)[b]
POPC	SUV	12 ± 3	5.1 ± 0.2	64 ± 2
	LUV	—[c]	—[c]	2500 ± 700
DMPC	SUV	2 ± 6	19 ± 4	38 ± 4
POPG	SUV	121 ± 30	0.0102 ± 0.005	1.2 ± 0.1
POPA	SUV	67 ± 19	0.028 ± 0.009	1.9 ± 0.1
POPMe	SUV	120 ± 18	0.022 ± 0.016	1.8 ± 1.1
	LUV	—[c]	—[c]	3000 ± 1000
POPS	SUV	79 ± 22	0.068 ± 0.037	5.4 ± 2.3

[a] Dissociation constants extracted using a Langmuir adsorption isotherm: n is the number of lipids per site, K_d is the binding constant to an individual site, and nK_d is the binding in terms of bulk phospholipid concentration. [b] Binding of PI-PLC to unilamellar vesicles measured in 10 mM Tris-HCl, pH 7.5, at 22 °C: n is the number of lipid molecules per binding site, K_d is the apparent binding constant for this site, and nK_d is the apparent dissociation constant in terms of bulk phospholipid concentrations; the value indicated is the average nK_d from different binding experiments for a given phospholipid vesicle. For these experiments, the bulk phospholipid concentration ranged between 0 and 0.2 mM for SUVs and between 0 and 2 mM for LUVs. [c] Binding was much weaker so that values for n and K_d could not be reliably extracted. Therefore, the binding of PI-PLC to these vesicles was analyzed by a simple partitioning isotherm [$E_B = E_T[L_0]/([L_0] + K_d)$, where $K_d = nK_d$].

vesicles, the presence of EDTA was critical to remove any excess Ca^{2+} that remained from preparation of PA from PC by phospholipase D. nK_d values for PI-PLC binding to the anionic lipid vesicles ranged from 1 to 5 μM with the number of phospholipids per site between 70 and 120 and K_d ranging from 0.01 to 0.07 μM.

PI-PLC exhibited considerably weaker noncooperative binding to PC SUVs (Figure 1B,C). Analysis of the dependence of E_B on $[L_0]$ yielded nK_d values of 64 ± 2 μM for POPC and 38 ± 4 μM for DMPC at pH 7.5. At 22 °C, the binding of PI-PLC to the DMPC vesicles was occurring right around the T_m (26 °C for LUVs and 2−3 °C lower for SUVs) in the binding assay (22 °C). The 2-fold tighter binding could reflect a preferential interaction with a surface of mixed gel−liquid crystalline patches. The values obtained with this vesicle binding assay for both POPC and DMPC SUVs were comparable to apparent K_d values of 20−80 μM measured for PC surfaces by fluorescence methods (*8*, *11*). The differences in the binding of PI-PLC to zwitterionic PC versus anionic lipids were more pronounced when n and K_d were compared. The average n for PI-PLC binding to POPC SUVs was 12 ± 3 and $K_d = 5.1 \pm 0.2$ μM. Thus, for PC interfaces, K_d, the binding constant to the multilipid site, was about 200-fold weaker than for anionic lipid interfaces. Furthermore, the number of PC molecules per site was much smaller than the number of anionic phospholipids per site.

Modulation of PI-PLC Binding to Surfaces. A variety of factors can often modulate peripheral membrane protein binding to surfaces. Vesicle curvature appears to be critical for many phospholipases interacting with bilayers, since these enzymes usually exhibit higher specific activities toward substrate packed in SUVs (≤300 Å diameter) compared to LUVs (*1*). It is thought that smaller, highly curved vesicles offer the enzyme better access for binding an individual molecule in the active site. However, bulk partitioning of the enzyme to a surface can also be affected. The phase state of the bilayer, gel or liquid crystalline, as well as an additive such as cholesterol can also affect binding and catalysis.

