Articles

Listeria monocytogenes Phosphatidylinositol-Specific Phospholipase C: Kinetic Activation and Homing in on Different Interfaces†

Wei Chen,‡ Howard Goldfine,§ Bharath Ananthanarayanan,|| Wonhwa Cho,|| and Mary F. Roberts*,‡

Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, and Department of Chemistry, University of Illinois, Chicago, Illinois 60607

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ABSTRACT: The phosphatidylinositol-specific phospholipase C (PI-PLC) from *Listeria monocytogenes* forms aggregates with anionic lipids leading to low activity. The specific activity of the enzyme can be enhanced by dilution of the protein or by addition of both zwitterionic and neutral amphiphiles (e.g., diheptanoylphosphatidylcholine or Triton X-100) or 0.1−0.2 M inorganic salts. Activation by amphiphiles occurs with both micellar (phosphatidylinositol dispersed in detergents) and monomeric [dibutroylphosphatidylinositol (diC_4PI)] phosphotransferase substrates and inositol 1,2-(cyclic)-phosphate (cIP), the phosphodiesterase substrate. The presence of zwitterionic and neutral amphiphiles (to which the protein binds weakly) dilutes the surface concentration of the interfacial anionic substrate and thereby reduces the level of enzyme−phospholipid particle aggregation. Zwitterionic amphiphiles also can bind directly to the protein and enhance catalysis since they enhance both diC_4PI and cIP hydrolysis. In contrast to activation by amphiphiles, the rate enhancement by salt occurs for only the phosphotransferase step of the reaction. Added salt has a synergistic effect with zwitterionic phospholipids, leading to high specific activities for PI cleavage with only moderate dilution of the anionic substrate in the interface. This kinetic activation correlates with weakening of strong PI-PLC hydrophobic interactions with the interface as monitored by a decrease in the maximum monolayer surface pressure for insertion of the protein. Several point mutations of surface hydrophobic residues (W49A, L51A, L235A, and F237W) can dramatically alter the unusual kinetics of this secreted enzyme. The high affinity of PI-PLC for anionic phospholipids along with a strong hydrophobic interaction, which gives rise to the unusual kinetic behavior, is considered in terms of how it might contribute to the role of this phospholipase in *L. monocytogenes* infectivity.

Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC)[1] catalyzes the calcium-independent hydrolysis of PI in two steps: (i) an intramolecular phosphotransferase reaction at a phospholipid aggregate surface to produce diacylglycerol (DAG) and water-soluble inositol 1,2-(cyclic)-phosphate (cIP) followed by (ii) a cyclic phosphodiesterase reaction in which cIP is hydrolyzed to inositol 1-phosphate. The second reaction occurs with a soluble monomeric substrate and is much slower (with both a lower V_{max} and a significantly higher K_m) than the first one (*1, 2*). Previous studies of the PI-PLC from *Bacillus* sp. highlight several kinetic properties common to a wide range of phospholipase enzymes. That bacterial PI-PLC exhibits (i) "interfacial activation", an enhanced V_{max}/K_m, toward aggregated PI compared to monomeric PI (*3*−*5*), (ii) "surface dilution inhibition", a decrease in specific activity as the surface concentration of the substrate is diluted with detergents or other phospholipids while the total substrate concentration is kept constant (*2*), and (iii) "scooting mode catalysis", in which enzyme completes several rounds of substrate turnover at the substrate interface before dissociating from the particle (*5*). The *Bacillus* sp. PI-PLC is also active toward GPI linkages (*6*).

PI-PLC from *Listeria monocytogenes*, a ubiquitous foodborne intracellular pathogen of humans and animals (*7, 8*), plays a role as a virulence factor for the organism by aiding escape of the bacterium from the vacuoles in macrophages

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* To whom correspondence should be addressed. Phone: (617) 552-3616. Fax: (617) 552-2705. E-mail: mary.roberts@bc.edu.

‡ Boston College.

§ University of Pennsylvania School of Medicine.

|| University of Illinois.

[1] Abbreviations: cIP, inositol 1,2-(cyclic)-phosphate; CMC, critical micelle concentration; DAG, diacylglycerol; diC_4PC, dibutyroylphosphatidylcholine; diC_6PC, dihexanoylphosphatidylcholine; diC_7PC, diheptanoylphosphatidylcholine; DOPMe, 1,2-dioleoylphosphatidylmethanol; GPI, glycosylphosphatidylinositol; I-1-P, D-myoinositol 1-phosphate; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; SUV, small unilamellar vesicle; TIM barrel, triose phosphate isomerase; TX-100, Triton X-100.

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(*9*). PI-PLC aids in the escape of the single-membrane primary vacuole (*9*−*11*) and in the disruption of the inner membrane of the double-membrane spreading vacuole (*12*). A broad-range PLC that primarily targets PC also plays a role in the infectivity of this organism that is distinct from that of the PI-PLC (*13*−*15*). The *L. monocytogenes* PI-PLC appears to share the same general base and acid catalytic mechanism as *Bacillus* sp. PI-PLC (*16, 17*), and the enzyme has been shown to be activated by short-chain phosphatidylcholine (PC) molecules as well (*18*). There are, however, several major differences between the *L. monocytogenes* PI-PLC and *Bacillus* homologues, namely, a high pI (>9), kinetic activation by salts, and relatively weak activity toward GPI anchors (*17, 19*).

In this work, water-soluble synthetic short-chain phosphatidylinositol and cIP along with PI/detergent mixed micelles and PI/PC vesicles were used as substrates to improve our understanding of how salts and other phospholipids affect both steps of PI hydrolysis by the *L. monocytogenes* PI-PLC. The results indicate that *L. monocytogenes* PI-PLC binds tightly to anionic phospholipids (e.g., PI and PG) and tends to form aggregated complexes with those anionic lipids. The enzymatic specific activity is much lower when these complexes form. The two types of activators previously shown for this enzyme work by different mechanisms. Neutral amphiphiles, which enhance both steps of catalysis regardless of the aggregation state of the substrate, bind directly, though weakly, to the protein and enhance its catalytic ability (at sufficiently high mole fractions, the amphiphiles can prevent the enzyme from forming the aggregated complexes). In contrast, moderate ionic strength (e.g., salts) affects only the phosphotransferase reaction and then is most efficient when an activating interface is present. The latter is likely to be the result of altering the surface electrostatics and reducing the formation of aggregated complexes, and possibly altering the residence time of the enzyme on interfaces. Several point mutations of surface hydrophobic residues show that the unusual kinetic behaviors have a strong hydrophobic component. A model rationalizing the role of both types of activators in modulating the enzyme activity in situ is presented.

MATERIALS AND METHODS

Chemicals. Short-chain dibutyroylphosphatidylinositol (diC_4PI) was acquired from Echelon Biochemicals. Other phospholipids, including the long-chain lipids POPC, POPS, POPG, and DOPMe, PI from bovine liver, and the short-chain phospholipids diC_7PC, and diC_6PC, were obtained from Avanti Polar Lipids and used without further purification. Crude soybean PI (50%) was used for the enzymatic generation of cIP as described previously (*2*) by taking advantage of the fact that the phosphotransferase (PI cleavage) activity is much higher than the phosphodiesterase (cIP hydrolysis) activity for bacterial PI-PLC. Carboxyfluorescein (a mixture of 5- and 6-carboxyfluorescein) was acquired from Eastman Kodak Co. Citraconic anhydride (2-methylmaleic anhydride) was purchased from Pierce. Other reagents, including Triton X-100 and D_2O (99.9%), were purchased from Sigma.

L. monocytogenes PI-PLC Expression and Purification. The IMPACT-CN system (New England Biolabs) was used to construct the plasmid for expression of this PI-PLC protein as a fusion protein with both a chitin binding domain for ease of purification and inducible self-cleavage activity to remove the fusion tag (*20, 21*). The *plcA* gene without the region encoding the signal sequence was inserted into the pTYB11 N-terminal fusion vector between *Sap*I and *Eco*RI restriction sites following the manufacturer's instructions. The entire DNA sequence of the inserted target gene was determined and compared with that of pBS1462 (our original source of the *plcA* gene) to ensure no PCR-induced errors were introduced. The recombinant plasmid, IMPACT-Y, was transformed into *Escherichia coli* ER2566 cells for overexpression. An aliquot of the overnight culture was used to inoculate fresh LB medium with 100 μg/mL ampicillin in a rotary shaker (200 rpm) at 37 °C. Expression of recombinant PI-PLC was induced by adding IPTG at a final concentration of 0.8 mM when the OD_{600} of growing culture was between 0.7 and 0.8. After induction, the culture was incubated at 16 °C for 20 h, harvested by centrifugation, and stored at −80 °C. If needed, the frozen cell pellets were thawed at room temperature, resuspended in 50 mL of ice-cold column buffer [20 mM Tris-HCl and 0.5 M NaCl (pH 8.4)], sonicated on ice, and centrifuged to remove the cell debris. The cell extract supernatant of ER2566 harboring IMPACT-Y had high PI-PLC activity, albeit no obvious band of 88 kDa on the SDS−PAGE gel. Recombinant *L. monocytogenes* PI-PLC was then obtained following the purification protocol in the manufacturer's instruction manual. All protein concentrations were determined by a Lowry assay. The best yield for recombinant *L. monocytogenes* PI-PLC was ∼6 mg/L. The stock solution could be stored at 4 °C without activity loss for ∼1 month. SDS−PAGE analysis showed that the purity of PI-PLC after the chitin column was more than 85% and that PI-PLC was contaminated with a band around 50 kDa (the intein tag judging from the SDS−PAGE gel of chitin beads after elution of PI-PLC). If needed, a SP Sepharose Fast Flow (Amersham Pharmacia Biotech) column was used to further purify PI-PLC right after dialysis. The dialyzed eluate from the chitin column was applied at 2 mL/min onto the strong cation exchange column (∼25 mL) equilibrated with 100 mL of starting buffer [50 mM sodium phosphate buffer (pH 7.0)]. The protein was eluted using a NaCl gradient from 0 to 0.5 M in 50 mM sodium phosphate (pH 7.0) at rate of 2 mL/min, dialyzed to remove the high salts, filtered, and concentrated. Only the PI-PLC band was observed on the SDS−PAGE gel after these two columns.

A series of mutant *plcA* genes harboring mutations encoding W49A, L151A, L235A, F237A, F237W, and W49A/F237A were constructed using the Quik-Change site-directed mutagenesis kit. All mutant *plcA* genes were sequenced to confirm that the correct mutations were introduced. Each recombinant IMPACT-Y plasmid was transformed into *E. coli* ER2566 competent cells for expression of the mutant protein and purification as described above.

Citraconic Anhydride Modification of PI-PLC. To alter the surface charge of the *L. monocytogenes* PI-PLC, the protein was treated with citraconic anhydride. Chemical modification with this reagent changes the positive charges of lysine residues on the protein surface to negative ones. Sodium phosphate, 0.5 M at pH 8.5, was used to provide adequate buffer capacity. The PI-PLC to be modified was purified with two columns (chitin column and SP Sepharose

Fast Flow column), dialyzed against reaction buffer, and concentrated; 200 μL of PI-PLC (6.5 mg/mL) was incubated with 50 μL of citraconic anhydride (113 mM, diluted with reaction buffer) in 1 mL of reaction buffer at room temperature for 3 h, after which the reaction mixture was dialyzed extensively against 20 mM Tris-HCl (pH 7.0) to quench the reaction. The change in pI for the modified protein was measured via two-dimensional gel electrophoresis separating the protein by isoelectric point and then mass. The two batches of citraconylated protein showed that the pI changed from the 9−10 region for the unmodified enzyme (which migrated as a single peak) to the 7−9 region (two distinct peaks were observed for the modified enzyme).

