Engineering of the Nonspecific Phospholipase C from *Bacillus cereus*: Replacement of Glutamic Acid-4 by Alanine Results in Loss of Interfacial Catalysis and Enhanced Phosphomonoesterase Activity†

Cristina A. Tan and Mary F. Roberts*

Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02167

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ABSTRACT: The nonspecific phospholipase C from *Bacillus cereus* is a zinc metalloenzyme that catalyzes the hydrolysis of phospholipids to yield diacylglycerol and a phosphate monoester. Glu-4 has been proposed as a potential candidate for the general base in the hydrolysis reaction and was shown to interact with the substrate headgroup. Site-specific mutagenesis studies suggest that Glu-4 is important for substrate binding but not for catalysis. This residue is also critical for the enzyme's preference for a phosphodiester substrate. PA, both monomeric and micellar, is shown to be a poor substrate and inhibitor of wild-type PLC. When Glu-4 was mutated to an alanine, a significant increase in PA hydrolysis and a decrease in PC hydrolysis were observed. Unlike the wild type, kinetic studies suggest that the Glu-4→Ala mutant does not exhibit interfacial activation and processive catalysis. Glu-4 is part of a highly flexible loop flanking the entrance to the active site, suggesting that this loop might constitute an interfacial binding recognition site. This is the first evidence for the presence of an interfacial binding site distinct from the active site in the nonspecific PLC.

Phospholipases are small, water-soluble enzymes whose natural substrates are membrane phospholipids (*1*). Hydrolysis of phospholipids by phospholipase C (PLC)[1] produces diacylglycerol and a phosphate monoester. While the PLC enzymes specific for phosphosphatidylinositols have clear roles in signal transduction pathways by generating the second messengers diacylglycerol (which activates protein kinase C) and inositol trisphosphate (involved in intracellular Ca^{2+} mobilization), nonspecific PLC enzymes may also have roles in the control of cell growth and tumor transformation (*2*−*4*). Mammalian nonspecific PLCs have not been purified, and the genes encoding such enzymes have not been cloned. In *Bacillus cereus*, PLC is an exocellular zinc metalloenzyme, and experiments have shown that the PLC from *B. cereus* is useful in mimicking the action of mammalian nonspecific PLC activities. Clark and coworkers have shown that antibodies to *B. cereus* PLC cross-reacted with the mammalian activity, suggesting an antigenic and structural similarity between the two enzymes (*5*). The bacterial PLC has also been shown to initiate the expected cellular responses upon exogenous addition to mammalian cell cultures, possibly implying functional similarities (*3*, *4*). In addition to the physiological activities of the broad-range *B. cereus* PLC applied to cells exogenously, Johansen and co-workers (*6*) have shown that mammalian cells stably transfected with the gene for this enzyme acquire a transformed phenotype.

One of the most interesting features of phospholipases including the bacterial PLC is that they exhibit "interfacial activation," preferentially binding and catalyzing the hydrolysis of aggregated substrates (*1*). This activation is normally monitored using short-chain phospholipid substrates that are present in monomolecular form below the critical micelle concentration (CMC), but readily form micelles when the concentration exceeds the CMC (*7*). The interfacial mechanism of PLC is not well understood, unlike that of phospholipase A_2. For the nonspecific PLC from *B. cereus*, the observed interfacial activation is 2−3-fold (*8*). For comparison, for PLA_2, the rate of enhancement (based on the specific activity of the enzyme) is 20−100-fold upon micellization of the substrate. The mechanism responsible for observed rate increases upon aggregation of substrate may not be unique to all phospholipases but may be quite different depending on the specific phospholipase.

