Expression and Site-Directed Mutagenesis of the Phosphatidylcholine-Preferring Phospholipase C of *Bacillus cereus*: Probing the Role of the Active Site Glu146[†]

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Received June 4, 1996[⊗]

ABSTRACT: A series of site-specific mutants of the phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc}) was prepared in which the glutamic acid residue at position 146 was replaced with glutamine, aspartic acid, histidine, and leucine to elucidate what role Glu146 might play in catalysis. An expression system for the native enzyme in *Escherichia coli* was first developed to provide PLC_{Bc} that was fused via an intervening factor Xa protease recognition sequence at its N-terminus to maltose binding protein (MBP). This MBP−PLC_{Bc} fusion protein was isolated at levels of 50−70 mg/L of culture; selective trypsin digestion of the MBP−PLC_{Bc} fusion protein followed by chromatographic purification yielded recombinant PLC_{Bc} at levels of ca. 10 mg/L. Polymerase chain reaction (PCR) mutagenesis on the PLC_{Bc} gene (*plc*) was then used to replace the Glu146 codon with those for glutamine (E146Q), aspartic acid (E146D), histidine (E146H), and leucine (E146L). The catalytic efficiency of the E146Q mutant was 1.6% that of native PLC_{Bc}, while the other mutants each possessed activities of 0.2−0.3% of the wild type. The k_{cat}/K_m vs pH profiles for both E146Q and native PLC_{Bc} have ascending acidic limbs, suggesting that Glu146 does not serve as the general base in the hydrolysis reaction. As measured by circular dichroism, all of the mutant proteins contained less helical structure and underwent denaturation at lower temperatures than the wild type in the order: wild type > E146Q > E146D ≈ E146H ≈ E146L. Atomic absorption analyses indicated that the mutant proteins also exhibited lower Zn^{2+} content than the wild type. Thus, the Glu146 residue in PLC_{Bc} stabilizes the secondary and tertiary structure of the enzyme and serves as a critical ligand for Zn2, but it does not appear to have any specific catalytic role.

Isozymes of the phospholipase C (PLC)[1] family catalyze the hydrolysis of the phosphodiester bond in phospholipids to give diacylglycerol (DAG) and a phosphorylated head group (Berridge, 1984; Titball, 1993). The PC-preferring PLC from *Bacillus cereus* (PLC_{Bc}) has been extensively investigated and currently stands as the best characterized of the "broad spectrum" PLC enzymes. The lack of structural and mechanistic information for mammalian PC-PLCs has accorded PLC_{Bc} added significance. Antibodies raised against PLC_{Bc} cross-react with its mammalian analog (Clark *et al.*, 1986), indicating that PLC_{Bc} may be a potential model for mammalian PLC-type enzymes. There have also been tentative links between PC-PLC and tumor growth (de Herreros *et al.*, 1991), so compounds that inhibit this enzyme may be potential anticancer agents. Although not definitely established, there is evidence that the *in vivo* function of PLC_{Bc} itself is tied to a cellular phosphate retrieval system (Guddal *et al.*, 1989).

PLC_{Bc} (EC 3.1.4.3) is a monomeric, 28.5 kDa, metalloenzyme that has been cloned and weakly expressed in *Escherichia coli* (Johansen *et al.*, 1988). PLC_{Bc} bears extensive sequence similarity (39%) with the PC-preferring PLC of *Listeria monocytogenes* (Vazquez-Boland *et al.*, 1992), another "broad-spectrum" bacterial enzyme that hydrolyzes phospholipids in membrane structures (Goldfine *et al.*, 1993). The three-dimensional structure of PLC_{Bc} in its free form (Hough *et al.*, 1989) and complexed with several ions (Hansen *et al.*, 1992) showed that the enzyme had three zinc ions (Zn1, Zn2, Zn3) in the active site. Although such trimetal centers are relatively uncommon, the alkaline phosphatase of *E. coli* (Kim & Wyckoff, 1991) possesses two zinc ions and a magnesium ion, and the P1 nuclease of *Penicillium citrinium* (Volbeda *et al.*, 1991) contains three zinc ions. Comparison of the coordination geometries of the three zinc ions and the internuclear distances between these zinc ions and their associated amino acid ligands reveals that the three-dimensional structures of the active sites of P1 nuclease and PLC_{Bc} are remarkably similar.