PI-PLC Size As Determined by Laser Light Scattering. The native protein molecular weight of purified *L. monocytogenes* PI-PLC was determined by laser light scattering (LS) (*22*) at the HHMI Biopolymer Facility and W. M. Keck Foundation Biotechnology Resource Laboratory. Because the protein interacted with the column matrix dramatically, it was analyzed by a microbatch approach in which the sample from an injector loop was eluted through the system and the UV/LS/refractive index signals were monitored while the buffer was continuously delivered from the HPLC system. Conditions (1.1 mL protein sample, 500 μL sample loop, and 0.3 mL/min for 80 s) were established to provide a reading "plateau" (~0.2 mL, free of air and particle) in all three detectors. Transferrin with an extinction coefficient comparable to that of *L. monocytogenes* PI-PLC was used as a standard, and the same calibration constant was applied to analyze the PI-PLC sample. The concentrations of PI-PLC during the reading plateau were measured with the UV absorbance at 280 nm (extinction coefficient of 33710 M^{-1} cm^{-1}, 1 cm length). The molecular weight was determined by solving the equation that relates the excess scattered light to the concentration of solute and the weight-average molecular weight by ASTRA calculations (http://info.med.yale.edu/wmkeck/6_16_98/Astra2a.htm).

Vesicle Preparation. The appropriate amount of a lipid stock solution in chloroform was placed in a 20 mL glass scintillation vial; the chloroform was removed with a rotary evaporator. The resulting lipid film was dissolved in 5 mL of water, frozen on dry ice, lyophilized overnight, and rehydrated with the appropriate buffer. Lipids supplied as a powder were dissolved in the buffer directly. To prepare small unilamellar vesicles, the chilled phospholipid suspensions were sonicated on ice using a Branson sonifier W-150 ultrasonic cell disruptor with a 1 cm diameter probe until maximum clarity was achieved. Vesicle preparations were usually centrifuged in a benchtop centrifuge (14000 rpm for 3 min) to remove any large particles.

PI-PLC Enzymatic Activity. The specific activity of PI-PLC was measured using ^{31}P NMR spectroscopy (202.3 MHz on a Varian Inova 500 spectrometer) and parameters previously reported (*1, 2*). Two types of assays (end point and continuous time point) were used for phosphotransferase activity (PI as the substrate) and phosphodiesterase activity (cIP as the substrate), respectively. Except for diC_4PI, experiments were conducted in duplicate, and the average specific activities are reported; the errors in specific activities were typically <15%.

For long-chain PI from bovine liver (8 mM), dispersed in diC_7PC or Triton X-100 (TX-100) micelles, or in small unilamellar vesicles (SUVs) in the absence and presence of POPC, in 50 mM HEPES (pH 7.0) containing 0.5 mg/mL BSA, enzyme (covering a range of 0.01−7.2 μg/mL) was added to the 200 μL assay mixture and incubated at 25 °C. The reaction was quenched by the addition of 400 μL of chloroform at appropriate incubation times (from 1 min to a few hours) which were chosen so that less than 20% PI cleavage occurred. The protein concentrations were kept constant within the same series of assays as protein concentration also affects the specific activity of *L. monocytogenes* PI-PLC. The cIP content in the aqueous phase (separated from the organic layer using centrifugation at 14000 rpm for 6 min) was quantified in the ^{31}P NMR spectrum using added glucose 6-phosphate (1 mM) as an internal standard. Triton X-100 and salt effects were also measured using the water-soluble substrate diC_4PI. In this assay, 100 μL assay mixtures containing 2 mM diC_4PI and 1.6 μg/mL enzyme were used.

In the continuous time point assay for cIP hydrolysis, the release of I-1-P product was monitored as a function of time until 20% of the cIP was converted to I-1-P using a ^{31}P NMR experiment in which the preacquisition delay was arrayed. The reaction was initiated by adding 3.6 or 7.2 μg/mL PI-PLC to the 400 μL assay mixture in a NMR tube typically containing 10 mM cIP in the absence and presence of different additives (diC_7PC, POPC, DOPMe, POPS, and salts) in 50 mM imidazole buffer (pH 7.0), incubated at 25 °C, and monitored for a period of time, typically from at least 30 min to a few hours. The rate of cIP hydrolysis was calculated from the slope of I-1-P production as a function of incubation time.

Monolayer Penetration Assay. Lipid monolayers were formed by spreading 5−10 μL of the appropriate lipids (POPC, DOPMe, or a mixture of 70% POPC and 30% DOPMe) dissolved in an ethanol/hexane mixture (1:9, v/v) onto 10 mL of 50 mM HEPES (pH 7.0), with KCl at three different concentrations (0, 0.16, and 0.5 M) contained in a circular Teflon trough (4 cm diameter × 1 cm depth). The surface pressure (π) of the subphase (the solution in the trough) was measured using a Wilhelmy plate attached to a computer-controlled tensiometer as described for the *Bacillus thuringiensis* PI-PLC (*23*). The subphase was gently mixed with a magnetic stir bar, and the monolayer was allowed to equilibrate until a stable surface pressure (defined as the initial surface pressure, π_0) was obtained. Then a protein solution was injected into the subphase through a small hole in the side of the trough without disruption of the monolayer. The change in surface pressure ($\Delta\pi$) was measured as a function of time. Since the value of $\Delta\pi$ depends on the protein concentration in the low concentration range, the protein concentration in the subphase was kept sufficiently high (>3 μg/mL for PI-PLC) to ensure the observed $\Delta\pi$ represented a maximal value (*24*). In general, $\Delta\pi$ is inversely proportional to the π_0 of the lipid monolayer, and extrapolations of the $\Delta\pi$ versus π_0 plot to $\pi_0 = 0$ and $\Delta\pi = 0$ yield the maximum $\Delta\pi$ obtainable and the critical surface pressure (π_c) that specifies an upper limit of π_0 into which a protein can penetrate, respectively.

Fluorescence Assays for Vesicle Leakage. Vesicle leakage promoted by the bound *L. monocytogenes* PI-PLC was monitored by the release of entrapped carboxyfluorescein (*25*). Vesicles were prepared by sonication of the appropriate

lipid (POPC or DOPMe) at 20 mM with a solution of 100 mM carboxyfluorescein in 10 mM HEPES (pH 7.1). The nonencapsulated carboxyfluorescein was removed by rapid filtration of the vesicle solution through a Sephadex G-10 column at room temperature. POPC prepared with 150 mM KCl in the buffer along with the carboxyfluorescein was also prepared. The carboxyfluorescein-loaded vesicles were used immediately after gel filtration. Vesicle integrity in the absence or presence of PI-PLC protein was monitored with a Fluorolog R-3 spectrofluorimeter using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. All the carboxyfluorescein assays were conducted in the dark with a narrow excitation slit width (1 nm). The percent leakage at time t, L_t, was expressed relative to the initial fluorescence, F_o, and the maximum fluorescence, F_{TX}, obtained after complete lysis of the vesicles by the addition of 25 mM Triton X-100: $L_t = (F_t - F_0)/(F_{TX} - F_0) \times 100\%$.

Precipitation and Turbidity Assays. We assessed aggregation and precipitation of PI-PLC with SUVs by incubating protein (0.036 mM) with pure POPG, pure POPC, and POPG/POPC (1:1 or 1:25) SUVs and then separating any precipitate by centrifugation (15000 rpm for 10 min with a benchtop centrifuge). We measured the relative partitioning of the protein in the precipitate versus what remains in solution by first lyophilizing both precipitate and solution and then examining relative amounts of protein in each phase by SDS−PAGE.

The time course for aggregation of DOPMe (0.86 mM)/POPC (3.1 mM) SUVs [in 50 mM HEPES (pH 7.0)] promoted by different concentrations of PI-PLC (0.15, 0.75, and 1.5 mg/mL) was monitored by the increase in the optical density at 350 nm of the solution after the addition of protein (*26*). Monitoring the OD_{350} of corresponding vesicles without protein provided baseline turbidity.

^{31}P NMR Line Width Assay. Changes in phospholipid ^{31}P line widths were used to monitor the binding of diC_6PC and diC_7PC to *L. monocytogenes* PI-PLC (2 mg/mL). The line width of the PC resonance was measured at various concentrations (0.1−20 mM) in the absence and presence of enzyme. Line width changes upon micellization of both of these short-chain lipids were small, so that the difference in line width caused by the presence of enzyme was initially assumed to reflect the amount of ligand bound to the enzyme in fast exchange with free ligand. As discussed previously (*27*), the difference in line width in the presence and absence of ligand at a given ligand concentration ($\Delta\upsilon_{obs} - \Delta\upsilon_o$) is a function of the total concentration of the enzyme (E_T) and ligand ($[PC]_T$), the bound line width for the E•PC complex ($\Delta\upsilon_b$), the dissociation constant (K_d), and n, the number of ligands bound per enzyme molecule. If there are multiple, independent binding sites for the PC (to form E•PC_n) leading to the change in line width and if [PC] ≫ [E•PC], then the following equation holds:

$$\Delta\upsilon_{obs} - \Delta\upsilon_o = \frac{nE_T[PC]_T^{n-1} \times (\Delta\upsilon_b - \Delta\upsilon_o)}{K_d + [PC]_T^n}$$

RESULTS

Dependence of L. monocytogenes PI-PLC Specific Activity on Protein Concentration. Previously, the pH behavior for the *L. monocytogenes* PI-PLC phosphotransferase reaction was shown to vary slightly with the detergent matrix for the PI. A sharp optimum pH of 7.0 was observed toward PI solubilized in deoxycholate micelles with <30% activity at pH 6.5 and 7.5 (the suspension of PI in deoxycholate was no longer clear at pH <6.5), while a broader optimum pH range, from 5.5 to 6.5, was observed for the enzyme acting on PI in TX-100 micelles (*17*). For the sake of comparison, we tested the effect of pH on the phosphodiesterase reaction in the absence of detergent. The enzyme was optimally active toward cIP at pH 7.0, with 67 and 57% maximum activity at pH 6.5 and 7.5, respectively (Figure 1A). Thus, pH 7.0 was used for both phosphotransferase and cyclic phosphodiesterase kinetic assays.

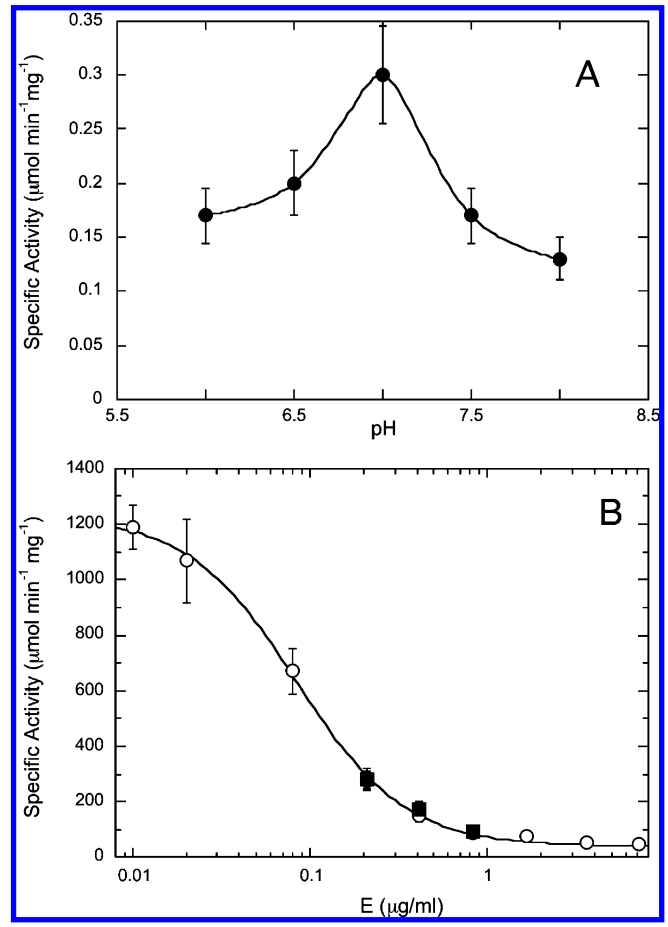

FIGURE 1: (A) Dependence of cIP hydrolysis by *L. monocytogenes* PI-PLC (3.6 μg/mL) on the pH of the solution. (B) Dependence of *L. monocytogenes* PI-PLC phosphotransferase specific activity on enzyme concentration (micrograms per milliliter): PI (8 mM) solubilized in either 32 mM TX-100 (○) or 32 mM diC_7PC (■).