The crystal structure of PLC from *B. cereus* with various inhibitors revealed how a phospholipid monomer can bind at the active site (*9*−*11*). However, these structures cannot provide a clear picture of how this enzyme interacts with the lipid−water interface. In the structure of native PLC, a region in the enzyme that has the highest temperature factor is a loop that includes Glu-4 (12). In addition, this residue was proposed to activate a water molecule that will attack the phosphorus atom of the substrate (*13*, *14*). An acidic residue at position 4 is conserved in phospholipase C from several organisms: residue 4 in PLC from *Listeria monocytogenes* (*15*) and *Pseudomonas aeruginosa* (*16*) as well

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

[1] Abbreviations: PLC, phospholipase C; PLA_2, phospholipase A_2; PI-PLC, phosphatidylinositol-specific phospholipase C; AP, alkaline phosphatase; CMC, critical micelle concentration; diC_6PC, dihexanoylphosphatidylcholine; diC_7PC, diheptanoylphosphatidylcholine; diC_6PA, dihexanoylphosphatidic acid; HEPES, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid; NMR, nuclear magnetic resonance.

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Table 1: Kinetic Parameters of Recombinant Wild Type and E4A Phospholipase C toward Phosphodiester Substrates[a]

	diC_6PC (monomer)[b]				diC_7PC (micelle)[b,c]			
enzyme	V_{max}	K_m	k_{cat}	k_{cat}/K_m	V_{max}	K_m^{app}	k_{cat}	k_{cat}/K_m
wild type	856	0.19	407	2142	1748	0.03[d]	830	27667
E4A	624	1.12	296	265	866	1.09	411	377

[a] Units are mM for K_m, μmol min^{-1} mg^{-1} for V_{max}, and s^{-1} for k_{cat}; the calculation of k_{cat} is based on the PLC molecular mass of 28.5 kDa. [b] pH-stat assays used 2−10 mM diC_6PC and 2−10 mM for diC_7PC. All rates were determined in duplicate. At high substrate concentrations, the estimated average experimental uncertainty in the rates was 8%; at low substrate concentrations, the uncertainty was 7%. Errors in K_m extracted from a Michaelis−Menten treatment of monomeric substrate for both wild type and E4A and micellar substrate for E4A were estimated to be less than 20%. The CMC of diC_6PC is 14 mM so that it was monomeric under assay conditions while the CMC of diC_7PC is 1.2 mM so that it was micellar under the assay conditions. [c] The K_m for diC_7PC is an apparent value based on a Michaelis−Menten treatment and a phase separation model of monomeric and micellar species (8). [d] Errors in extrapolating the apparent K_m for micelles are relatively large; the K_m for diC_7PC with wild-type PLC isolated from *B. cereus* is 0.02 ± 0.01 mM (*18*).

as *B. cereus* is occupied by aspartic acid. However, in other bacteria (notably *Clostridium* strains), this residue is a lysine (*17*). Perhaps this indicates that a charged residue at this position is important for catalysis or substrate binding. We have used site-specific mutagenesis to replace Glu-4 with alanine in the cloned *B. cereus* enzyme. The mutant enzyme E4A was expressed in *E. coli*, and the effects of this mutation on the kinetic parameters were examined. A key observation is that the E4A mutant does not exhibit interfacial catalysis. Moreover, this mutant PLC shows an enhanced activity toward a phosphomonoester substrate.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were purchased from New England Biolabs (Cambridge, MA). The ligation kit was from Novagen (Madison, WI), the phagemid site-specific mutagenesis kit was from Bio-Rad, the Sequenase version 2.0 DNA sequencing kit and [^{35}S]dATP were obtained from Amersham Corp. (Cleveland, OH). Oligonucleotide primers for mutagenesis and sequencing were obtained from Operon Technologies (Alameda, CA). Q-Sepharose Fast Flow was purchased from Pharmacia (Piscataway, NJ). Ultrapure urea was obtained from USB (Cleveland, OH). Barbital buffer and clostripain were obtained from Sigma (St. Louis, MO). All other reagents were of analytical or molecular biology grade. Recombinant plasmid pMR1, consisting of the cDNA clone of the *B. cereus* PLC gene with the signal sequence, cloned into the *Nde*I/*Eco*RI sites of pET 23a (*18*), was used.