We prepared and kinetically evaluated a series of substrate analogues of PLC_{Bc} (Martin *et al.*, 1994), and the X-ray structure of PLC_{Bc} complexed with one of the inhibitors thus identified revealed a number of features that are relevant to substrate binding (Hansen *et al.*, 1993). This structure also provides important data that may be employed to formulate a catalytic mechanism and to assign specific roles to different active site residues. Such assignments must, of course, be

[†] This research was supported by Grant GM 42763 from the National Institutes of Health and by the Robert A. Welch Foundation.

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[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1996.

[1] Abbreviations: A_{280}, absorption at 280 nm; cmc, critical micelle concentration; DAG, diacylglycerol; DMG, dimethylglutaric acid; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; kDa, kilodalton(s); MBP, maltose binding protein; PC, phosphatidylcholine; PCR, polymerase chain reaction; PLC, phospholipase C; PLC_{Bc}, PC-preferring phospholipase C from *Bacillus cereus*; E146Q, mutant of PLC_{Bc} in which Glu146 is changed to Gln; E146D, mutant of PLC_{Bc} in which Glu146 is changed to Asp; E146H, mutant of PLC_{Bc} in which Glu146 is changed to His; E146L, mutant of PLC_{Bc} in which Glu146 is changed to Leu; PLD, phospholipase D; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(96)01316-5 CCC: $12.00

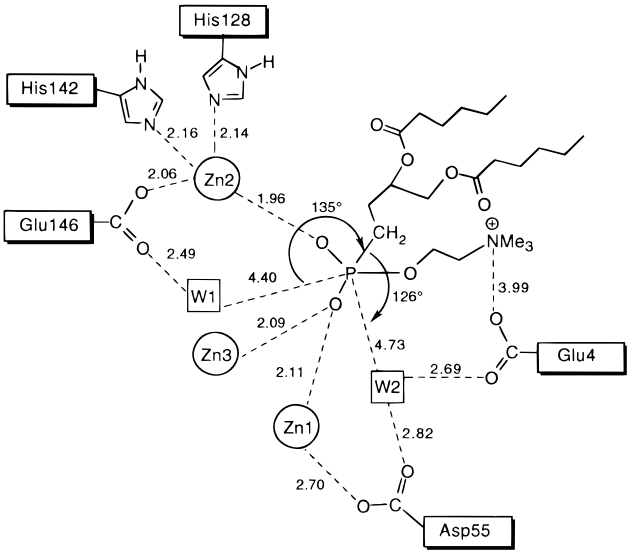

FIGURE 1: Summary of distance interactions of two potential nucleophilic waters present in the crystal structure of PLC_{Bc} complexed with a phosphonate inhibitor. The distance from the zincs to the water molecules is between 4.6 and 7.1 Å.

regarded as tentative because the structures observed in these crystallographic studies correspond to static and inactive complexes, and as such they do not necessarily reveal the true details of catalysis. Recognizing this caveat, we initiated studies directed toward elucidating the mechanism of the PLC_{Bc}-catalyzed hydrolysis of phospholipids with the objective of identifying the general base that activates the water for nucleophilic attack on phosphorus. In analogy with mechanisms that have been invoked for *E. coli* alkaline phosphatase (Butler-Ransohoff *et al.*, 1992) and *staphylococcal* nuclease (SNase) (Judice *et al.*, 1993), the nucleophilic water might be activated by a zinc ion. However, careful scrutiny of the X-ray structure of the PLC_{Bc}−inhibitor complex (Hansen *et al.*, 1993) reveals that there are no water molecules in the first coordination sphere of any of the zinc ions, so it is possible that there is no direct zinc activation of the attacking water molecule; an amino acid side chain would then be involved. In this context, it is relevant to note that the water molecule that served as the fifth ligand for both Zn1 and Zn3 in the ligand-free form of PLC_{Bc} is replaced with one of the nonbridging phosphoryl oxygens upon complexation with the inhibitor; the pentacoordinate geometry of these zinc ions is thus maintained. On the other hand, two water molecules are coordinated with Zn2 in the native enzyme, and although both are lost upon complex formation, only one is replaced with one of the nonbridging phosphoryl oxygens, making Zn2 tetracoordinate.