PI can be presented to PI-PLC in a range of different matrices: micelles, unilamellar vesicles, or water/organic solvent comixtures (*1, 2, 4, 28, 29*). Differences in PI-PLC activity toward the different types of interfaces can often provide insights into what factors control the activity of this enzyme. However, *L. monocytogenes* PI-PLC exhibited an added complication in that protein concentration also affected enzyme activity. Since the assay for phosphotransferase activity is not continuous, we monitored the generation of cIP from PI/TX-100 mixed micelles at various time points to avoid the influence of a lag or burst in product formation. As shown in Figure 1B, the phosphotransferase specific activity of *L. monocytogenes* PI-PLC toward PI (8 mM) in TX-100 (32 mM) mixed micelles increased dramatically from

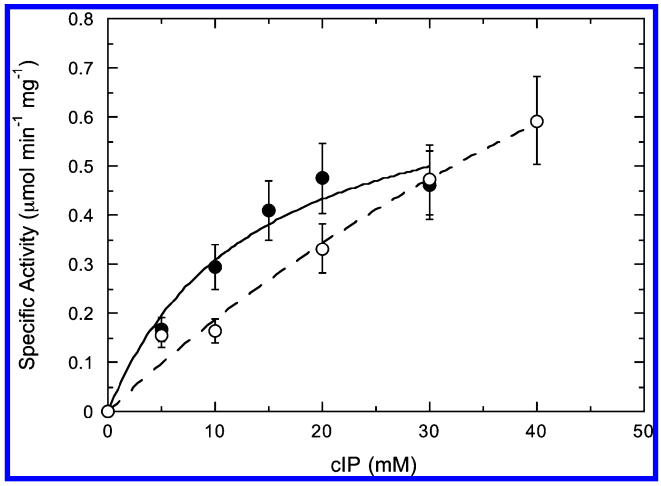

FIGURE 2: Effect of cIP concentration on *L. monocytogenes* PI-PLC phosphodiesterase specific activity: (●) 3.6 and (○) 7.2 μg/mL PI-PLC. Assay conditions included 50 mM imidazole, pH 7.0, and variable cIP concentrations.

150 to 1200 μmol min^{-1} mg^{-1} as the protein was diluted from 0.4 to 0.01 μg/mL. Further dilution of the protein had only a weak effect on specific activity. A similar dependence of specific activity on protein concentration was also observed with PI/diC_7PC mixed micelles. To rule out any possible inhibitors from the expression/purification system, purified recombinant *L. monocytogenes* PI-PLC was further purified with cation exchange or gel filtration (with a high salt concentration) chromatography, and the concentration dependence of specific activity on enzyme concentration still persisted.

The loss of activity with high enzyme concentrations has been discussed by Wang (*30*) for an association−dissociation enzyme system. PI-PLC could be aggregating in solution with the monomer being more active than the oligomer. However, this PI-PLC interacted anomalously with gel filtration resins even in the presence of 0.5 M NaCl, so that another method was needed to assess the size of the protein in solution. Laser light scattering, with some modification from the standard protocol, was used to characterize the native molecular mass of the protein. The average molecular mass for *L. monocytogenes* PI-PLC was determined to be 28.2 kDa at a concentration of 0.14 mg/mL, well above that used in the kinetic assays. This is quite close to the predicted molecular mass of 33 kDa determined from the sequence. Thus, this PI-PLC exists as a monomer in solution in the absence of detergent. If the protein aggregation does account for the unusual dependence of specific activity on protein concentration, the oligomerization of PI-PLC must occur only in the presence of an interface and/or the anionic substrates.

The phosphodiesterase activity of *L. monocytogenes* PI-PLC toward 5−40 mM cIP without detergent was investigated at two different protein concentrations. Because cIP is a poorer substrate than PI, considerably higher enzyme concentrations (3.6 or 7.2 μg/mL) were needed. In this high range of enzyme concentration, the activity was low and varied little between these two protein concentrations. However, there was a very significant difference in kinetic parameters extracted for the two different enzyme concentrations (Figure 2). In both cases, the data could be fit to the Michaelis−Menten equation: $V_{max} = 0.7 \pm 0.1$ μmol min^{-1} mg^{-1} and $K_m = 12.5 \pm 5.0$ mM at the lower PI-PLC concentration (3.6 μg/mL), and $V_{max} = 2.0 \pm 1.0$ μmol min^{-1} mg^{-1} and $K_m = 100 \pm 62$ mM at double the enzyme concentration. Although the errors in V_{max} and K_m are large for the higher protein concentration, the major effect of the higher enzyme concentration appears to be an increase in K_m for water-soluble substrate cIP. Since *L. monocytogenes* PI-PLC exists as a monomer in solution as shown by the laser light scattering measurement, the enzyme may aggregate with the anionic substrate present (in this case, soluble cIP). Such aggregation would appear to specifically increase the apparent K_m.

Effect of Detergent or Vesicle Interface on PLC Activities. The *Bacillus* sp. PI-PLC enzymes exhibit a specific activation of both phosphotransferase and phosphodiesterase steps by interfaces containing phosphatidylcholine (*2*). It has been suggested that such activation also occurs for the *L. monocytogenes* PI-PLC (*18*). Therefore, we examined the effect of micelle matrix on the phosphotransferase activity of recombinant *L. monocytogenes* PI-PLC toward PI dispersed in TX-100. The enzyme concentration, as well as detergent, was varied. TX-100 is a nonionic detergent that clarifies PI bilayers at >2:1 TX-100:PI ratios. If the surface concentration of PI is important for *L. monocytogenes* PI-PLC activity, then increasing the TX-100 concentration at a fixed PI concentration (8 mM) will eventually "inhibit" the enzyme leading to lower specific activity. Such surface dilution inhibition has been documented for the *B. thuringiensis* PI-PLC (*2*). However, *L. monocytogenes* PI-PLC presented different kinetic patterns depending on the protein concentration (Figure 3A). For 0.4 μg/mL enzyme, the enzyme exhibited no decrease in specific activity up to $X_{det} = 0.94$ (16:1 TX-100:PI). In fact, the enzyme phosphotransferase specific activity increased dramatically as the surface concentration of the detergent increased above 0.90. The same amount of PI-PLC exhibited the same specific activities toward PI presented in diC_7PC micelles, suggesting that the diC_7PC and TX-100 detergents were essentially equivalent. A similar trend, but with higher specific activities, was observed when the protein concentration in the assay was decreased 2-fold.

At a much more dilute concentration, 0.02 μg/mL, a more typical "surface dilution curve" was observed with the maximum phosphotransferase specific activity around $X_{det} = 0.78$ (3.5:1 TX-100:PI). This detergent:substrate ratio for optimal activity is higher than what is needed to minimally solubilize PI bilayers (2:1 TX-100:PI). It has been reported that PI in predominantly PC bilayers is partially demixed (*31*). A similar demixing in mixed micelles could occur unless an excess of detergent is used. Alternatively, this could reflect a distinct activation by the detergent. At the very low concentration of this PI-PLC, the specific activity of *L. monocytogenes* PI-PLC decreased above $X_{det} = 0.80$ as might be expected for a regime in which the surface concentration of substrate influences the enzyme action. The dilute enzyme is very sensitive to the mole fraction of PI in the interface. Comparing the activity curves of three protein concentrations in Figure 3A shows that a significantly increased concentration of TX-100 could compensate for the lower specific activity at high enzyme concentrations, since comparable high activities (1155 and 1260 μmol min^{-1} mg^{-1}) were achieved for all the protein concentrations examined. If the protein concentration decreased further, a lower optimum

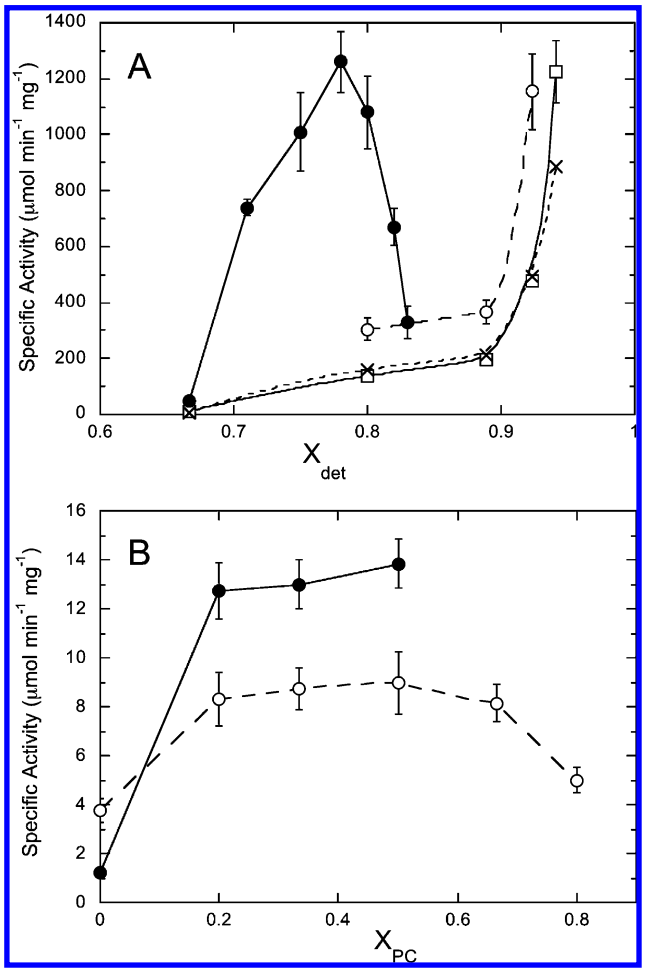

FIGURE 3: (A) Dependence of *L. monocytogenes* PI-PLC phosphotransferase specific activity on the mole fraction of detergent: 0.4 μg/mL PI-PLC with 8 mM PI solubilized in TX-100 (□) or diC$_7$PC (×) and 0.02 (●) or 0.2 μg/mL (○) PI-PLC with the PI solubilized in TX-100. (B) Dependence of phosphotransferase specific activity on the mole fraction of POPC in PI (8 mM)/POPC SUVs with 0.4 (●) or 1.6 μg/mL (○) enzyme.

TX-100:PI ratio might be expected. However, 0.02 μg/mL is the limit of our assay system. Using [^{3}H]PI as the substrate, the phosphotransferase activity of PI-PLC was previously measured at 0.003 μg/mL enzyme. The optimal *L. monocytogenes* PI-PLC specific activity (~2900 μmol min^{-1} mg^{-1}) toward 0.08 mM PI micelles dispersed in TX-100 or deoxycholate was reported at a TX-100:PI ratio of 10:1 or deoxycholate:PI ratio of 30:1 (*17*). The higher specific activity compared to what we observe with low PI-PLC or moderate PI-PLC concentrations with high mole fractions of detergent (X_{det}) is likely due to the addition of ammonium sulfate in the previous report.