Novablue and BL21(DE3)pLysS were obtained from Novagen. CJ236 and the M13KO7 helper phage were obtained from M. J. Hehir, Boston College.

Preparation of the Mutant PLC. Site-specific mutagenesis was performed using the Kunkel method (*19*). Uracil-containing single-stranded DNA was obtained by infection of CJ236 containing a phagemid with helper phage M13KO7. Mutants were verified directly by dideoxy sequencing of single-stranded DNA isolated from several candidates. The region flanked by the *Nde*I and *Eco*RI sites was sequenced. The mutant plasmid was digested with *Nde*I and *Eco*RI and subcloned into a similarly digested pMR1 to create pMR5 which carries the gene for E4A.

To express E4A, plasmid pMR5 was transformed into *E. coli* strain BL21(DE3)pLysS. Induction of T7 RNA polymerase was initiated by addition of IPTG to a final concentration of 0.4 mM. After growing the cells for an additional 2−3 h, the cells were harvested. The wild type and E4A mutant PLC were purified to homogeneity as described before (*18*). The E4A concentration was determined using the Bio-Rad version of Bradford assay with the wild-type PLC as standard (*18*).

Kinetic Analyses. Phospholipids (diC_7PC, diC_6PC, diC_6-PA) were obtained from Avanti and used without further purification. pH-stat assays were used to monitor the activity of wild-type PLC and E4A by titration of the product monoester phosphoric acid liberated from the hydrolysis of phospholipid with NaOH as the titrant and an end point of pH 8.0, 25 °C. The concentration range used for diC_6PC was 2−10 mM so that it is monomeric under assay conditions [the CMC is 14 mM (*20*)]. The concentration range for diC_7-PC was 2−10 mM, ensuring micellar substrate under assay conditions (the CMC is 1.2 mM). In addition, to monitor the interfacial activation property of the mutant enzyme, the enzyme was assayed using diC_6PC covering both monomeric and micellar concentrations (0.5−40 mM diC_6PC). All rates were determined in duplicate. ^{31}P NMR spectroscopy was used to monitor the activity of wild-type and E4A PLC toward diC_6PA. Spectra were obtained with a Varian Unity 500 spectrometer operating at a frequency of 202.33 MHz. Acquisition conditions for a kinetic run included 16 380 Hz sweep width, 5.1 μs pulse width (57°), 1 s acquisition delay, 1.6 s accumulation time, ^{1}H-WALTZ decoupling, and 100 transients per time points. Under these conditions, the product phosphocholine (or P_i in the case of PA substrate) was not saturated. Hydrolysis of diC_6PA as a function of time was monitored for 2−3 h at 30 °C. DiC_6PA concentration ranged from 3 to 10 mM in 20 mM HEPES, pH 8.0.

RESULTS

The mutant enzyme was purified as described before for this PLC recombinant system (*18*). E4A was stable to heating to 50 °C, similar to the wild-type enzyme, and showed a single band in SDS−PAGE, with a yield of at least 30 mg/L culture. The kinetic properties of this mutant were determined toward monomeric and micellar phosphodiester substrates (PC) and a monomeric phosphomonoester (PA).

Kinetic Parameters toward Phosphatidylcholine. The E4A mutant remains fairly active toward monomeric and micellar PC substrates (Table 1). When monomeric PC was the substrate, the V_{max} was reduced about 30% and the K_m increased 5-fold compared to the value for the wild-type enzyme. The catalytic efficiency of E4A decreased 8-fold compared to the wild-type enzyme toward monomeric PC not only because of a slight decrease in k_{cat} but also more significantly by an increase in K_m. This drop in catalytic efficiency is more evident when the substrate is micellar PC