Each of the acidic residues Glu146 (Hansen *et al.*, 1993; Ikeda *et al.*, 1991), Asp55 (Sundell *et al.*, 1994), and Glu4 (Byberg *et al.*, 1992) has been proposed as a potential candidate for the general base in the hydrolysis reaction. Clues supporting the case for Glu146 as an important catalytic residue are found upon examination of the PLC_{Bc}−inhibitor complex (Figure 1). One of the side chain carboxyl oxygen atoms of Glu146 is 2.06 Å from Zn2, while the other oxygen is located 2.49 Å from an active site water (W1) that was tentatively identified as the attacking nucleophile (Hansen *et al.*, 1993). The reaction coordinate distance from the oxygen of this water to the phosphorus of the inhibitor is 4.40 Å, and the angle of nucleophilic attack, which is defined by the water oxygen, the phosphorus, and methylene group of the inhibitor, is about 135°. Such geometric parameters are consistent with those proposed for an associative nucleophilic displacement at phosphorus (Weber *et al.*, 1994; Mildvan, 1981). These observations suggest that Glu146 might be involved in either orienting or activating this putative nucleophile for attack on phosphorus in the first step of the hydrolysis. Similar arguments may be advanced for the candidacy of Asp55 as the general base. One of the side chain carboxyl oxygen atoms of Asp55 is 2.70 Å from Zn1, while the other oxygen is located 2.82 Å from an active site water (W2) that could serve as the putative nucleophile. The distance from the oxygen of this water to the phosphorus of the inhibitor is 4.73 Å, and the angle of nucleophilic attack is about 126°. The obvious dilemma for both Glu146 and Asp55 is that a side chain carboxyl group cannot simultaneously be a ligand for a zinc ion while serving as the general base for the reaction. Glu4 could also act as the general base to activate W2 as one of its side chain carboxyl oxygens is 2.69 Å from the oxygen of this water molecule. However, Glu4 is also positioned to help stabilize the positive charge on the choline moiety.

To delineate the function of Glu146 of PLC_{Bc} in binding and/or catalysis, we developed the first recombinant system for the high-level expression and purification of PLC_{Bc} and then used this expression system to prepare and characterize the series of the site-specific mutants E146Q, E146D, E146H, and E146L. These results are presented herein.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes, T4 DNA ligase, *Vent* polymerase, amylose resin, factor Xa, and the pMAL-c2 vector were all obtained from New England Biolabs (Beverly, MA). Ampli-*Taq* polymerase and AmpliWax PCR Gems were obtained from Perkin-Elmer (Norwalk, CT). Authentic isolated PLC_{Bc} (P-7147), TPCK-treated trypsin (T-8642), and soybean trypsin inhibitor beads bound to DITC glass (T-9024) were acquired from Sigma (St. Louis, MO). 1,2-Dihexanoyl-*sn*-glycero-3-phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). Q-Sepharose and chelating Sepharose were obtained from Pharmacia (Piscataway, NJ). All oligonucleotides were acquired from Integrated DNA Technologies (Coralville, IA), except for the *malE* primer, which was obtained from New England Biolabs. DNA sequencing was performed with the Sequenase 2.0 system from United States Biochemical (Cleveland, OH). QIAquick and QIAprep DNA purification kits were purchased from Qiagen (Chatsworth, CA). Geneclean II agarose gel purification kits were obtained from Bio 101 (Vista, CA).

Methods

Construction of MBP−PLC_{Bc} Expression Vectors. The plasmids used for expression of PLC_{Bc} are shown in Figure 2. The gene for PLC_{Bc} (*plc*), which contains 735 base pairs, along with the pMAL-c N-terminal linker sequence was transferred from pMALc/plc(Bst) (obtained from Professor T. Johansen, University of Tromsø) into pMAL-c2 to generate pMALc2-plc (Figure 2A). The construct pMAL-plc (Figure 2B), in which the sequence coding for the N-terminal region of sphingomyelinase has been excised, was