The activity of *L. monocytogenes* PI-PLC toward a fixed bulk concentration of PI (8 mM) diluted in a POPC vesicle matrix was also studied. Compared with PI/TX-100 micelles, unilamellar vesicles of long-chain PI are poor substrates for PI-PLC from *L. monocytogenes*, necessitating higher protein concentrations for these assays. At a protein concentration of 1.6 μg/mL, the enzyme phosphotransferase specific activity increased ~2.2-fold as the mole fraction of POPC increased from 0 to 0.2. Once the POPC mole fraction was greater than 0.2 (at a fixed PI concentration), the *L. monocytogenes* PI-PLC specific activity remained constant to a POPC mole fraction of 0.5 and then decreased as the

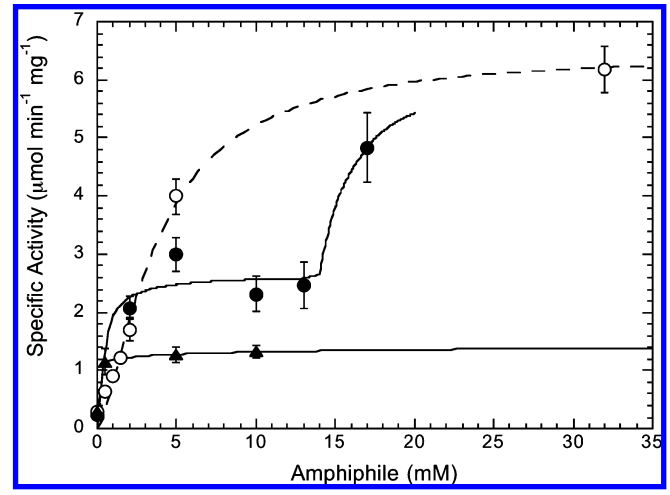

FIGURE 4: Effect of diC$_6$PC (●), diC$_7$PC (○), or POPC SUVs (▲) on cIP (10 mM) hydrolysis by 3.6 μg/mL *L. monocytogenes* PI-PLC.

mole fraction increased further to 0.8 (Figure 3B). Similar to that toward PI/TX-100 micelles, higher specific activities toward PI/POPC SUVs were also observed at a lower enzyme concentration (0.4 μg/mL); the activation with 0.2 mole fraction in the vesicle was also larger than with the higher enzyme concentration.

Examining amphiphile effects with monomeric substrate, in this case cIP, is one way of separating activator effects that alter the substrate versus those that alter the enzyme. With cIP at a fixed concentration, we can screen different additives for their effect on *L. monocytogenes* PI-PLC activity toward this monomeric substrate. Ryan and coworkers (*18*) have shown that diC$_6$PC at concentrations below its CMC activates this enzyme with respect to nonnatural water-soluble PI substrates. In our system, the plot of phosphodiesterase specific activity of *L. monocytogenes* PI-PLC versus diC$_6$PC concentration was bimodal (Figure 4). Below the CMC of diC$_6$PC (14 mM), there was an ~10-fold increase in activity upon the addition of diC$_6$PC; a second increase in specific activity was observed around the CMC of diC$_6$PC, with PI-PLC activity 20-fold higher than for cIP alone. A slightly more hydrophobic amphiphile, diC$_7$PC, also enhanced the activity of the enzyme toward the natural soluble substrate cIP (Figure 4). The bimodal dependence of specific activity on diC$_7$PC concentration is consistent with the PC interfaces being more potent activators than monomeric PC at concentrations where it is monomeric in the absence of protein. POPC SUVs also enhanced cIP hydrolysis (Table 1 and Figure 4), although the maximum activity was considerably lower than for diC$_7$PC or TX-100 activation. SUVs composed of anionic nonsubstrate phospholipids (POPS and DOPMe) strongly inhibited the cIP hydrolysis reaction (Table 1). Vesicles with 0.2 mole fraction POPS disproportionately reduced *L. monocytogenes* PI-PLC activity toward cIP. Therefore, the enzyme must bind to those anionic phospholipids more tightly than it binds to POPC, and this interaction prevents cIP binding and hydrolysis.

Unlike what was observed for *B. thuringiensis* PI-PLC, TX-100 was also an effective activator of cIP hydrolysis by *L. monocytogenes* PI-PLC. Comparable activities were obtained with 3 mM TX-100 or 5 mM diC$_7$PC (Table 1). Since the substrate cIP is monomeric, the amphiphile must activate *L. monocytogenes* by a direct interaction. However,

Table 1: Effect of Different Additives on cIP (10 mM) Hydrolysis Catalyzed by *L. monocytogenes* PI-PLC[a]

assay system	[PI-PLC] (μg/mL)	additive (mM)	relative activity[a]
cIP	7.2	TX-100 (3)	14.8
cIP	7.2	diC_7PC (5)	15.1
cIP	7.2	POPC (2)	4.38
cIP	7.2	DOPMe (1)	<0.1
cIP	7.2	POPS (1)	0.43
cIP	3.6	POPC (2)	7.94
cIP with 2 mM POPC	3.6	POPS (0.1)	0.31
cIP	7.2	$(NH_4)_2SO_4$ (150)	0.65
cIP with 5 mM diC_7PC	7.2	$(NH_4)_2SO_4$ (100)	0.83

[a] Assays measured in 50 mM HEPES (pH 7.0) at 25 °C. Relative activity is the ratio of the specific activity measured with the additive present compared to the activity in the absence of that additive (salts or lipids). For 10 mM cIP without additives, the enzyme specific activity was 0.29 μmol min^{-1} mg^{-1} for 3.6 μg/mL protein or 0.16 μmol min^{-1} mg^{-1} for 7.2 μg/mL protein.

whether in doing so it disfavors anionic ligand-induced protein aggregation that may be occurring or promotes a more active form of the enzyme is not clear.

Effect of Salts. Salt activation of PI cleavage by *L. monocytogenes* PI-PLC has been documented previously (*17*). Although no explanation was proposed, it was noted that in the absence of high salt, *L. monocytogenes* PI-PLC in solution behaved as a large aggregate on some gel filtration columns, but that 1 M $(NH_4)_2SO_4$ attenuated this behavior. Salts can enhance hydrophobic interactions (e.g., promote insertion of protein hydrophobic side chains into bilayers) as well as weaken electrostatic interactions that hold complexes together. With this in mind, we examined the effect of several different salts [$(NH_4)_2SO_4$, NH_4Cl, K_2SO_4, and KCl] on *L. monocytogenes* PI-PLC cleavage of PI in TX-100 or diC_7PC mixed micelles and hydrolysis of cIP. As shown in Table 2, the ionic strength, and not the salt identity, was important for *L. monocytogenes* PI-PLC activation toward PI in micelles. At a fixed enzyme concentration and 8 mM PI (solubilized in 32 mM TX-100), *L. monocytogenes* PI-PLC activity displayed a hyperbolic dependence on the concentration of $(NH_4)_2SO_4$. For 0.2 μg/mL *L. monocytogenes* PI-PLC, the apparent V_{max} was 2022 $\pm$ 202 μmol min^{-1} mg^{-1} with a $K_{0.5}$ (concentration of salt for half the maximum specific activity) of 38 $\pm$ 16 mM; for 1.6 μg/mL *L. monocytogenes* PI-PLC, the apparent V_{max} decreased somewhat to 1355 $\pm$ 256 μmol min^{-1} mg^{-1} with a comparable salt $K_{0.5}$ (42 $\pm$ 20 mM). The lower V_{max} extrapolated from the salt dependence of activity at an 8-fold higher *L. monocytogenes* PI-PLC concentration indicates that the presence of salt does not completely eliminate the unfavorable factors associated with high enzyme concentrations. The ratio of specific activity for 0.21 μg/mL PI-PLC compared to 1.6 μg/mL at 0, 20, and 100 mM $(NH_4)_2SO_4$, is 3.7, 1.6, and 1.5, respectively. These decreases suggest that $(NH_4)_2SO_4$ does partially compensate for the negative effects of high enzyme concentration.

PI-PLC cleavage of PI presented in SUVs was also activated by salt (Table 2). With KCl, the effect leveled off around 0.3 M salt. With PC in the SUV along with the PI, the addition of salt also led to a much larger increase in specific activity (Table 2).

In contrast to its activation of PI cleavage by *L. monocytogenes* PI-PLC, $(NH_4)_2SO_4$ (as well as other salts) inhibited cIP hydrolysis regardless of whether diC_7PC was present (Table 1). These results make it unlikely that salt biases the enzyme toward a more active conformation; otherwise, it should have enhanced cIP hydrolysis as well as PI cleavage. The added salt could alter the properties of substrate interfaces, making them more susceptible to *L. monocytogenes* PI-PLC, or it could alter the interaction of the enzyme with the interface. One way of testing these explanations is to examine the effect of salts and amphiphiles on cleavage of diC_4PI, a soluble phosphodiesterase substrate with no tendency to partition into aggregates as long as the substrate concentration is low [the CMC for this lipid is likely to be >150 mM since diC_4PC has a CMC of 250 mM (*32*) and other short-chain PI species exhibit CMC values comparable to the same chain length PC compound (*33*)]. As a way of ruling out any aggregation of the diC_4PI induced by the amphiphile or salt, we measured the ^{31}P line width of 2 mM diC_4PI in buffer in the absence and presence of 100 mM KCl, 100 mM TX-100, or both components. The line width, 1.6 $\pm$ 0.1 Hz, was unchanged with the additives.

Table 2: Effect of Inorganic Salts on the Specific Activity of *L. monocytogenes* PI-PLC toward the PI Substrate Presented in TX-100 Mixed Micelles, in SUVs in the Absence or Presence of POPC, or as Monomers with Added Amphiphiles

substrate	amphiphile	salt (M)	specific activity (μmol min^{-1} mg^{-1})
PI[a]	TX-100	–	274
	TX-100	$(NH_4)_2SO_4$ (0.10)	1430
	TX-100	NH_4Cl (0.15)	936
	TX-100	K_2SO_4 (0.10)	1120
	TX-100	KCl (0.15)	1030
PI[b]	–	–	1.3
	–	KCl (0.10)	54
	–	KCl (0.20)	330
	–	KCl (0.30)	511
	POPC	–	9.0
	POPC	$(NH_4)_2SO_4$ (0.10)	127
diC_4PI[c]	–	–	0.7[d]
	–	KCl (0.075)	1.2[d]
	TX-100	–	1.8[d]
	TX-100	KCl (0.075)	11.2
	diC_6PC	–	1.2
	diC_6PC	KCl (0.075)	10

[a] Assays toward 8 mM PI presented in TX-100 (32 mM) mixed micelles used 0.2 μg/mL PI-PLC in 50 mM HEPES (pH 7.0). All specific activities are the average of at least two samples; errors in these values are less than 15%. [b] Assays toward PI (8 mM) in SUVs in the absence or presence of an equimolar amount of POPC used 1.6 μg/mL *L. monocytogenes* PI-PLC; cIP was the sole product. [c] Monomeric diC_4PI (2 mM) in 50 mM HEPES (pH 7.0) treated with 1.6 μg/mL PI-PLC. Added amphiphiles such as TX-100 (32 mM) or diC_6PC (5 mM, monomeric in the absence of enzyme) had little effect on PI-PLC specific activity in the absence of added salt. [d] Both cIP and I-1-P were generated, so the specific activity represents the sum of both cIP and I-1-P production.