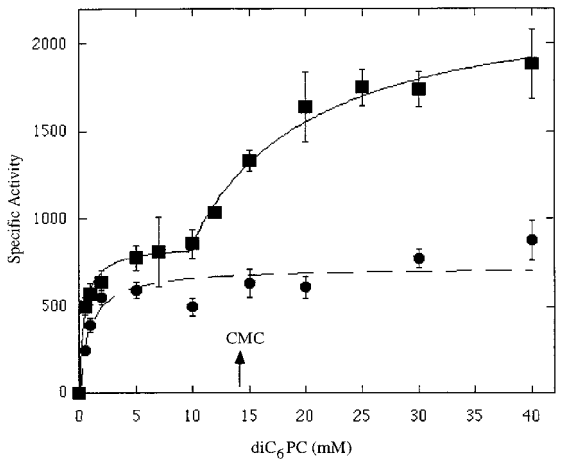

FIGURE 1: Activity of the E4A mutant (filled circles) and wild-type PLC (filled squares) toward diC_6PC (0.5−40 mM) measured using pH-stat assays. The arrow indicates the CMC of pure diC_6-PC. Error bars represent the standard deviations of duplicate or triplicate kinetic assays. The dashed line represents a Michaelis−Menten analysis for the E4A mutant below 10 mM substrate using the following parameters: V_{max} = 624 μmol min^{-1} mg^{-1} and K_m = 1.12 mM.

where the catalytic efficiency was reduced 73-fold compared to the wild-type enzyme. The k_{cat} exhibited only a slight decrease, but the apparent K_m increased 37-fold. Thus, the mutation at Glu-4 affected the substrate binding step more than the catalytic step when PC was the substrate.

More interestingly, in contrast to wild-type PLC, E4A did not exhibit significant interfacial activity. For wild-type enzyme, the V_{max} increased 2-fold while the K_m decreased 10-fold when the aggregation state of the substrate was changed from monomer to micelle when comparing activity toward diC_6PC and diC_7PC. This represented a 13-fold increase in catalytic efficiency of the wild-type enzyme when hydrolyzing micellar substrates. In contrast, both the K_m and V_{max} of E4A were not significantly different for monomeric and micellar PC substrates.

To further explore the reduced interfacial activation of the E4A mutant, kinetic experiments were carried out using diC_6-PC as monomer substrate and monitoring PLC activity at higher concentrations where micelles predominate (0.5−40 mM). Figure 1 shows the hydrolysis of diC_6PC over a wide range of concentrations by E4A and recombinant wild-type PLC. The CMC of pure diC_6PC is shown with an arrow. For the wild-type enzyme, this type of curve exhibits two sharp increases in enzyme activity, the first reflecting the K_m and V_{max} for monomeric diC_6PC, while the second reflects kinetic parameters for micellar diC_6PC (*8*). In contrast, for the E4A mutant, only one hyperbolic increase in enzyme activity was observed (Figure 1).

Kinetic Parameters toward Phosphatidic Acid. Monomeric and micellar PA are very poor substrates of wild-type PLC (*21*). Kinetics using PA as the substrate were measured using ^{31}P NMR spectroscopy because of the slow rate of hydrolysis of this lipid by PLC. A typical kinetic run lasted for 2−3 h. As seen in Table 2, the V_{max} of E4A toward phosphomonoester substrate, 2.51 μmol min^{-1} mg^{-1}, was increased about 2-fold compared to the value observed for the wild-type enzyme, 1.14 μmol min^{-1} mg^{-1}. The catalytic efficiency of the E4A mutant increased almost 5-fold compared to the value observed for wild-type PLC because

Table 2: Kinetic Parameters of Recombinant Wild Type and E4A Phospholipase C toward a Phosphomonoester Substrate, Dihexanoylphosphatidic Acid[a]

enzyme	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m
wild type	1.14	9.0	0.54	0.06
E4A	2.51	4.2	1.19	0.28