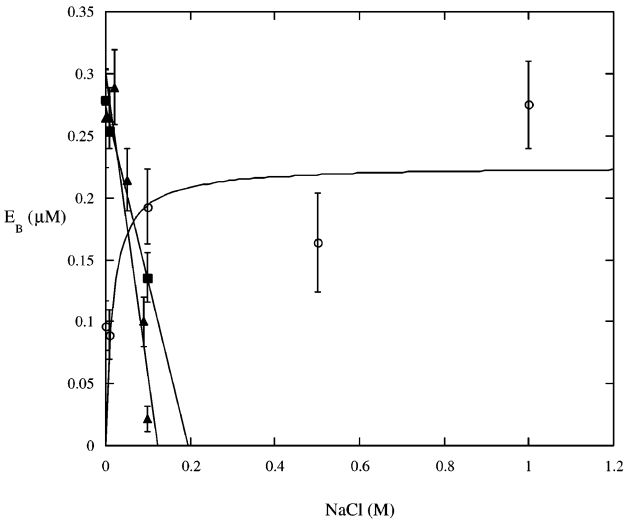

FIGURE 2: Dependence of wild-type PI-PLC (0.36 μM) binding to (○) POPC (40 μM), (▲) POPG (40 μM), and (■) POPMe (30 μM) SUVs in 10 mM Tris-HCl, pH 7.5, as a function of added NaCl.

PI-PLC binding to PC vesicles of large (SUV) and small (LUV) curvature (Table 1) was quite different and paralleled the relative activity of this enzyme toward PI substrate presented as vesicles. The enzyme partitioned poorly to liquid crystalline POPC LUVs ($nK_d = 2.5 \pm 0.7$ mM) versus SUVs ($nK_d = 64 \pm 2$ μM). Weaker binding of PI-PLC to anionic POPMe LUVs was also observed with $nK_d = 3 \pm 1$ mM (Table 1). Therefore, vesicle curvature is very important for PI-PLC interactions with a bilayer.

Cholesterol incorporation into bilayers affects phospholipid motions and could also modulate enzyme binding. PI-PLC partitioned to SUVs prepared with POPC (0.035 mM) and cholesterol (0.015 mM) to about the same extent as to vesicles of just POPC (0.05 mM), where the fractions of PI-PLC bound were 45% and 50% of the total enzyme concentration, respectively. If anything, the effective binding of PI-PLC is marginally tighter to the cholesterol-containing surface.

Vesicle binding studies were typically done with 10 mM buffer. If electrostatic interactions were critical to PI-PLC surface binding, one would expect that addition of NaCl would reduce binding of PI-PLC to interfaces. Interactions of protein with vesicles that have a strong hydrophobic component would be unaffected or possibly enhanced by increasing NaCl. As shown in Figure 2, PI-PLC binding to PC vesicles increased with increasing ionic strength. Addition of 100 mM NaCl roughly doubled the amount of protein that partitioned to the PC interface. In contrast, essentially all the PI-PLC could be stripped off PMe and PG SUVs by adding 200 mM NaCl. Therefore, binding of the enzyme to zwitterionic PC surfaces is dominated by hydrophobic interactions while binding to the anionic bilayers is dominated by electrostatic interactions.

The binding of PI-PLC to vesicle surfaces can also be affected by pH. Except for PA, none of the phospholipids examined have changes in ionization state between pH 6 and pH 8. Therefore, differences in the amount of enzyme that partitions to the surface reflect changes in charge distribution or conformation on the protein. For all of the surfaces

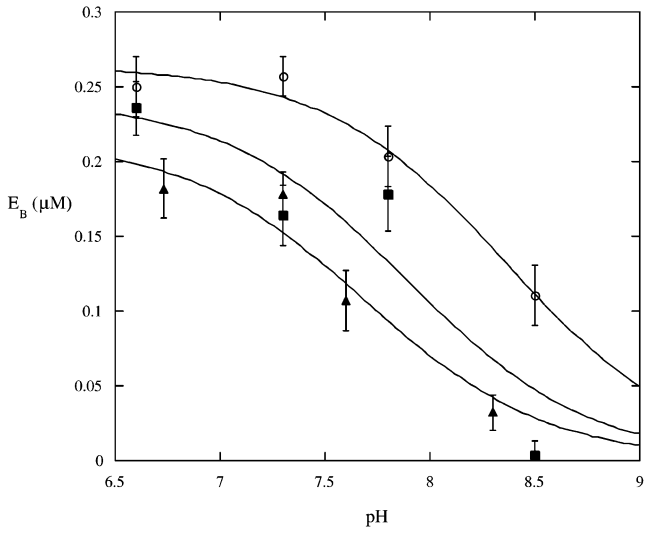

FIGURE 3: Dependence of wild-type PI-PLC (0.36 μM) binding to (○) POPC (40 μM), (▲) POPG (40 μM), and (■) POPMe (30 μM) SUVs as a function of pH. Buffers used were 50 mM MES between pH 6 and pH 7 and 50 mM Tris-HCl buffer at pH >7.