Keeping the diC_4PI concentration at 2 mM, we measured the effect of KCl and TX-100 on *L. monocytogenes* PI-PLC activity toward the soluble substrate (Table 2). With either 75 mM KCl or 32 mM TX-100 only, the specific activity of *L. monocytogenes* PI-PLC toward diC_4PI increased slightly (~1.6-fold). However, if both salt and TX-100 were present, there was an ~14-fold increase in *L. monocytogenes* PI-PLC activity toward diC_4PI. A similar synergistic effect was observed with monomeric diC_6PC added; a much larger activation was seen with both KCl and the amphiphile present. This synergistic effect cannot reflect alteration of the substrate interface because the substrate is monomeric.

This suggests that for the phosphotransferase reaction, the presence of an amphiphile is important for the kinetic activation observed with moderate ionic strength. KCl (0.1 M) could lower the CMC of diC_6PC slightly, but the effect in the absence of enzyme is not significant (*34*). However, the slight decrease in specific activity for *L. monocytogenes* PI-PLC acting on cIP with monomeric diC_6PC approaching the CMC (Figure 4) may suggest that the enzyme itself could nucleate "mini" micelle formation when it binds a diC_6PC molecule. Studies of *B. thuringiensis* PI-PLC showed that diC_6PC added at a monomeric concentration can bind to the protein, at a site distinct from the catalytic site, and activate the enzyme (*27*). DiC_4PI is a poor substrate for *L. monocytogenes* PI-PLC, necessitating long incubation times (2−5 h) if the same enzyme concentration is to be used in the absence and presence of salts and amphiphiles. With TX-100 present, final products included both cIP and I-1-P for incubation times longer than 1 h. However, if diC_6PC was added as the amphiphile, cIP was the only product even after 2 h. There is a difference when the *L. monocytogenes* PI-PLC interacts with TX-100 versus a short-chain PC molecule such that binding of diC_6PC to the protein promoted release of cIP from the active site, while with TX-100 as the amphiphile, both products were detected. Nonetheless, both amphiphiles effectively enhanced the enzyme activity.

Binding of PI-PLC to Interfaces. Short-chain PC molecules activate the *L. monocytogenes* PI-PLC toward cIP in the absence of salt, so there must be a direct interaction of the enzyme with the PC molecules. This can be probed by ^{31}P NMR line width studies (*27*). As shown in Figure 5, a threshold of diC_7PC ($\geq$1 mM) was needed before significant broadening of the phospholipid resonance (0.8 Hz maximum under the conditions used) was detected. As more PC was added, the line width difference in the presence and absence of *L. monocytogenes* PI-PLC decreased. This type of profile is typical for multiple PC molecules interacting with the enzyme (and occupation of all these sites needed before the measured spectral change occurs). As shown in Figure 5B, the line width difference for diC_7PC caused by the presence of *L. monocytogenes* PI-PLC (2 mg/mL) was consistent with an "*n*" value of 3−4, a $\Delta\upsilon_b - \Delta\upsilon_0$ difference of 5.5 ± 0.6 Hz, and an apparent K_d of 1.5 ± 0.6 mM. The analysis with diC_6PC binding to *L. monocytogenes* PI-PLC yielded the same *n*, a $\Delta\upsilon_b - \Delta\upsilon_0$ difference of 3.3 ± 0.8 Hz, and a K_d of 17 ± 10 mM (data not shown). In both cases, the "K_d" describing the interaction was essentially at the CMC of the short-chain PC. The line width for bound PC would suggest significant mobility consistent with a small aggregate or mini micelle with the protein.

Binding of *L. monocytogenes* PI-PLC to SUVs of POPC or anionic vesicles (DOPMe, POPG, etc.) was also examined using a filtration/centrifugation binding assay (*23*). *L. monocytogenes* PI-PLC (30 μg/mL or 0.9 μM) had weak affinity for POPC SUVs; essentially >90% of the protein was eluted through the filter upon centrifugation even after incubation with 1 mM POPC SUVs. In contrast, this bacterial PI-PLC bound very tightly to anionic phospholipid SUVs regardless of headgroup. For DOPMe concentrations of >10 μM, essentially all the protein was bound to the phospholipids and none passed through the filter upon centrifugation. The dramatically slower rate for the solution to pass through the filter suggested that large aggregates of the proteins and

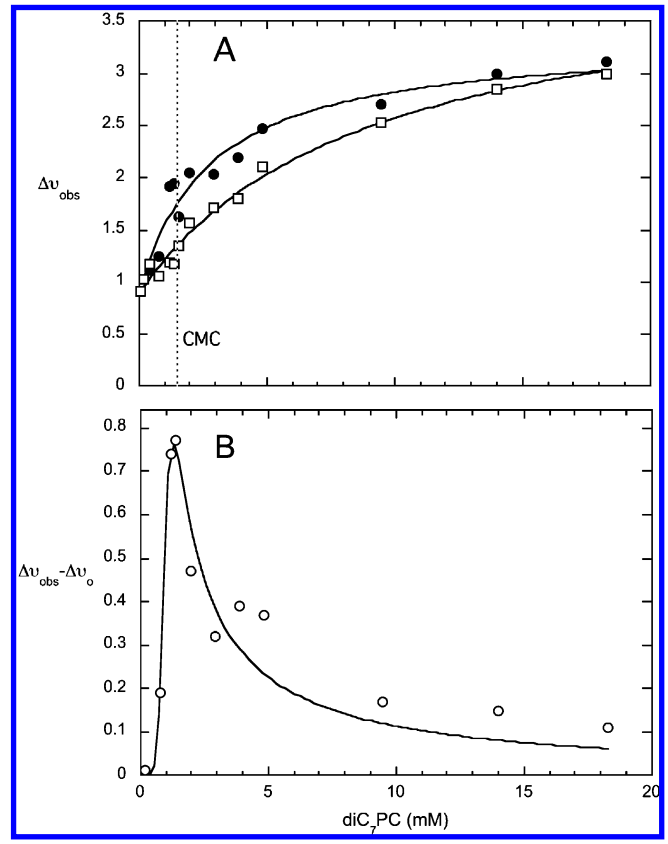

FIGURE 5: (A) ^{31}P line width (hertz) of diC_7PC in the absence (□) and presence (●) of *L. monocytogenes* PI-PLC. The dotted line indicates the CMC of pure diC_7PC. (B) Increase in line width (hertz) for diC_7PC induced by the inclusion of 2 mg/mL PI-PLC (differences in line width in panel A). The line represents the optimized fit to the equation $\Delta\upsilon_{obs} - \Delta\upsilon_0 = nE_T[PC]_T^{n-1}[(\Delta\upsilon_b - \Delta\upsilon_0)/(K_d + [PC]_T^n)]$ as described in Materials and Methods.

vesicles might be forming. To test this, we incubated 36 μM PI-PLC (a considerably higher concentration) with different concentrations of POPG, POPC, or mixed POPG/POPC SUVs (Figure 6A). There was a significant anionic lipid-induced precipitation of protein (Figure 6A). With 0.2 mM POPG and 0.036 mM protein, most of the protein (64 ± 10%) was associated with the precipitate. With the concentration of POPG reduced to 0.02 mM, a moderate amount of the protein (42 ± 5%) was still associated with the precipitate. When that amount of POPG was presented in 0.5 mM POPC, the amount of protein in the precipitate was significantly reduced (16 ± 3%). Mixing PI-PLC with pure POPC SUVs led to only 5 ± 3% of the protein in a precipitate, much of which could be due to a small fraction of larger vesicles with some PLC bound. If micelles as opposed to vesicles were examined, more PG was needed to precipitate the protein (data not shown). The amount of each lipid in the precipitate (extracted with methanol and chloroform) was measured with ^{31}P NMR, and the lipid composition in the precipitate for POPG/PC SUVs or micelles was very similar to that in solution. Therefore, increasing the PI-PLC specific activity as the level of detergent or PC is increased in the SUV assay system is likely to reflect (at least in part) disruption of a large, nonproductive aggregated complex of the enzyme with several vesicles that sequesters it from the substrate in other vesicles.

We monitored the progress of aggregation and precipitation by looking at the OD_{350} with continuous stirring (Figure

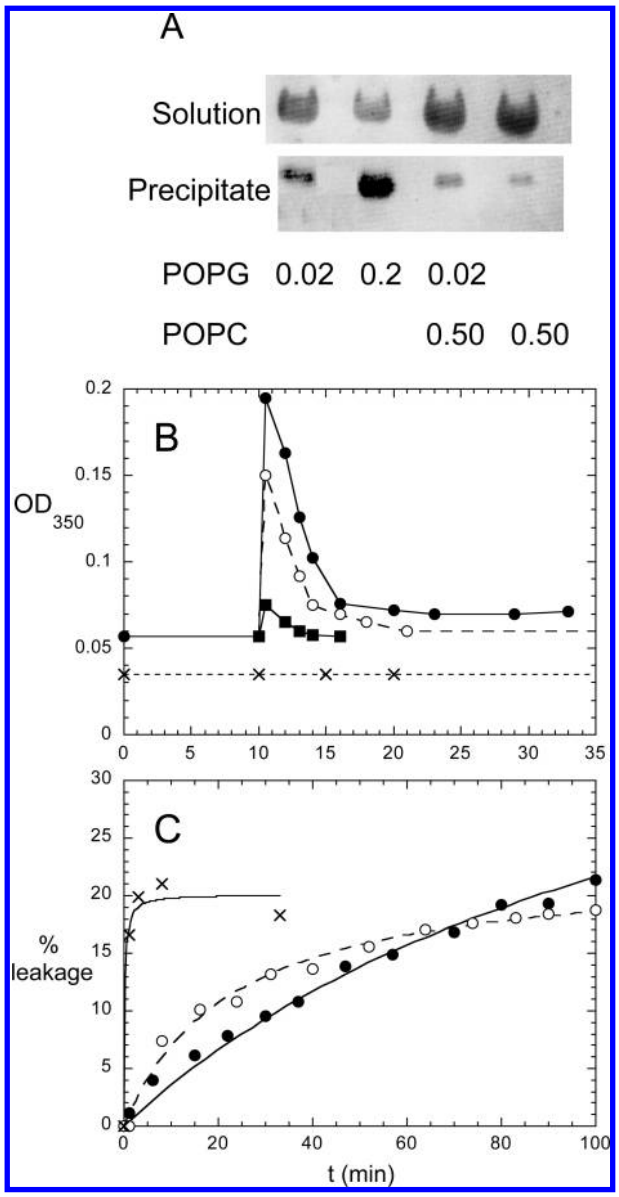

FIGURE 6: (A) Binding of *L. monocytogenes* PI-PLC (1.2 mg/mL, 36 μM) to SUVs can cause precipitation of the vesicles depending on composition. The numbers below each lane indicate the millimolar concentration of PG or PC in SUVs mixed with the protein. (B) Turbidity changes measured at 350 nm (OD_{350}) of 0.86 mM DOPMe/3.1 mM POPC cosonicated SUVs upon addition (at 10 min) of 0.15 (■), 0.75 (○), and 1.5 mg/mL (●) PI-PLC. For the sake of comparison, the effect of PI-PLC added to pure POPC SUVs is shown (×). (C) Carboxyfluorescein released (percent of total entrapped) from 20 mM POPC (○), 20 mM POPC with 150 mM KCl (●), and 20 mM DOPMe (×) vesicles mediated by *L. monocytogenes* PI-PLC (0.5 mg/mL). TX-100 (20 mM) was used to release all entrapped carboxyfluorescein.