[a] Determined by ^{31}P NMR assays using 3−10 mM diC_6PA in 20 mM HEPES, pH 8.0, 30 °C. The CMC of the diC_6PA under these conditions is 14 mM. Errors in K_m are estimated to be less than 20%. [b] Calculation of k_{cat} is based on a PLC molecular mass of 28.5 kDa.

of an increase in k_{cat} and a decrease in K_m, from 9 mM observed for the wild type to 4.2 mM observed for the E4A mutant. The slight differences in values of kinetic parameters between wild-type PLC and E4A toward the phosphomonoester substrate are made more significant by the observation that the E4A mutant has a reduced phosphodiesterase activity toward PC compared to wild type (Table 1).

DISCUSSION

Glu-4 Is Part of an Interfacial Binding Site in the Nonspecific PLC. The kinetic preference of an aggregated substrate versus a monomeric one appears to be a common property of water-soluble phospholipases. However, the mechanism for this activation is likely to depend on the type of phospholipase (*22*). Aggregation of a substrate monomer leads to only small changes in the average conformation of the phospholipid (*23*), a decreased polarity in the glycerol interface region, and an enhanced local concentration of substrate. While these effects may contribute to interfacial activation by enhancing substrate binding and water polarization, there is more evidence for changes in the enzyme leading to large changes in V_{max}. For 14 kDa phospholipase A_2 enzymes, the best studied of the phospholipases in terms of interfacial activation, it is clear that an interfacial binding region (*24, 25*) distinct from the active site helps anchor the enzyme to the substrate interface and allows for processive catalysis. The details of conformational changes in that enzyme have been deconvoluted by NMR and show that relatively small movements in the chain accompany surface binding when a substrate analogue is present (*26, 27*). A substrate interface provides an isoenergetic state for the 'dangling' acyl chains and allows residues at the entrance of the active site to change slightly, increasing the accessibility of the active site (*22*). In porcine PLA_2, residues located at the edge of the entrance to the active site (Arg-6, Leu-31, Tyr-69) are involved in interaction with monomeric and micellar substrates, and in most cases, modification of such residues resulted in reduction of enzymic activity or interfacial binding (*22*). In the case of human pancreatic lipase, a surface loop or 'flap' covers the active site and must be removed to allow substrate access. The large movement in the 'flap' might be related to interfacial activation for that interfacial enzyme (*28*).

Both bacterial and mammalian phosphatidylinositol-specific PLC (PI-PLC) enzymes also exhibit a preference for aggregated substrate (*29, 30*). For the PI-PLC from *B. thuringiensis*, the enhancement in specific activity is 5−6-fold for micellar versus monomeric PI (PI-PLC δ1 from rat brain shows a similar magnitude enhancement in activity); at this point, there is no concrete information on the

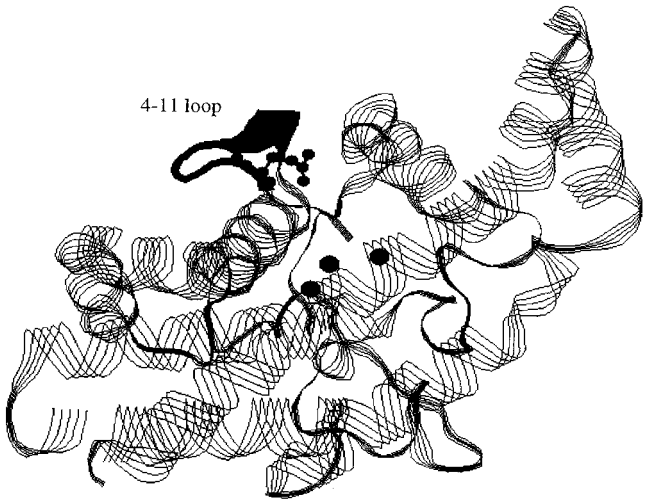

FIGURE 2: Schematic representation of PLC showing the 4−11 loop. Glu-4 is shown in a ball-and-stick representation. The three spheres are zinc ions.

molecular mechanism of this activation. However, a rather different type of interfacial activation of PI-PLC involves micellar or vesicle PC binding to an allosteric site on the enzyme and promoting an appropriate conformational change that enhances V_{max} and reduces K_m both for water-soluble inositol 1,2-cyclic phosphate (cIP), a soluble substrate for the enzyme, and for PI (*31*, *32*). There appears to be no analogue of this type of activation for the nonspecific PLC from *Bacillus* species.