examined (PC, PG, PMe), PI-PLC bound more tightly below pH 7 (Figure 3). At pH 8.5, there was little affinity of the enzyme for the two anionic surfaces. Binding to PC was reduced at this pH compared to pH ~7; however, significant protein still partitioned to the PC bilayer (roughly 50% of the maximum under these conditions). Acidification of the PI-PLC should make the protein less negatively charged (although the theoretical p*I* for the protein is 5.4, it is likely that cationic clusters on the enzyme mediate electrostatic interactions with anionic surfaces) and enhance interactions with an anionic surface. If the interaction with PC surfaces has a large hydrophobic component, the effect on vesicle partitioning of ionizing groups on the protein would be considerably less (as is seen in comparing PMe and PC binding at pH 8.5). The apparent pK_a that governs loss of surface binding is 7.7−7.9 for the anionic surfaces and 8.4 for PC.

Monolayer Studies of PI-PLC Binding to Surfaces. The penetration of wild-type *B. thuringiensis* PI-PLC into POPC and POPG monolayers was studied at pH 5.5 and 7.5 in order to understand the differential interfacial binding to zwitterionic and anionic interfaces at different pH values. Typically, a phospholipid monolayer of a given initial surface pressure (π_o) is spread at constant area, followed by injection of the protein into the subphase. Addition of the surface-active protein increases the surface pressure, depending on the extent to which the protein inserts into the monolayer, and this change ($\Delta\pi$) is generally inversely proportional to π_o. As shown in Figure 4, PI-PLC penetrates more effectively into a PC monolayer than a PG monolayer regardless of the pH. Although PI-PLC has higher binding affinity for anionic compared to zwitterionic vesicles, the monolayer penetration studies show that the enzyme exhibited more insertion into PC versus PG surfaces. The extrapolated π_c (the pressure above which the enzyme cannot penetrate the monolayer) for PI-PLC binding to a PC monolayer is 24−25 mN/m; this value is 18.6 mN/m for a POPG monolayer. The higher π_c and the increased slope for $\Delta\pi$ versus π_o for the POPC monolayer are consistent with dominance of hydrophobic interactions for PI-PLC binding to PC and with electrostatic

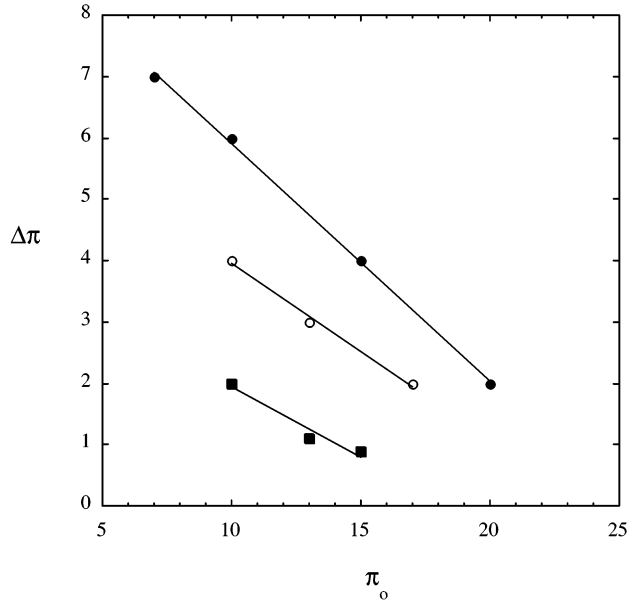

FIGURE 4: Effect of PI-PLC added to the subphase on the change in surface pressure of POPC at pH 5.5 (●) and pH 7.5 (○) and POPG at pH 5.5 (■). The initial surface pressure (mN/m) is π_o; $\Delta\pi$ represents the change (in mN/m) upon addition of enzyme.

interactions dominating the enzyme binding to anionic lipid surfaces. Additionally, the results show that the binding to monolayer interfaces is pH dependent with weaker binding at pH 7.5 compared to that at pH 5.5 for both PC and PG (in the latter case, the binding as monitored by $\Delta\pi$ was too weak at pH 7.5 to be measured). This result is consistent with what was observed for pH dependence of PI-PLC binding to vesicles.