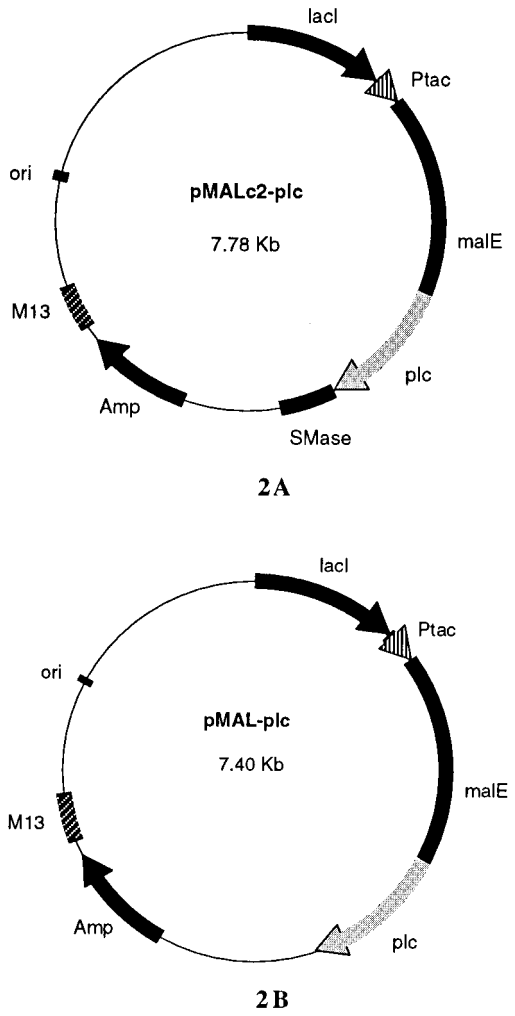

FIGURE 2: Vectors used for expression of PLC_{Bc}. pMALc2-plc differs from pMAL-plc only in that it contains an open reading frame after *plc*.

prepared by a two-primer PCR amplification with pMALc2-plc as template. Primer A (CAAAAATAAAGATATGAAACAAGC) (all primers are listed 5′ to 3′) was upstream of the unique *Nsi*I restriction site of *plc*, while the reverse primer (CGGAATTCCATTTGTATAGTAATAAAAAGGTC) was complementary to the inverted repeat immediately following *plc* with a noncomplementary *Eco*RI site at the 5′ end. The PCR product was purified (QIAquick spin PCR purification), and the resulting product and pMALc2-plc vector were subjected to restriction digest using *Nsi*I and *Eco*RI. Gel purification (Geneclean) of the fragments followed by ligation provided pMAL-plc (Figure 2B), which was transformed into *E. coli* DH5α.

Oligonucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed by PCR using several different primer strategies. Standard two-primer PCR mutagenesis, in which a single forward primer incorporated both the *Nsi*I site and the mutagenic codon at residue 146 of the indicated DNA template, was used to generate the Asp (E146D), Glu (E146Q), and His (E146H) mutants. The mutagenic region of the primer is underlined.

For the E146D mutagenesis, the forward mutagenic primer was CCAACCGATGCATGCGGCAAACTTTACAAATCTTTCATATCCACAAGGATTCCATTCTAAATATGACAACTTTGTAGATACG, and the complementary primer downstream of a unique *Hin*d III site was primer B, CGACGGCCAGTGCCAAGCTTGCC. A standard 100 μL PCR was performed using pMALc2-plc as the template with Ampli*Taq* polymerase according to the "hot-start" protocol, and the product was cloned into the TA vector pCRII (Invitrogen). Transformation and subsequent blue-white screening on X-gal/IPTG plates yielded a clone that was cultured and ultimately determined by DNA sequencing to harbor the E146D mutant. After plasmid isolation (QIAprep spin plasmid mini-prep), this vector was subjected to restriction digest using *Nsi*I and *Hin*dIII, and the products were gel-purified and subcloned into pMALc2-plc to give pMALc2-plc(E146D).

A four-primer PCR method (Aiyar & Leis, 1993) was employed with pMALc2-plc as template using a completely degenerate codon within the mutagenic primer to generate a random assortment of mutants at position 146. The mutagenic primer used with primer B to generate PCR product 1 was GGATTCCATTCTAAATATNNNAACTTTGTAGATACG. PCR product 2 was created with primer A and GAATGTATCCAGCAGTAACAC. These two products were mixed in a 5:1 ratio of PCR product 1 to PCR product 2 and used with primer A and primer B in a third PCR reaction. The PCR product was cloned into the TA vector pCRII, and several positive transformants were cultured and screened by DNA sequencing. One of these clones was determined to possess the E146L mutant, and the *Nsi*I-*Sal*I region bearing the mutation was subcloned into pMALc2-plc to provide pMALc2-plc(E146L).

A degenerate mutagenic primer, CCAACCGATGCATGCGGCAAACTTTACAAATCTTTCATAT CCACAAGGATTCCATTCTAAATATCASAACTTTGTAGATACG, was used to provide both E146Q and E146H. The codon at residue 146 was CAS (S = G and C), which encoded for either Gln or His. Employing pMAL-plc as the template, a standard 100 μL PCR was performed using *Vent* polymerase with the "hot-start" protocol. The PCR product was purified (QIAquick) and subjected to a double restriction digest using *Nsi*I and *Eco*RI; gel purification provided the mutagenic fragment that was subcloned into pMAL-plc. DNA sequencing screening of several transformants was used to identify both the E146Q and E146H mutants. The entire genes of all four mutants were sequenced to ensure that no undesired mutations had been introduced during the mutagenesis.