6B). After adding different concentrations of *L. monocytogenes* PI-PLC to DOPMe/POPC (1:3.6) SUVs, we observed an instant and very dramatic increase in optical density, corresponding to the formation of the precipitate. The optical density decreased over time but did not settle back to the initial value; the limiting OD_{350} was highest for the highest concentration of protein. Similar behavior was also observed for incubation of this PI-PLC with POPG/POPC SUVs, but not for pure PC vesicles. Since the precipitate could be dispersed by adding salt at concentrations (0.1−0.3 M) comparable to those used in the kinetics, it does not reflect vesicle fusion.

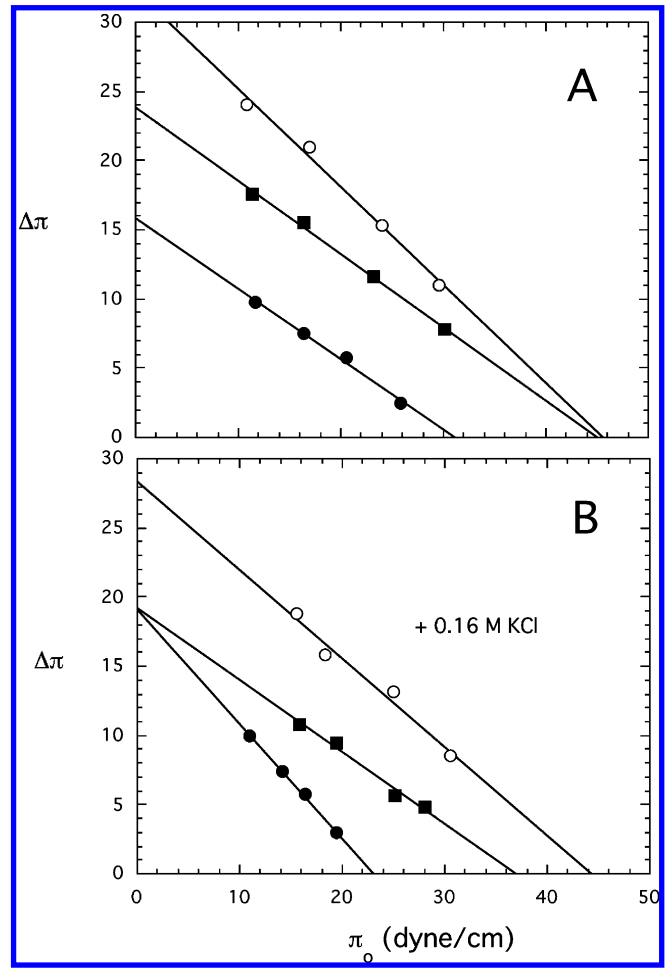

FIGURE 7: Effect of *L. monocytogenes* PI-PLC on the surface pressure of DOPMe (○), POPC (●), and POPC/DOPMe (7:3) (■) monolayers in the (A) absence and (B) presence of 0.16 M KCl.

The release of carboxyfluorescein trapped in POPC/POPG vesicles upon addition of protein was monitored to check the stability of the vesicles in the presence of PI-PLC (Figure 6C). *L. monocytogenes* PI-PLC caused <25% carboxyfluorescein leakage (compared to total leakage from TX-100) within 2 h (the reaction time course used for most assays) for three vesicle systems [POPC, POPC with KCl, and DOPMe (as the nonhydrolyzable substrate analogue)]. The initial release rate of carboxyfluorescein increased in the order of the affinity of the enzyme for the interface (DOPMe > POPC > POPC/KCl). The more tightly the enzyme binds to a given vesicle surface, the longer the enzyme stays on that structure (a nonproductive aggregated complex in the case of the pre-DOPMe SUVs) and less chance it will dissociate and bind to another vesicle.

Monolayer studies provide insight into how PC and likely other neutral amphiphiles as well as salt contribute to the kinetic activation of the enzyme. *L. monocytogenes* PI-PLC binds tightly to anionic monolayers of DOPMe (Figure 7A), and π_C, the threshold pressure above which the enzyme cannot insert into the membrane, is high (46 dyn/cm) for this protein under these conditions. The strong interaction with an anionic surface may not be surprising given the high pI for this protein and the potent inhibition of cIP hydrolysis by anionic phospholipid vesicles (Table 2). The addition of KCl at a concentration comparable to its effective $K_{0.5}$ for kinetic activation (0.16 M) had little effect on π_C for the

Table 3: Effect of Citraconic Anhydride Modification of Lys Residues on the Kinetic Activity of Recombinant *L. monocytogenes* PI-PLC for PI Cleavage

[PI-PLC] (μg/mL)	X_{det}	[KCl] (M)	specific activity[a] (μmol min^{-1} mg^{-1}) control	CA-1[b]	CA-2[b]
0.2	0.80	0.0	303	165	109
1.6	0.80	0.0	60.6	82.5	32.7
0.2	0.92	0.0	1150		534
0.2	0.80	0.15	1030	990	403

[a] The phosphotransferase activity was measured toward 8 mM PI solubilized in 32 or 96 mM TX-100; specific activities of duplicate assays were within 20%. [b] Two different batches of citraconylated PI-PLC were prepared. Approximately five to seven lysine residues of the 29 total were modified according to two-dimensional gels.

enzyme binding to a DOPMe monolayer, indicating binding and insertion in the anionic interface were not dramatically affected by this concentration of salt (Figure 7B). The slope of the plot decreased, suggesting that the portion of protein maximally inserted into a membrane had decreased in the higher-ionic strength salt solution. Binding of *L. monocytogenes* PI-PLC to POPC membranes was characterized by a considerably smaller π_C (31 dyn/cm) that was further reduced in the presence of 0.16 M KCl (to 23 dyn/cm). Binding of *L. monocytogenes* PI-PLC to a mixed monolayer of 70 mol % PC and 30 mol % DOPMe in the absence of KCl resembled the binding curve for a pure DOPMe monolayer (Figure 7A); π_C was unaltered, while the maximum $\Delta\pi$ extrapolated decreased, reflecting the PC content (24 vs 32 dyn/cm). The addition of 0.16 M KCl to this mixed monolayer had a pronounced effect on *L. monocytogenes* PI-PLC binding. π_C was decreased to 37 dyn/cm, while $\Delta\pi$ was the same as that extrapolated from *L. monocytogenes* PI-PLC binding to a PC monolayer. When the KCl concentration in the solution was increased to 0.5 M, the protein exhibited very minimal binding irrespective of any lipid composition, indicating the driving force for the *L. monocytogenes* PI-PLC to reach the lipid surface has a large electrostatic component. The synergistic effect of PC and KCl in weakening the interaction of *L. monocytogenes* PI-PLC with the monolayer correlates with higher enzymatic activity observed with PI/PC SUVs upon addition of KCl.

Citraconylation of PI-PLC. The high pI for *L. monocytogenes* PI-PLC coupled with the aggregation and monolayer results suggested that strong interactions with anionic phospholipids can be detrimental to enzyme action. Citraconic anhydride was used to modify a portion of the Lys residues on the protein surface to see if surface charge had a dominant effect on the kinetic behavior. This reagent converts surface Lys residues to amides with a terminal carboxylate. For each Lys modified, the charge is decreased by 2. The pI values of two separate batches of modified enzyme were checked with a two-dimensional gel. Two distinct spots were detected with the pI between pH 7 and 9. Given the 28 Lys residues in the protein, this shift is consistent with approximately five to seven Lys residues modified (estimated by changing several Lys residues to Glu and computing the pI). A kinetic analysis of PI cleavage in the TX-100 mixed micelle system (Table 3) showed that the modified enzymes still could be activated to approximately the same extent by salt and amphiphiles. However, the effect of protein concentration was diminished compared to that of the control enzyme, suggesting that the high cationic character of the protein is a factor in the unusual dependence of specific activity on enzyme concentration. The smaller effects on salt and amphiphile activation indicate that either (i) Arg and/or remaining unmodified Lys residues account for this kinetic activation or (ii) a hydrophobic interaction with the *L. monocytogenes* PI-PLC is also an important contributor to the activation by salts and amphiphiles.

Site-Specific Mutations of PI-PLC Surface Hydrophobic Residues. Since the unusual dependence of *L. monocytogenes* PI-PLC activity on the enzyme concentration, amphiphiles, and salts persisted after citraconylation, hydrophobic interactions of the protein with interfaces may be responsible for the unusual kinetic behavior. For *Bacillus* sp. PI-PLC, several hydrophobic amino acid residues from short α-helix B and some loops, arranged in a semicircle around the active site cleft, form a mobile hydrophobic ridge fully exposed to solvent. Among the exposed residues, two tryptophans, Trp47 in α-helix B (PIKQV<u>W</u>G) and Trp242 in one particular loop (SGGTA<u>W</u>N), were shown to be important for the enzyme to bind to both activating zwitterionic and substrate anionic interfaces (*35*). The structure of *L. monocytogenes* PI-PLC also consists of a short α-helix B and an analogous rim loop (*36*). Although the degree of sequence similarity is low for these two regions, the structure-based sequence alignment of *L. monocytogenes* versus *Bacillus cereus* PI-PLC did provide several candidates. Both Trp49 and Leu51 in α-helix B (IT<u>W</u>TLTKP) and Leu235 and Phe237 in the loop (SATSLT<u>F</u>) were chosen as targets for mutagenesis (the location of these residues in the structure is shown in Figure 8A).

Most of the mutant enzymes constructed had reasonable activity and were much more active than the wild type at high protein concentrations (>1 μg/mL). Only F237A exhibited an increase in specific activity as the protein concentration decreased, behavior like that of the wild-type enzyme (Figure 8B). All the rest either showed little change or a decrease in specific activity as the protein became very dilute. The latter effect could reflect some surface unfolding of a protein under very dilute conditions. W49A and W49A/F237A exhibited very low specific activities for cleavage of PI in TX-100 micelles compared to the recombinant wild-type enzyme (Figure 8C), consistent with Trp49 having a different role than in the *B. thuringiensis* enzyme. In the *L. monocytogenes* PI-PLC crystal structure, Trp49 is tucked in toward the active site, where it might help align substrate, rather than situated facing the external medium like Trp47 in the *B. cereus* PI-PLC (*36*). In the *B. cereus* PI-PLC, Trp47 is critical to anchoring the protein to interfaces. However, in the *L. monocytogenes* PI-PLC, an intact helix B would not orient Trp49 for insertion into the membrane. The position and proximity of Trp49 to the active site may explain why its replacement with a small residue alters the efficiency of the enzyme. At a concentration of 0.82 μg/mL, the specific activity of W49A toward PI was only 2% of that of the wild-type enzyme. These two fairly inactive enzymes also exhibited a very distinct decrease in specific activity as the enzyme was diluted in the assay, the opposite of the trends for the wild-type enzyme. Incubation of 10 mM cIP and 5 mM TX-100 with 7.2 μg/mL W49A or W49A/F237A for 24 h did not generate any ^{31}P NMR-detectable I-1-P. Clearly, modifying key hydrophobic groups in helix B and the active