PC and PA Binding to PI-PLC and Changes in Secondary Structure. CD spectra of PI-PLC (~0.3 mg/mL in 10 mM borate, pH 8) in the presence of SUVs or short-chain phospholipid micelles of different lipids were recorded in the far-UV region (between 190 and 300 nm) to assess any effects of interfacial binding on PI-PLC secondary structure. The distorted $(\alpha\beta)_6$-barrel of PI-PLC is a rigid structure so only small changes in secondary structure would be expected when protein binds to monomers, micelles, and vesicles. The presence of 1 or 2 mM POPC SUVs caused a small (but reproducible) increase in β-sheet at the expense of α-helix (Figure 5 and Table 2). Smaller magnitude changes were also observed for PMe but not for PA. Micellar phospholipids, diC_6PC (5 and 20 mM) and diC_7PC (1 and 2 mM), showed a similar trend, notably an increase in β-sheet (1.8 ± 0.5%), but the other major change appeared to be in random coil (which decreased) rather than α-helix. The small increase in β-sheet content would correspond to an additional four to six residues involved in β-sheet formation. Whether or not this change is critical to the activation of the enzyme will require more detailed structural analyses of the protein bound to interfaces.

Comparison of pH Dependence of Vesicle Binding to PI-PLC Activity. The pH dependence of PI-PLC binding to vesicles can be compared to the pH profile of both phosphotransferase and cyclic phosphodiesterase reactions (Figure 6). The drop in activity around pH 8 for PI (solubilized in diC_7PC) cleavage is consistent with the drop in binding affinity to activating surfaces. Specific activity of the enzyme

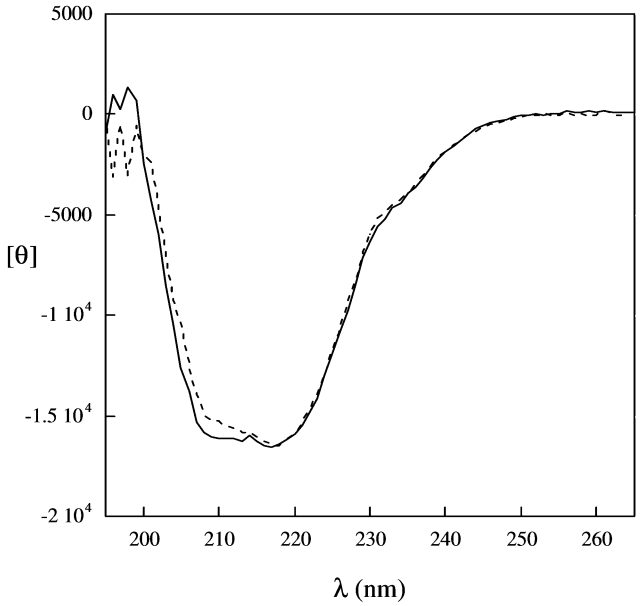

FIGURE 5: CD spectra of PI-PLC (0.25 mg/mL) in the absence (—) and presence (---) of 1 mM POPC SUVs in 10 mM borate, pH 8.0.

Table 2: Effect of SUVs (1−2 mM Total Phospholipid) on the Secondary Structure of *B. thuringiensis* PI-PLC As Determined by CDNN Analysis of CD Spectra[a]

secondary structure	% change[b]		
	+PC	+PMe	+PA
α-helix	−1.9 ± 0.1	−1.1 ± 0.3	0.25 ± 0.13
β-sheet	1.3 ± 0.1	0.8 ± 0.1	0.0 ± 0.1
β-turn	−0.07 ± 0.03	0.0 ± 0.1	0.05 ± 0.03
random coil	0.7 ± 0.1	0.3 ± 0.3	−0.4 ± 0.1

[a] Secondary structure estimation using CDNN and the wavelength range 195−260 nm (*17*−*19*); the SUVs were prepared by sonication. [b] Deconvolution of the CD spectrum suggests 25.5% α-helix, 24.4% *β*-sheet, 17.8% *β*-turn, and 32.3% random coil.