Unlike PLA_2 or lipase, the nonspecific phospholipase C from *B. cereus* has a relatively open and accessible substrate binding site. The structure of the enzyme appears to be very rigid (*9*). In fact, residues 4−11 in the N-terminal loop of native PLC form one of the few regions in the enzyme whose temperature factor lies above 15 $Å^2$. This region is shown in Figure 2 with Glu-4 highlighted. The 4−11 loop flanks the entrance to the active site. In the crystal structure of PLC with phosphate bound, the most pronounced change in the enzyme structure is in this N-terminal loop, where residues 2−8 undergo a parallel displacement away from the metal ions (*9*). The E4A mutant remains a fairly active enzyme despite a significant increase in K_m, which suggests that Glu-4 plays an important role in zwitterionic substrate binding. However, the loss of interfacial activation suggests that this residue helps differentiate micellar from monomeric substrate. Other site-specific mutants including N134A and E146Q still display the kinetic preference for micellar substrate (C. Tan and M. F. Roberts, unpublished results). It is tempting to speculate that the 4−11 loop forms an interfacial binding recognition site. This supports earlier work using vanadate, a potent competitive inhibitor of PLC, which implied that a discrete surface binding step must occur, although nothing was known about the location of such a site (*21*). Whether it involves electrostatic interactions (as PLA_2 seems to) or more direct contacts between the interface and the enzyme, this nonactive site surface binding would be critical to processive catalysis by PLC. Since the E4A mutant does not exhibit interfacial activation, processive catalysis is unlikely. In other words, phospholipase C E4A does not work in a scooting mode but dissociates from the interface between binding and hydrolyzing successive substrate molecules.

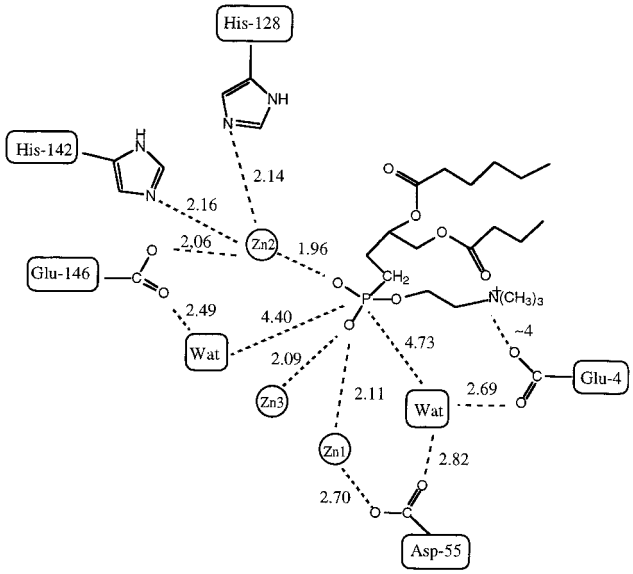

FIGURE 3: Crystal structure of PLC complexed with a phosphonate inhibitor showing the distance interactions of potential nucleophilic water molecules. One of the water molecules is 2.69 Å away from Glu-4. The figure is based on those presented in references (*11*) and (*37*).