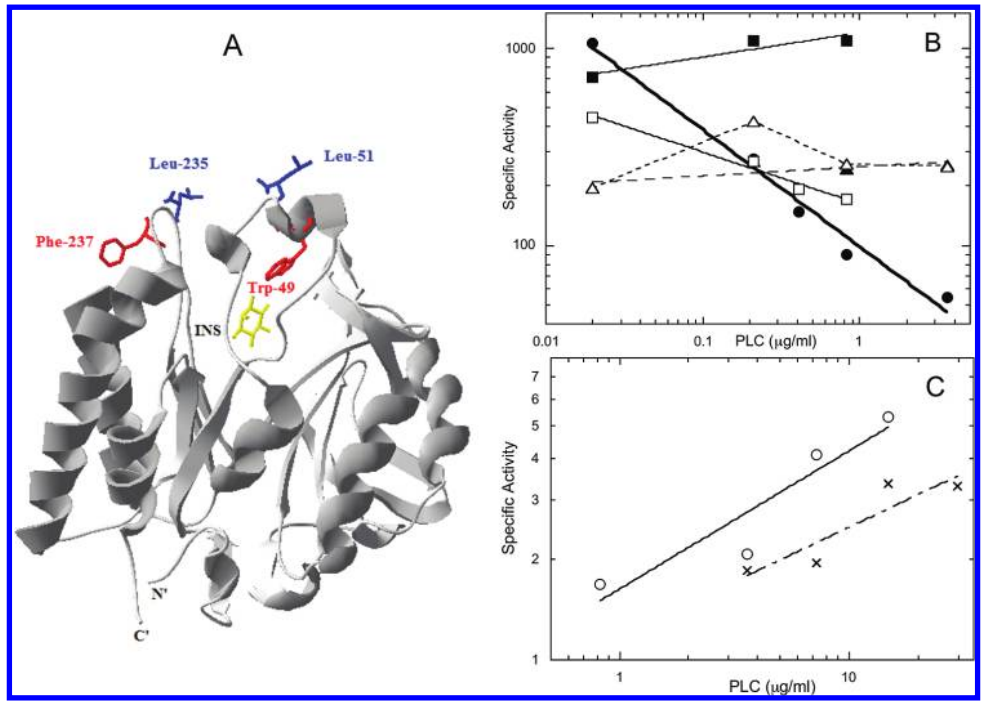

FIGURE 8: (A) Ribbon diagram of the *L. monocytogenes* PI-PLC showing the surface hydrophobic groups mutated (the two aromatic residues colored red and the two Leu residues blue; myo-inositol colored yellow is shown in the active site). (B) Dependence of mutant enzyme phosphotransferase specific activity (8 mM PI/32 mM TX-100 corresponding to $X_{det} = 0.8$) on enzyme concentrations: wild type (WT) (●), L51A (▲), L235A (△), F237A (□), and F237W (■). (C) Effect of protein concentration on PI (8 mM PI; $X_{det} = 0.8$) cleavage by W49A (○) and W49A/F237A (×). For panel B, the errors in activities are less than 20% for each specific activity; for panel C, errors were as high as 30% for the lowest concentrations of enzyme used.

site loop reduces or removes the unusual concentration dependence of wild-type *L. monocytogenes* PI-PLC.

The surface dilution profiles of the more active mutant enzymes (all assayed with a fixed PI-PLC concentration of 0.2 μg/mL and 8 mM PI) were also different from that of the wild type. At a fixed total PI concentration, all enzymes exhibited an increase in specific activity as the mole fraction detergent increased from 0.67 to 0.80 (detergent:PI ratio changing from 2:1 to 4:1), suggesting the larger amount of TX-100 was needed to adequately solubilize the PI in this system (emphasized in Figure 9A). However, the further 3-fold increase in specific activity for this concentration of the wild-type enzyme as the mole fraction of detergent was increased above 0.9 (Figure 9B) was not observed for any of the mutant enzymes.

We also examined these mutant enzymes for the effect of salt on the phosphotransferase reaction and amphiphile effects on cIP hydrolysis. Added KCl (0.15 M) still enhanced PI-PLC cleavage of PI in TX-100 micelles for the mutant enzymes (Figure 10A), although KCl effects were much more modest for W49A (1.8-fold increase), L51A (1.6-fold), and L235A (1.4-fold) compared to those for the wild type, F237A, and F237W. In contrast to the salt activation of phosphotransferase activity, all mutant enzymes exhibited a large and similar magnitude increase in the specific activity for cIP hydrolysis with TX-100 micelles present (Figure 10B).

DISCUSSION

Mechanisms for the Activation of L. monocytogenes PI-PLC. Activation of the *B. thuringiensis* PI-PLC by phosphatidylcholine interfaces has been studied in detail (*2, 23, 27*−*29, 35*). Binding of *B. thuringiensis* PI-PLC to PC interfaces, which appears to have a strong hydrophobic component (*23*), alters the enzyme conformation so that it is a better catalyst in both phosphotransferase and cyclic phosphodiesterase reactions. The activation is specific for zwitterionic phospholipids (*2*). The *L. monocytogenes* PI-PLC has a structure similar to that of the *B. cereus* enzyme (*36*), which might suggest very similar catalytic and possibly regulatory behavior. While the catalytic mechanism may be essentially the same (*16*), the regulation of activity by amphiphiles and ionic strength is quite different for the more cationic protein.

Activators for *L. monocytogenes* PI-PLC exist in two classes: (i) neutral amphiphiles (PC and TX-100) and (ii) moderate salt concentrations. The first of these directly bind (albeit weakly) to the enzyme (e.g., diC_7PC binding to *L. monocytogenes* PI-PLC as monitored by ^{31}P line width changes) and alter the interaction of the enzyme with its anionic substrates. As with *B. thuringiensis* PI-PLC (*4*), adding a micellar activator to an assay system with monomer substrates is more effective than adding a monomeric activator. However, micelles of a neutral amphiphile such as TX-100 are just as effective as diC_7PC, suggesting the amphiphile binding may not involve a specific site on the enzyme. The affinities of the two PI-PC enzymes for anionic versus zwitterionic interfaces are exact opposites. *L. monocytogenes* PI-PLC binds tightly to surfaces high in anionic phospholipids with much weaker interactions with zwitterionic PC interfaces, while *B. thuringiensis* PI-PLC prefers zwitterionic lipids. Furthermore, the *L. monocytogenes* PI-PLC tends to form large aggregates with vesicles or micelles rich in anionic lipids. As revealed from its crystal structure (*36*), there are many basic residues clustered on the bottom side of the *L. monocytogenes* PI-PLC TIM barrel that are

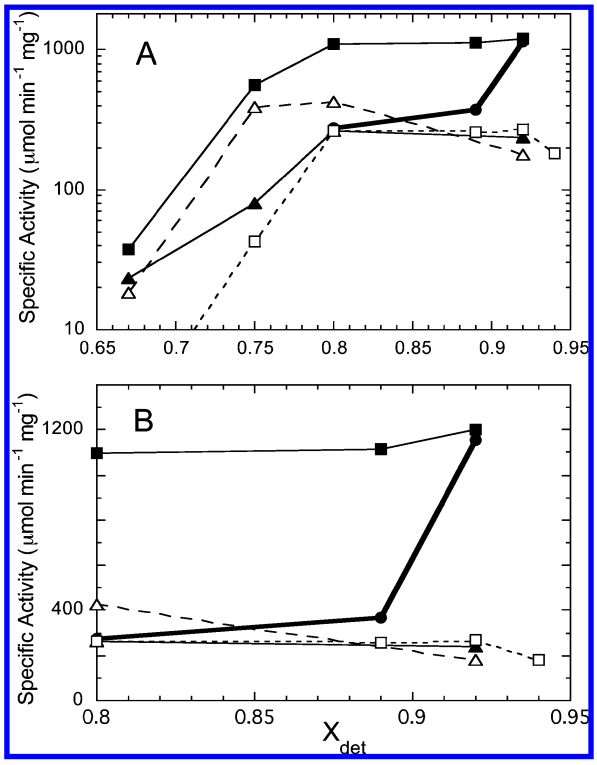

FIGURE 9: Effect of detergent (Triton X-100) mole fraction, X_{det}, on mutated PI-PLC specific activities with fixed PI (8 mM) and enzyme (0.2 μg/mL) concentrations: wild type (●) with only data for $X_{det} \geq 0.8$ shown, L51A (▲), L235A (△), F237A (□), and F237W (■). The errors in activities are less than 20% for each specific activity. In panel A, the data from an X_{det} of 0.67 (16 mM TX-100) to 0.92 (80 mM TX-100) are presented, but with specific activity on a semilog scale to emphasize the similar behavior for the mutant proteins. In panel B, the data for the region for X_{det} values of ≥ 0.80 are presented to emphasize that none of the mutant proteins show a significant increase above $X_{det} = 0.9$ as seen for the wild-type enzyme at the same concentration.

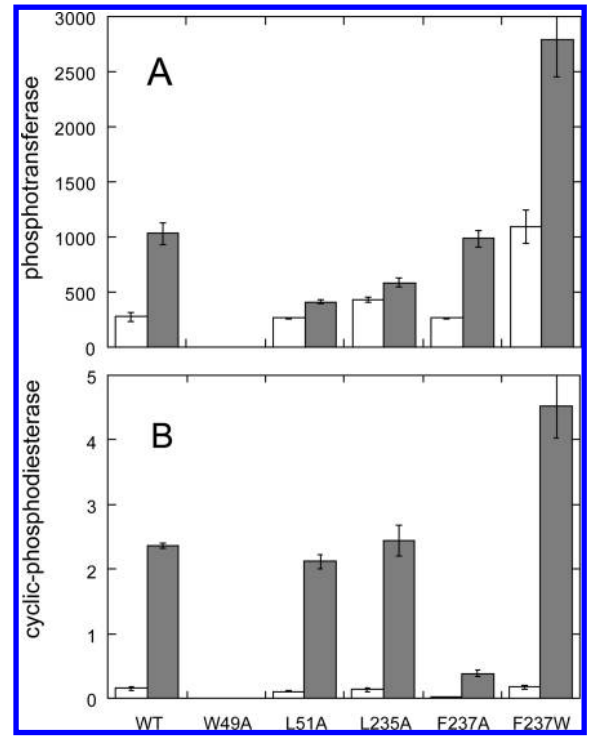

FIGURE 10: (A) Effect of 0.15 M KCl on cleavage of PI (8 mM) in TX-100 (32 mM) mixed micelles with 0.2 μg/mL wild type or different interfacial mutant enzymes. (B) Effect of 3 mM TX-100 on the hydrolysis of cIP by *L. monocytogenes* wild-type and mutant enzymes. In each panel, the specific activity is shown in the absence (white bars) and presence (gray bars) of the additive. Errors in activities are less than 20% for each specific activity.

far from the rather hydrophobic opening to the positively charged active site. With PI dispersed in a variety of aggregates (mixed micelles or vesicles), the electrostatic interaction between the positive residues of enzyme and anionic substrate drives the enzyme to the interface where it will bind tightly. The basic residues on the face of the protein opposite the active site will cause the clustering of nearby negatively charged lipid surfaces (illustrated schematically in Figure 11A). For vesicles, this could cause aggregation of PI in the same vesicle or more likely cause aggregation with other highly anionic vesicles, forming a complex in which the enzyme is sequestered from other substrate-containing vesicles. A significantly increased surface concentration of zwitterionic/neutral amphiphiles disperses the anionic substrate and shields charges on the protein from sparser anionic lipid patches on the same or other particles. Protein−protein interactions must also contribute to this trapping of the enzyme since dramatically reducing the protein concentration also increases enzyme specific activity (Figure 11B).