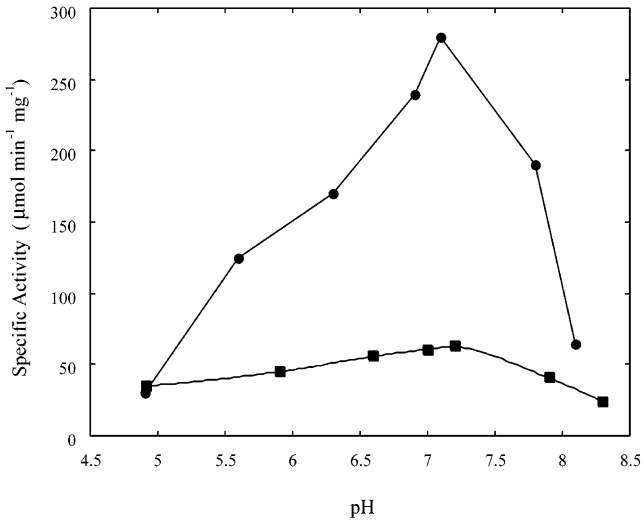

FIGURE 6: PI-PLC activity toward (●) PI SUV (5 mM) and (■) cIP (5 mM) as a function of pH. 56 ng and 0.54 *μ*g of enzyme were used, respectively. Buffers used at different pH values were 50 mM MES for assaying PI-PLC activity between pH 4.9 and pH 6.5, and 50 mM Tris-HCl buffer between pH 7.0 and pH 8.3.

for cIP hydrolysis was also reduced. Whether the decrease reflects impaired binding of the enzyme to activating

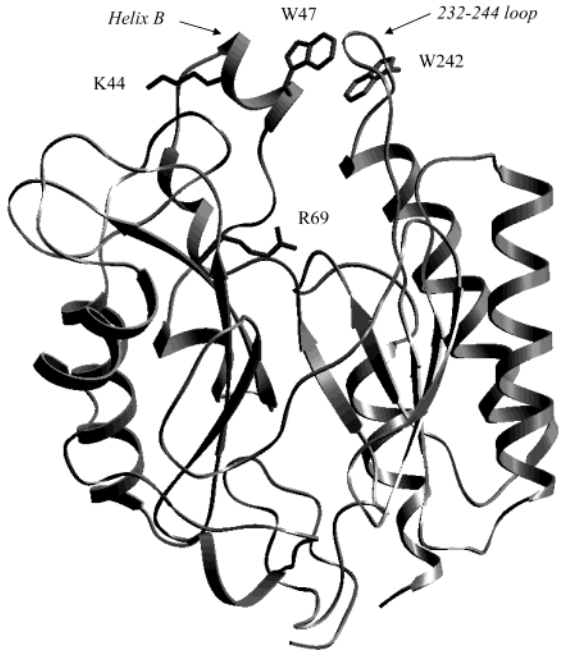

FIGURE 7: Ribbon diagram of PI-PLC showing rim tryptophans (Trp47 in helix B and Trp242 in the rim loop) as well as Lys44 (also in helix B) and Arg69 at the bottom of the active site.

Table 3: Apparent Dissociation Constants (nK_d) for PI-PLC Mutants Binding to POPC and POPMe SUVs

PI-PLC	POPC nK_d (*μ*M)	ΔΔ*G* (kJ/mol)	POPMe nK_d (*μ*M)	ΔΔ*G* (kJ/mol)
WT	64 ± 2		1.8 ± 1.1	
K44A	87 ± 5	0.8	4.0 ± 1.2	2.0
W47A	3200 ± 500[a]	9.7	1.2 ± 0.4	−1.1
W242A	8600 ± 600[a]	12.1	2.3 ± 0.9	0.6
W47A/W242A	>25000[a]	>15	167 ± 35	11.2
R69D	18 ± 2	−3.1	59 ± 5	8.6

[a] Taken from Feng et al. (*16*).

interfaces (change in ionization states of enzyme groups that allow the enzyme to bind to the interface) or impaired catalysis (change in ionization state of key active site residues) is unclear. On the acidic side, the enhanced binding to surfaces is also not likely to contribute significantly since the cIP pH profile parallels that of PI cleavage.