Glu-4 Does Not Activate the Water Nucleophile. The hydrolysis of phospholipid ester bonds is thought to proceed via an associative pathway which involves a nucleophilic attack of water on the phosphorus atom and proceeds through a trigonal bipyramidal transition state (*9*, *21*). Inspection of the crystal structure of native PLC revealed no water molecule that could be activated directly by a zinc ion so that an amino acid side chain might be involved. The amino acid side chains that could function as a general base and lie within 7 Å of the phosphorus atom are Glu-4, Asp-55, and Glu-146 (Figure 3). Water molecules in appropriate positions have been identified in the native enzyme and in the Tris-inhibited and phosphonate-inhibited PLC structures (*10*, *11*). In the crystal structure of PLC with a phosphonate inhibitor (Figure 3), one of the side chain carboxyl groups of Glu-4 is 2.69 Å from the oxygen of a water molecule (*14*). Glu-4 was suggested to act as the general base based on an earlier observation that PLC contains one carboxyl group that is essential for catalytic activity and chemical modification of this group did not result in loss of the Zn^{2+} ions (*13*, *33*). The results presented in the present work strongly suggest that another amino acid must be acting as a general base. Mutation of Glu-4 to alanine resulted in only a slight drop in V_{max} compared to the value observed for wild-type PLC. Keeping in mind that the PLC−phosphonate complex is nonproductive, it is possible that the positions of water and the substrate in the active site during catalysis might be different.

The E4A Mutant Exhibits a Greater Phosphomonoesterase Activity Than Wild-Type PLC. The conformation of the substrate phospholipid bound to the active site of PLC remains unclear despite the crystal structures. In the X-ray structure of PLC with a nonhydrolyzable substrate analogue (a phosphonate where the oxygen atom in the phosphodiester bond of PC that is cleaved has been replaced by a methylene group), the phosphonate is in an unusual and strained conformation with the choline headgroup nearly parallel to the acyl chains (Figure 3). The phosphonolipid is not a

particularly good inhibitor, suggesting that the interactions of PLC with the replaced oxygen are important for substrate binding (*11*, *34*). A modeling study suggested that the orientation of the bound phospholipid is in a normal low-energy conformation, similar to that of a phospholipid in the crystalline state (*13*). Both of these studies, however, agreed on the orientation of the bound choline headgroup. In the modeling study, the positively charged nitrogen atom of the choline headgroup lies within 4.6 Å from one of the carboxylate oxygen atoms of Glu-4, similar to that observed in the crystal structure with phosphonate. Thus, it is likely that the positive charge on the choline group is stabilized by electrostatic interactions.

Alkaline phosphatase (AP) from *E. coli* is a phosphomonoesterase whose active site is remarkably similar to that of PLC (*35*). Inspection of the enzyme's active site suggests that if a phospholipid is to bind to AP, the amino acid residue that will interact with the headgroup is Arg-166. Perhaps, the positive charge of Arg-166 is responsible for this enzyme's phosphomonoesterase activity in a similar way that Glu-4 is responsible for the higher phosphodiesterase activity of PLC toward phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, which all contain a positively charged nitrogen, compared to phosphatidylglycerol, phosphatidylmethanol, and phosphatidic acid substrates (*36*). When Glu-4 of PLC was mutated to an alanine, a significant although not dramatic increase in PA hydrolysis and a decrease in PC hydrolysis were observed. The K_m for the dianionic PA also was decreased compared to the K_m of the wild type for that phospholipid, enhancing PLC phosphomonoesterase efficiency by nearly a factor of 5.

In summary, we have used site-specific mutagenesis to assess the importance of Glu-4 in PLC. The bulk of the evidence suggests that Glu-4 is important in substrate binding and specificity but not in catalysis. Glu-4 is located in a flexible loop which may constitute an interfacial binding recognition site. It would be interesting to see the effect of mutating or deleting other residues involved in this loop on the interfacial activity of PLC. Likewise, mutating Glu-4 to positively charged residues such as arginine or lysine (not easy with the present expression system using clostripain to remove the signal sequence) may further enhance its phosphomonoesterase activity.

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