Expression and Purification of Native and Mutant MBP−PLC_{Bc} Fusion Proteins. A 5−10 mL culture of LB/amp (100 μg/mL)/0.2% glucose was inoculated with a single recombinant colony of *E. coli* DH5α bearing the desired construct [pMAL-plc (for native PLC), pMALc2-plc(E146D), pMALc2-plc(E146L), pMAL-plc(E146Q), or pMAL-plc(E146H)] from a fresh streak plate and incubated overnight at 37 °C, with shaking at 225 rpm. This culture was then used to inoculate larger cultures (400 mL or 800 mL) at ratios of 1:100 or 1:200, and the resulting culture was incubated at 30 °C, with shaking at 225 rpm until reaching an OD_{600} of 0.6−0.7. At this point, fusion protein was induced by adding filtered IPTG to 0.3 mM, and the cultures were allowed to grow for 15 h. The cultures were then centrifuged at 4000*g*, at 4 °C for 20 min. The supernatant was decanted, and the cell pellet was frozen at −20 °C overnight. The pellet was resuspended in buffer A (20 mM Tris-HCl, 0.1 mM $ZnSO_4$, pH 7.3) at a ratio of 50 mL of buffer per 1 L of original liquid culture. This mixture was then lysed by passing twice through a French pressure cell (SLM Aminco, Rochester, NY) at a rate

of about 2 mL/min (15 000 psi). The lysate was clarified by centrifugation at 35000*g* at 4 °C for 30 min. The soluble fraction after centrifugation is shown in Figure 3, lane 1. The supernatant of the lysate was applied directly to an amylose resin column (ca. 5 mL column volume) under gravimetric flow. After application of the lysate, the column was washed with 10 column volumes of buffer A, and then the fusion protein was eluted with 3 column volumes of buffer A + 10 mM maltose (Figure 3, lane 2). Fusion protein was quantified by measuring the A_{280}, which was compared to calibrated values from a determination of the fusion protein using the Bradford assay.

Cleavage of Fusion Proteins and Purification of Native and Mutant PLC$_{Bc}$ Proteins. The MBP−PLC$_{Bc}$ fusion protein was cleaved by shaking (90 rpm) for 40 min with trypsin at 1% (w/w), at room temperature (Figure 3, lane 3). Trypsin inhibitor beads (0.5 g) were added to stop the cleavage reaction, and the mixture was shaken (200 rpm) at room temperature. After 1 h, the beads were removed by filtration and fresh beads added, and the mixture was shaken for 1 h as before. Depending upon the original concentration of fusion protein, a precipitate occasionally formed. Under these circumstances, urea was added to 5 M. After removing the second batch of beads by filtration, the resulting solution was applied to a Q-Sepharose column (8 mL of resin) eluting with buffer A under gravimetric flow. PLC$_{Bc}$ was collected in the immediate eluant from the column (Figure 3, lane 4). The protein solution was then applied to an IMAC column with Cu^{2+} as the immobilized metal. After being washed with 5 column volumes of buffer B (500 mM NaCl, 50 mM Tris−acetate, pH 7.5), PLC$_{Bc}$ was eluted with 3 column volumes of buffer B + 10 mM imidazole (Figure 3, lane 5). Dialysis vs 1.0 mM 3,3-dimethylglutaric acid (DMG), 0.1 mM $ZnSO_4$, pH 7.3 (1:100, 3 h, 2 times, 4 °C), afforded recombinant PLC$_{Bc}$ with maximal activity in the coupled chromogenic assay (Hergenrother *et al.*, 1995).

Protein Quantitation. Protein quantitation was performed using a modified Bradford protocol, as follows. Initial protein concentrations were diluted to the 10 μg/mL range to ensure linearity in the assay. A 120 μL volume of the protein solution (in 1.0 mM DMG, 0.1 mM $ZnSO_4$, pH 7.3) was added to 30 μL of the Bradford reagent (Bio-Rad) in 96-well plates. At $t = 5$ min, the plates were read at A_{595}. The absorbancies were calibrated to micrograms of PLC$_{Bc}$, via a calibration curve produced using PLC$_{Bc}$ obtained from Sigma. Three different dilutions (120 μL, 90 μL, and 60 μL) were run with DMG buffer making up the volume to 120 μL. At each of these three concentrations, three different samples were run. An average of the absorbancies was taken, and based on this, a microgram per milliliter value was calculated for each point. These three microgram per milliliter values were then averaged to obtain the final value. The three different dilutions generally differed by no more than 2.5%.