Probing the Role of Cationic Residues in PI-PLC Binding to Vesicles. The pH dependence of PI-PLC binding to vesicles suggested that one or more cationic side chains were important for surface binding. The rim of the α*β*-barrel has a single positively charged residue, K44 (Figure 7). This residue has been suggested to be critical for interaction with anionic surfaces (*21*). Since the interaction of PI-PLC with anionic bilayers has a large electrostatic component, we can explore the role of this cationic side chain in vesicle binding. Overexpression and purification of K44A were similar to WT recombinant protein. The kinetic characteristics of the mutant were quite similar to WT [diC_7PC micelles activate 6 mM cIP hydrolysis 10-fold, and PI/diC_7PC (1:6) mixed micelles are better substrates than PI/TX-100 (1:2) mixed micelles (135 ± 10 versus 95 ± 7 *μ*mol min^{-1} mg^{-1}, respectively)]. Interestingly, the nK_d for K44A binding to PC and PMe surfaces was only about 2-fold higher than that of WT (Table 3). The increase in nK_d corresponds to at most a 2 kJ/mol decrease in the free energy for binding of K44A

to anionic surfaces (Table 3). This suggests that this single positive charge at the rim of the $\alpha\beta$-barrel [and the closest positive charge to helix B and the 232−244 loop, which are implicated in surface binding (*16*)] is not the major contributor to the strong electrostatic interactions of PI-PLC with anionic surfaces. Other positively charged side chains in the enzyme must contribute to the strong interaction with anionic surfaces.

As with other peripheral proteins (*22*, *23*), the binding of PI-PLC to anionic lipids is likely the net result of electrostatic interactions of more than one positively charged residue on the protein. Earlier studies showed that anionic lipids bind to the active site (*8*) and cause a decrease in intrinsic fluorescence (as well as inhibit enzyme activity). Thus, the positively charged residues in the open active site might mediate PI-PLC electrostatic interactions with anionic lipids. Arg69, one of the positively charged residues present in the active site (see Figure 7 for location of this residue), is part of the general acid complex and is hypothesized to stabilize the increased negative charge of the transition state (*24*, *25*). Previous mutation of Arg69 to alanine, glutamate, and methionine led to a 10^3-10^4-fold decrease in the catalytic activity (*24*). We changed the positively charged residue to aspartate, in effect reducing the net positive charge at the active site by two. This protein (R69D) like the previously studied mutants (*24*) had a low specific activity toward PI dispersed in diC_7PC ($\sim 10^4$-fold lower activity than WT). The affinity of R69D for vesicles was considerably altered from WT PI-PLC with a 30-fold weaker partitioning (nK_d) to PMe SUVs and a 3-fold higher affinity to PC SUVs (Table 3). The reduction of affinity for anionic vesicles was even more dramatic when examining the n and K_d values: n decreased from $\sim$100 to $\sim$30 and K_d increased from 0.02 to 0.6 μM. $\Delta\Delta G$ estimated from nK_d for this substitution is $\sim$9 kJ/mol (Table 3), suggesting that the positively charged environment of the active site does contribute to electrostatic interactions of the enzyme with anionic surfaces.

Role of Tryptophan Residues in PI-PLC Binding to Interfaces. Tryptophans have been shown to be important in binding of peripheral membrane proteins at the water/lipid interface (*26*). Of the seven tryptophan residues in PI-PLC, two of these, Trp47 and Trp242 located in helix B and a loop composed of residues 237−243 (Figure 7), are critical for PI-PLC binding to zwitterionic interfaces (*16*). Partitioning of W47A and W242A into zwitterionic PC SUVs was much weaker than WT PI-PLC (Table 3), and basically no significant binding of the W47A/W242A mutant was observed. If binding of PI-PLC to anionic surfaces is really dominated by electrostatic interactions, then one could predict that these PC binding impaired mutants would still exhibit tight binding to anionic vesicles. With that in mind, we tested the tryptophan mutants for their ability to bind to PMe SUVs. Both single tryptophan mutants (W47A, W242A) showed tight binding (as monitored by nK_d) to the anionic surface comparable to that observed for the wild type (Table 3). $\Delta\Delta G$ extracted from the change in nK_d for the mutant compared to WT indicates <1 kJ/mol difference in binding energetics (Table 3). However, the W47A/W242A mutant showed a much weaker binding affinity for PMe than WT: $\Delta\Delta G \sim 11$ kJ/mol, a value comparable to the $\Delta\Delta G$ estimated for single tryptophan mutants binding to PC surfaces. The difference is likely to reflect removal of a tryptophan side chain that was inserted into the membrane. This suggests that PI-PLC binding to anionic interfaces has a hydrophobic component, where at least one of the rim tryptophan residues contributes to this binding.