Increasing the solution ionic strength also relieves formation of the trapped enzyme by weakening the hydrophobic interaction between enzyme and anionic surfaces. That cIP hydrolysis by *L. monocytogenes* PI-PLC is not affected by salt indicates this activator is altering the way the protein interacts with anionic interfaces. The kinetic and biophysical studies presented here show that increased ionic strength alters the way *L. monocytogenes* PI-PLC binds to anionic interfaces. The monolayer studies support the idea that KCl weakens the interaction of the protein with negatively charged surfaces. Increased ionic strength reduces the maximum penetration ($\Delta\pi$) of the enzyme and π_C to a greater extent when PC is present in the interface. These results are critical in explaining the synergistic effects of POPC and KCl on PI-PLC cleavage of PI in vesicles. Clearly, high π_C and $\Delta\pi$ values correlate with the lower activity of *L. monocytogenes* PI-PLC. High salt is also likely to affect PI behavior in the membrane by suppressing any demixing of PI and neutral and zwitteronic amphiphiles (*31*), an effect that may also contribute to enhanced activity.

However, the soluble substrates, diC_4PI for the phosphotransferase reaction and cIP, provide an interesting twist in understanding how the two types of activators work. DiC_4PI cleavage is not dramatically enhanced by *L. monocytogenes* PI-PLC binding to the same concentration of diC_6PC or TX-100 micelles used in cIP assays. A large increase in the enzyme specific activity is observed with only both the amphiphile and a moderate (0.075 M) KCl concentration. The difference between diC_4PI and cIP is that the first has acyl chains, short but with some hydrophobic character. There are multiple cationic sites on the enzyme that could bind this anionic phospholipid, leading to inhibited enzyme. cIP may have a much weaker affinity for these secondary sites. Amphiphile alone may not compete well at these secondary sites, so that activation by amphiphile at low ionic strength is weak. Increased salt alone (0.1−0.2 M) may not displace these molecules from the protein. However, the

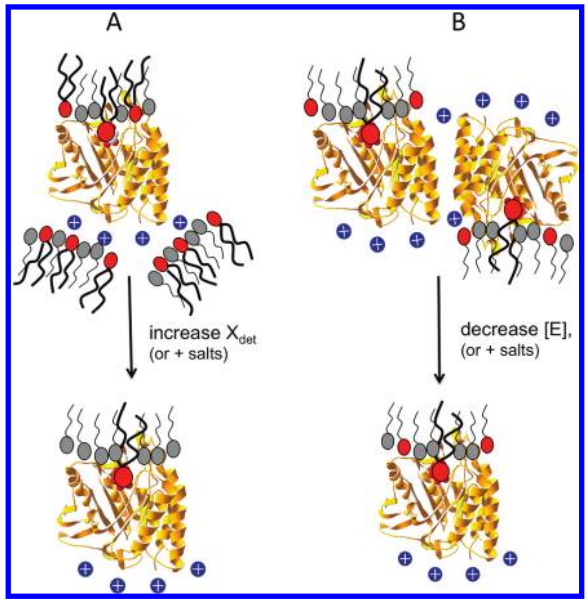

FIGURE 11: Schematic representation of the interactions of *L. monocytogenes* PI-PLC with mixed phospholipid interfaces (red for an anionic phospholipid such as PI substrate, gray for a neutral detergent or lipid). In panel A, protein oriented at the interface can interact with the anionic phospholipids in a separate particle (as well as the anionic lipids within a given particle), trapping the enzyme in aggregates that cannot easily exchange product for substrate in other regions of a particle or even other aggregates. This leads to low specific activity. If sufficient amphiphiles (or high salt) are added, these nonproductive interactions are suppressed and high specific activity is realized. In panel B, the high concentrations of protein together with the moderate surface concentration of anionic substrate form aggregates that again sequester the enzyme in complexes leading to low specific activity. Decreasing the enzyme concentration as well as adding salts suppresses nonproductive complex formation.

presence of an activating amphiphile and added salt might be more effective at weakening electrostatic interactions of diC_4PI with secondary sites and allowing the enzyme with bound substrate to bind to the activating amphiphile surface. This would lead to high specific activity toward diC_4PI only observed in the presence of both amphiphile and salt. In contrast, the enzyme would be adequately activated by the amphiphile toward cIP because this substrate molecule does not interact with other sites on the enzyme.

Testing the Importance of Hydrophobic Residues to PI-PLC Kinetic Behavior. The biophysical studies with the wild-type enzyme suggest that hydrophobic interactions as well as electrostatic interactions with negatively charged surfaces contribute to the unusual kinetic behavior of this enzyme. Modifying enzyme surface charge by citraconylation, which should at least partially affect any kinetic effects linked to the high cationic character of this protein, had little effect on surface dilution or salt effects. The only significant effect was that enzyme with a reduced positive charge exhibited a smaller increase in specific activity as the protein was diluted. The PI-PLC mutants we constructed all modified hydrophobic groups at the barrel rim in features that might be linked to membrane binding. Except for removal of Trp49 (which is the one residue in this series facing inward toward the active site and possibly involved in orienting or promoting proper access of the substrates), the PI-PLC mutant enzymes we constructed were all quite active and, most importantly, lost the strikingly large increase in specific activity with enzyme dilution or with surface dilution of the PI in mixed micelles. Alanine substitutions clearly will weaken hydrophobic interactions, and most of the mutants were not as active as the wild type if maximum specific activities are compared regardless of protein concentration (Figure 8). The kinetic behavior of these specific mutant enzymes contrasts strongly with that observed for the citraconylated enzyme. Both the enzyme concentration dependence and apparent surface dilution inhibition are dramatically reduced for all mutants with altered surface hydrophobic side chains (except W49A which is fairly inactive). Neither trend is abolished when the enzyme surface charge is reduced by modification of the lysines.

The behavior of the mutant enzymes also indicates that salt activation is distinct from the enzyme dilution and surface dilution activation. The level of KCl activation for W49A, L51A, and L235A is significantly reduced. In contrast, citraconylated enzyme exhibits a strong KCl activation. If added salt weakens the protein binding to the interface (an observation supported by the monolayer studies), then reducing the level of binding by removing hydrophobic residues should weaken the salt effect as observed with these mutant enzymes (they are already weaker binders).

Perhaps the most interesting mutation is F237W. Replacement of hydrophobic Phe237 with tryptophan, which prefers interfacial regions, may help to alter the conformation of the protein on a target membrane. F237W PI-PLC, even at high concentrations, has high specific activity comparable to that of dilute unaltered recombinant PI-PLC. This kinetic behavior suggests that at higher concentrations, F237W does not form the aggregates with anionic lipid-rich vesicles that are disrupted by excess detergent and salt, although it is still activated (to about the same extent as the wild type) by salt. Substitution with alanine generates a mutant that most approaches wild-type PI-PLC in its dependence on enzyme concentration. These results suggest that Phe237 is a key residue in the very tight binding of PI-PLC to membranes containing anionic phospholipids. One could suggest that the high activity of F237W at higher enzyme concentrations exists because it still binds to membranes, but the Trp alters the orientation by perhaps not inserting to the same depth as a Phe side chain. The F237W protein may bind more weakly than the wild type. The net result is that the enzyme is not trapped in underproductive precipitates, aggregates, etc. Future studies directed at vesicle binding (with mixtures of anionic phospholipids and PC) of wild-type and mutant proteins under conditions with very low concentrations of proteins should be extremely useful for unraveling how the specific residues mutated contribute to membrane partitioning. The phenylalanine at this particular position may be the key to tightly anchoring the *L. monocytogenes* PI-PLC on the inner leaflet of the plasma membrane of the host cell or onto the inner leaflet of the inner bilayer in the secondary vacuole where it can cleave PI to generate DAG.

Biological Relevance of Amphiphile Regulation of L. monocytogenes PI-PLC Activity? Several bacteria, both pathogenic and nonpathogenic, secrete PI-PLC enzymes into the media. The biological role of this enzyme, along with other nonspecific phospholipases, is to aid in survival of the organism, particularly as it infects mammalian cells. Those PI-PLCs that cleave GPI-anchored proteins, such as the PI-PLC secreted by *B. thuringiensis*, *B. cereus*, and *Staphylo-*

coccus aureus, are well poised to target those membrane components in the extracellular leaflet of the plasma membrane, which is rich in the zwitterionic lipids PC and sphingomyelin. The specific PC binding and activation of the *Bacillus* PI-PLC enzymes would help ensure that they bind the target cell membrane where they could catalyze the cleavage of GPI anchors. However, secreted PI-PLC enzymes whose targets are PI and not GPI anchors must have a different role in bacterial survival since PI is not found in the external leaflet of most organisms. In fact, they should not be targeted to PC-rich membranes but should have high affinity for the inner leaflet of the plasma membrane of the target cell.

In *L. monocytogenes* infections, the bacteria are surrounded by two types of vacuolar membranes at different stages and the PI-PLC is involved in both stages (*11, 37*−*42*). Upon initial infection of the host cell, the bacterium is surrounded by a single-membrane vacuole. In macrophages, both listeriolysin O and *L. monocytogenes* PI-PLC expression are upregulated in this phagosome (*43*). From the phagosome, *L. monocytogenes* PI-PLC could presumably gain access to host PI by means of phagosomal permeabilization and eventual destruction. Activation of host PKCβ by means of DAG generated from intracellular PI and elevated intracellular calcium may play a role in escape from this phagosome (*40, 42*). The inner leaflet of the primary vacuole membrane is presumably like the external leaflet of the plasma membrane, rich in PC or sphingomyelin with a low content of anionic phospholipids. Since the *L. monocytogenes* PI-PLC has weak affinity for PC (and presumably for sphingomyelin and PE as well) at the moderate ionic strengths in cells, it will stay in the vacuolar fluid and be dispersed into the cytoplasm. Once there, it will home in on the negatively charged components, including PI, of the target plasma membrane. It should be noted that intracellular concentrations of a typical mammalian cell are 5−15 mM NaCl, 140 mM KCl, 0.5 mM Mg^{2+}, and 0.1 mM Ca^{2+}, an ionic strength similar to what activates *L. monocytogenes* PI-PLC toward mixed PI/PC SUVs. Once at the plasma membrane, the bacterial PI-PLC will generate DAG required to activate host pathways. However, too much PI cleaved would be detrimental to the host and the replication efforts of the bacterium. Moderation of host PI cleavage by this bacterial PI-PLC could occur when the tightly bound PI-PLC is sequestered with other host anionic lipids and can no longer easily access more PI.

PI-PLC also plays an important role in escape from a secondary vacuole for cell-to-cell spreading of these bacteria (*12*). In this compartment, the bacterium is in a double membrane where the inner bilayer is derived from the primary cell and the outer bilayer from the secondary cell. The inner leaflet of the inner membrane will contain PE, PS, and PI, the last of these being the substrate for the PI-PLC. Thus, the PI-PLC enzyme secreted into the secondary vacuolar fluid will aid in dissolving this inner bilayer by hydrolysis of the PI and likely disruption and fusion of the bilayer after sufficient DAG is produced. The activity of the non-specific phospholipase/sphingomyelinase (PC-PLC) will generate even larger amounts of DAG plus ceramide, potentially leading to membrane fusion (*44*), and also cause complete disruption of the inner membrane (*12*). The high affinity of the wild-type enzyme for anionic phospholipids will ensure that it localizes on this membrane surface. Once the inner membrane is dissolved, PI-PLC faces the outer leaflet of the outer membrane, rich in PC and SPM, and will not bind tightly. In the escape from the secondary vacuole, there is fusion with early endosomes and a decrease in pH. While this may activate the nonspecific PC-PLC and listeriolysin, the fairly narrow pH range of PI-PLC suggests that it would not be very active if the pH dropped much. Therefore, it must contribute to disruption of the inner membrane prior to a more extreme pH drop (to ~5.5) and activation of LLO. Several of the mutant enzymes we have constructed may help sort out the relevance of the PI-PLC at different stages of infection by *L. monocytogenes*.

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