Kinetic Measurements. Activity assays of recombinant and mutant PLC$_{Bc}$ proteins were performed at pH 7.3 as previously described (Hergenrother *et al.*, 1995). Briefly, the phosphorylcholine product of PLC$_{Bc}$-catalyzed hydrolysis was converted to a chromogenic dye in 96-well plates via alkaline phosphatase, choline oxidase, peroxidase, phenol, and 4-aminoantipyrine. V_{max} and K_m values were obtained from the equations describing the Lineweaver−Burk plots of the data. Wild-type PLC$_{Bc}$ assays were conducted with the enzyme concentration at 5 nM (21.5 ng/well). Assays of mutant proteins were conducted in the same manner as the wild type with the exception that larger quantities of protein (ranging from 20× to 200× with respect to recombinant native PLC$_{Bc}$) were used. Thus, the E146Q mutant was assayed at 20−50 times the wild-type concentration, whereas the E146H, E146D, and E146L mutants were assayed at 75−200 times the wild-type concentration. The substrate 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine was routinely assayed from 0.7 mM to 5.0 mM. Higher concentrations were avoided due to the change in kinetics of PLC$_{Bc}$ as the cmc of the substrate is approached (El-Sayed *et al.*, 1985). The recombinant proteins were assayed from a minimum of three different protein preparations, each of which was derived from a distinct single colony from a streak plate. All protein preparations were tested on at least two different occasions, and 1 day to 1 month after isolation. This was especially significant in the case of the E146Q mutant to ensure that no deamidation (Wright, 1991) occurred to generate wild-type PLC$_{Bc}$.

pH Dependence. The dependence of activity (k_{cat}/K_m) on pH was measured for wild-type recombinant PLC$_{Bc}$ and for the E146Q mutant. The range studied was from pH 5.0 to pH 8.0 in 167 mM DMG, 0.1 mM $ZnSO_4$. For the E146Q experiments, assays at pH 5.0 and 5.5 were performed with the mutant at 1.0 μM. Studies at pH 6.0 were conducted with E146Q at 675 nM, and experiments at pH 6.5, 7.0, and 8.0 were carried out with E146Q at 250 nM. For the wild-type recombinant PLC$_{Bc}$, measurements at pH 5.0, 5.5, and 6.0 were performed with PLC$_{Bc}$ at 30 nM, and measurements at pH 6.5, 7.0, and 8.0 with PLC$_{Bc}$ at 5 nM.

Circular Dichroism Studies. Circular dichroism was performed on mutant and wild-type PLC$_{Bc}$ on a Jasco J600. Samples were run at 25 °C in a water-jacketed cylindrical quartz cell with a 1 cm path length at 0.020 mg/mL in 1.0 mM DMG, 0.1 mM $ZnSO_4$, pH 7.3. The parameters used are as follows: bandwidth = 1.0 nm, slit width = auto, sensitivity = 50 mdeg, time constant = 1 s, scan speed = 20 nm/s. Raw data were converted to ellipticity as reported (Little, 1978), using 116 as the mean residue weight. Temperature dependent studies were performed by raising the temperature at 2.5 °C intervals and then measuring the spectra after a 2 min equilibration time. Ellipticity at 222 nm as a function of temperature was used to monitor the denaturation of the proteins.

Analysis of Zinc Content. The zinc content of the mutant and wild-type PLC$_{Bc}$ enzymes was quantified by flame atomic absorption on a Perkin-Elmer 4000 atomic absorption spectrophotometer using the absorbance peak at 212.8 nm. After purification of the recombinant proteins, samples containing between 40 and 100 μg/mL protein were dialyzed against 1.0 mM DMG, pH 7.3, 3 times, 1:1000, for 3 h each time, at 4 °C. The buffer from the last dialysis was used as a blank and consistently showed no zinc present. Analyses were performed in triplicate using at least two different enzyme preparations.