DISCUSSION

PI-PLC enzymes are soluble peripheral membrane proteins that catalyze the hydrolysis of the phosphodiester linkage of phosphatidylinositols. In order for PI-PLC enzymes to catalyze this reaction, the protein must interact with substrate present in the two-dimensional interface (PI or GPI-anchored proteins for PI-PLC enzymes from bacterial organisms, PIP_2 for mammalian PI-PLC enzymes). The protein can interact with an interface composed of substrate and/or nonsubstrate molecules in a step distinct from extraction of a single lipid molecule for binding in the active site. These non active site binding interactions of PI-PLC with the membrane interface have the potential to modulate the catalytic activity. The crystal structure of PI-PLC from *B. cereus* with *myo*-inositol bound identified the residues involved in the interaction of the protein with the polar moiety of the substrate (*2*). However, the details of the binding and orientation of PI hydrophobic acyl chains could only be hypothesized. General models of peripheral protein binding to lipid interfaces (*22*, *26*) suggest a two-stage binding, where a peripheral protein would first be driven to the lipid surface by electrostatic forces, followed by formation of tight membrane−protein complexes that are stabilized by hydrophobic interactions (i.e., partial penetration of the protein into the membrane) and/or electrostatic interactions and hydrogen bonding.

The binding behavior of *B. thuringiensis* PI-PLC to interfaces is consistent with a two-stage binding model. Binding of PI-PLC to anionic lipids (PMe, PG, and presumably substrate PI) is dominated by electrostatic interactions while binding to zwitterionic PC surfaces is dominated by hydrophobic interactions. However, interactions with anionic surfaces also have a hydrophobic component (e.g., some penetration based on the monolayer results), and interaction with PC is likely to have some small electrostatic contribution since R69D has tighter interactions with that zwitterionic surface than WT PI-PLC (possibly charge repulsion between the choline positive charge and active site cationic side chains). The partitioning of PI-PLC to zwitterionic PC was consistently weaker than toward anionic interfaces and involved fewer lipid molecules defined as a site. PI-PLC showed similar affinities and high n values for the different anionic lipids studied (POPMe, POPG, POPS, and POPA), consistent with the dominance of electrostatic interactions. Thus, a distinction between the mode of binding to zwitterionic and anionic lipid surfaces can be established.

Other evidence for differences in the modes of binding to anionic versus a zwitterionic surface comes from studying the role of the tryptophan residues on the binding affinity to interfaces. It has been observed in several peripheral membrane proteins that tryptophans have a role in lipid/protein binding (*26*). For phospholipase A_2, both electrostatic interactions and insertion of two tryptophan residues into the membrane interface are important for membrane binding (*23*). For PI-PLC, Trp47 and Trp242 located at the top of the rim (in helix B and loop 237−242) appear to be critical for insertion into the bilayer (*16*). The removal of one of

these bulky hydrophobic side chains drastically weakened the binding of the enzyme to PC SUVs but had basically no effect on protein binding electrostatically to anionic surfaces. Removal of both rim tryptophans basically abolished binding to PC. However, the protein could still interact relatively well with anionic surfaces. The observation that removing these tryptophan residues had a smaller effect on binding of PI-PLC to PMe than to PC vesicles is consistent with dominance of electrostatic interactions for the anionic surfaces. The weaker binding affinity of W47A/W242A for PMe vesicles suggests that side chains in PI-PLC are partially inserted in anionic surfaces as well, although the extent of insertion must be less than PC since the protein can be washed off the vesicle surface with moderate NaCl. Evidence for greater insertion of PI-PLC into PC interfaces compared to anionic phospholipid interfaces is also provided by the monolayer studies.

The importance of hydrophobic rim residues for optimal catalysis of a PI-PLC has also been seen with mammalian PLCδ1 (27). That work showed that modification of hydrophobic ridge residues (Leu320, Phe360, and Trp555) reduced activity roughly 3-fold (in both PIP_2/cholate and dodecyl maltoside mixed micelles), consistent with the type of activity changes seen with *B. thuringiensis* rim tryptophan single mutants (*16*). However, with the mammalian enzyme, those PLCδ1 mutants became less dependent on the surface pressure when analyzed for monolayer activity (suggesting better binding to surfaces). Indeed, PLCδ1 Phe360A was independent of monolayer surface pressure, and the interpretation was that this aromatic group had a negative effect on binding and insertion into membranes (*27*). For that mutant, evaluation of the strength of it partitioning to bilayers would be particularly informative in light of what we see with the bacterial PI-PLC.