RESULTS

Plasmid Construction. The plasmids used for the expression of PLC$_{Bc}$ were pMALc2-plc and pMAL-plc (Figure 2A,B). These plasmids differ only in that pMALc2-plc contains an open reading frame after the PLC$_{Bc}$ gene. Use

Table 1: Kinetic Parameters for PLC_{Bc} Proteins[a]

	V_{max} [μmol/(min·mg)]	k_{cat} (1/s)	K_m (mM)	k_{cat}/K_m [1/(s·mM)]
PLC_{Bc} (Sigma)	1250 ± 75	575	1.7 ± 0.1	338
PLC_{Bc} (recomb)	1200 ± 75	570	1.7 ± 0.2	335
E146Q	18 ± 4	8.6	1.6 ± 0.3	5.4
E146D	2.5 ± 0.5	1.2	1.1 ± 0.3	1.1
E146H	2.0 ± 0.5	1.0	1.4 ± 0.3	0.7
E146L	2.7 ± 0.5	1.3	1.6 ± 0.3	0.8

[a] Kinetic parameters for the substrate 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine were measured at pH 7.3 in the coupled chromogenic assay (Hergenrother *et al.*, 1995), with k_{cat} values calculated using a molecular mass = 28.5 kDa.

of the streamlined pMAL-plc construct typically gave about 25% greater isolated yield of fusion protein than pMALc2-plc. The E146D and E146L mutations were created using the pMALc2-plc template, whereas the E146Q and E146H mutants were prepared from the pMAL-plc template.

Protein Expression and Purification. Growth and induction with IPTG at 30 °C resulted in a fusion protein yield of ca. 60 mg/L. The MBP−PLC_{Bc} fusion protein was isolated by passing the post-French press supernatant over an amylose resin followed by elution with 10 mM maltose. Cultures grown and induced at 37 °C yielded only 20 mg/L fusion protein. The MBP−PLC_{Bc} fusion protein was then cleaved with trypsin, which was removed with trypsin inhibitor bound to glass beads. Factor Xa also cleaved the fusion protein, albeit much slower and with no more selectivity. The beads were removed by filtration, and the solution was passed over a Q-Sepharose ion exchange column, which retained the MBP. A Q-Sepharose column was used instead of the usual amylose resin column because the latter did not quantitatively bind the MBP. Passing the post-Q-Sepharose eluant over an IMAC resin column charged with Cu^{2+} retained PLC_{Bc} while the minor degradation products eluted; an IMAC/Zn^{2+} column did not selectively bind PLC_{Bc}. Subsequent elution of the column with 10 mM imidazole afforded PLC_{Bc}. The wild-type enzyme was isolated at levels of ca. 8−10 mg/L of culture, E146Q at ca. 2.5 mg/L, and E146D, E146H, and E146L at ca. 1.0 mg/L. N-Terminal analysis confirmed the authenticity of the first 30 residues of recombinant wild-type PLC_{Bc}. The purified recombinant PLC_{Bc} exhibited activity identical to that of commercially available PLC_{Bc} (Sigma) (Table 1).

The gel in Figure 3 reveals that there is a small amount of an approximately 26 kDa band present as an impurity, and gel scanning followed by densitometry shows that this band represents only about 5% of the total protein. This band was observed at approximately the same levels in samples of PLC_{Bc} isolated from its natural source *B. cereus* (Sigma) and in all of the recombinant wild-type and mutant PLC_{Bc} proteins isolated from *E. coli*. Blotting and subsequent N-terminal analysis of the 26 kDa band showed that its N-terminus was identical to that of PLC_{Bc}, suggesting that it is a truncated form of PLC_{Bc} in which a segment of about 2.5 kDa has been removed from the C-terminal end.

Effects of Mutations at Glu146. The activities of the four Glu146 PLC_{Bc} mutants are shown in Table 1. The catalytic efficiency (k_{cat}/K_m) for the E146Q mutant was reduced by 60-fold with respect to the wild-type recombinant enzyme, whereas the efficiencies for the E146D, E146L, and E146H mutants were about 300−500-fold lower than wild type. This