Are there changes in the protein conformation upon binding to interfaces? From the CD analysis, the addition of PC interfaces (vesicles and micelles) consistently introduced a small change of secondary structure of PI-PLC (enhanced β-sheet). This change was smaller with both PMe and PA vesicle surfaces. The increase in β-sheet corresponds to four to six residues adopting a β-sheet structure. While the $\alpha\beta$-barrel of PI-PLC is fairly rigid, loops or helical regions near the rim could be induced to create strand extensions upon binding of the enzyme to surfaces. We tentatively suggest that this small change in secondary structure elements is related to the ability of phospholipids to activate PI-PLC.

The effect of pH on the binding of PI-PLC to different interfaces (both anionic and zwitterionic) is related to changes in the ionization state of one (or more) residue on the protein. The pK_a that governs the loss of binding affinity is between 7.5 and 7.8 for the two anionic surfaces and 8.4 to 8.5 for PC. Since PI-PLC has no cysteines, the likely candidates are histidines and possibly lysines. The pK_a values for histidine residues have already been determined by NMR titration methods (*28*, *29*). *B. cereus* as well as *B. thuringiensis* PI-PLC enzymes contain six histidine residues, two of which (His32 and His82) are critical for catalysis. His61 and His81 did not titrate over the pH range of 4.0−9.0, consistent with the crystal structure data that showed that these two histidines are buried. These two residues are unlikely to be responsible for the pH dependence of binding to interfaces. The crystal structure also showed that His92 and His227 are on the surface of the protein. The pK_a values of these two residues were determined as 5.4−5.6 and 6.8−6.9, respectively. These two exposed histidine residues are unlikely to be involved in the observed pH effect, since they are located close to the other end of the barrel, away from where interfacial binding occurs. The two histidine candidates that are likely to be involved are His32 and His82 whose pK_a values were determined as 7.4−7.6 and 6.9−7.0, respectively (*28*, *29*). His32 and His82 could then play a dual role in interfacial binding as well as in catalysis. On the top of the hydrophobic rim, Lys44 in helix B was suggested as a potential site for electrostatic interactions (*3*, *21*). However, K44A bound to PC and PMe vesicles to almost the same extent as WT PI-PLC. Therefore, its contribution to the electrostatic interactions of PI-PLC with interfaces is small. Other positively charged residues have been suggested to be involved in electrostatic interactions such as Lys122 and Lys201 present in sheet VI and helix E, respectively (*3*). We suggest that the positively charged residues in the solvent-accessible active site also act as an ensemble to interact with anionic surfaces. While the histidines are likely to be critical for binding, other nontitratable charged active site residues may be important. For example, replacement of Arg69, which as part of the general acid triad stabilizes the negative charge of the transition state (*24*), with a negatively charged residue (aspartate) weakened PI-PLC binding to PMe interfaces 15−20-fold.

Understanding the differences in how PI-PLC binds to PC and anionic interfaces sheds light on the mechanism by which PC kinetically activates the enzyme. For the phosphotransferase reaction, PI-PLC is first driven to the PI membrane through electrostatic interactions. Since PI-PLC penetration in this interface is small (it appears that only one of the two available rim tryptophans inserts into the bilayer), enzymatic activity is not likely to be optimal. Introducing PC into the interface will promote insertion of both Trp47 and Trp242 into the surface and induce a small increase in β-sheet in the process. This serves to anchor the protein to the largely PI interface for more efficient catalysis (presumably processive). Partial insertion of rim tryptophans could affect water structure in the active site or the arrangement of catalytic residues which in turn promotes catalysis. Such effects must occur since PC interfaces also enhance the cyclic phosphodiesterase reaction of PI-PLC toward cIP, a compound with no tendency to partition to interfaces. Previous work has shown that there is a small activation of cIP hydrolysis by anionic short-chain phospholipids below their CMC (*12*). However, this activation is never as potent as that by short-chain PC added below its CMC. The observation that many anionic phospholipids inhibit the catalytic activity of PI-PLC might correlate with their binding nonspecifically and electrostatically in the active site, forming strong interactions with cationic residues that are critical in the catalytic mechanism (e.g., Arg69). PC, which has no significant affinity for the PI-PLC active site, promotes insertion of both Trp47 and Trp242 inserting into the PC bilayer. Since the conformation of helix B and the loop (237−243) might be critical for the binding of the acyl chains of PI in the active site (*16*), partitioning of PI-PLC into PC surfaces might change the orientation of these two regions, leading to an enhancement of PI-PLC catalytic activity.

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