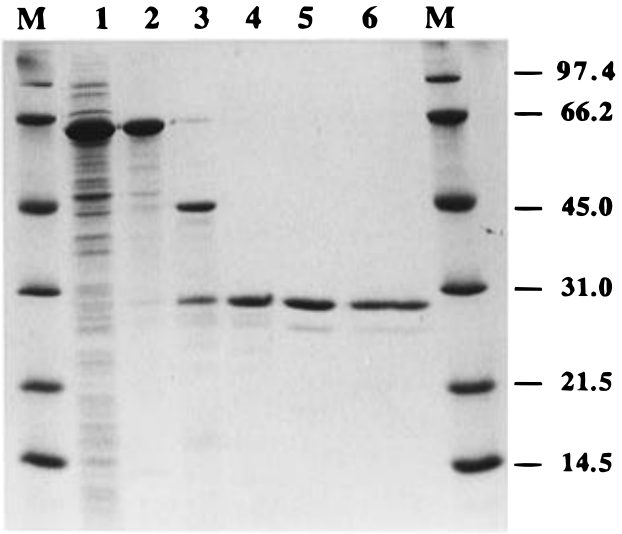

FIGURE 3: SDS−PAGE gel (15%) showing the purification of PLC_{Bc} from a MBP−PLC_{Bc} fusion protein. M, molecular weight markers (Bio-Rad); lane 1, soluble fraction after French press cell lysis; lane 2, eluate from amylose resin; lane 3 trypsin digest of fusion protein; lane 4, eluate from Q-Sepharose column; lane 5, eluate from IMAC (Cu^{2+}) resin; lane 6, PLC_{Bc} commercially available from Sigma; M, molecular weight markers (Bio-Rad).

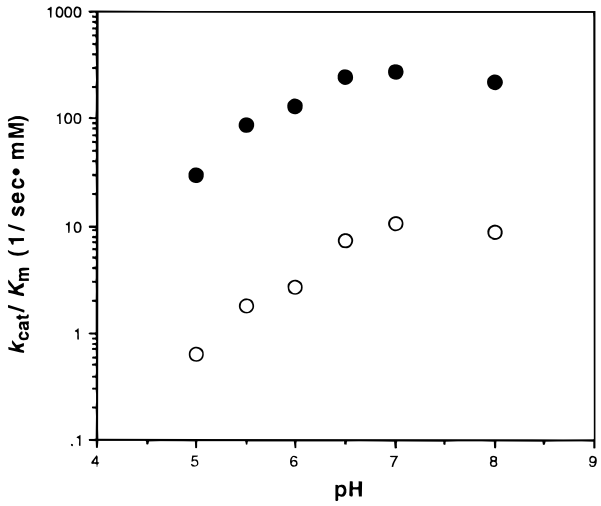

FIGURE 4: k_{cat}/K_m as a function of pH for wild-type recombinant PLC_{Bc} (closed circles) and the E146Q mutant (open circles).

decrease was due primarily to a lowering of the k_{cat}s of the mutants, as the K_m remained basically unchanged. The kinetic parameters for the wild-type and mutant enzymes were routinely determined in the presence of 0.1 mM $ZnSO_4$. However, the kinetic results obtained for wild-type and mutant PLC_{Bc}s at $ZnSO_4$ concentrations up to 1.0 mM were identical to those measured at 0.1 mM $ZnSO_4$.

pH Dependence. The pH vs k_{cat}/K_m graph for wild-type PLC_{Bc} and the E146Q mutant is shown in Figure 4. The pH vs rate experiments were performed 4 times from two separate enzyme preparations.

Circular Dichroism. The far-UV CD spectra of the recombinant proteins were measured at 25 °C and compared with that of wild-type PLC_{Bc} (Figure 5). The spectrum of the E146Q mutant is similar to wild type, but those for the other three mutants are considerably different, indicating that replacing Glu146 with Asp, His, and Leu results in the loss of a substantial degree of helical structure. Qualitatively, the percent of α-helix in the five proteins follows the order

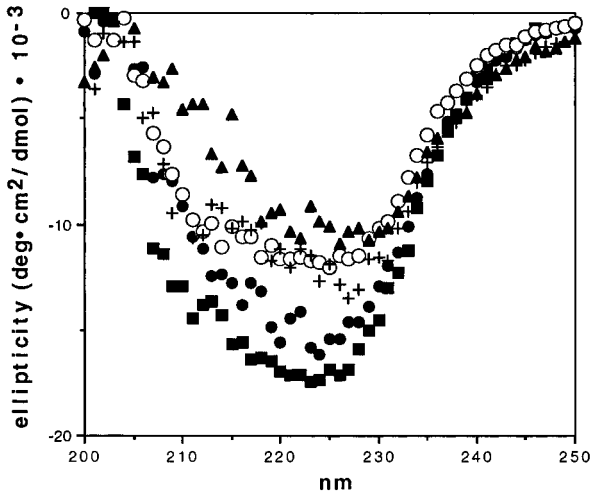

FIGURE 5: CD spectra of recombinant proteins at 25 °C. Wild-type (closed squares), E146Q (closed circles), E146D (open circles), E146H (closed triangles), and E146L (crosses) spectra were taken at a protein concentration of 20 μg/mL.

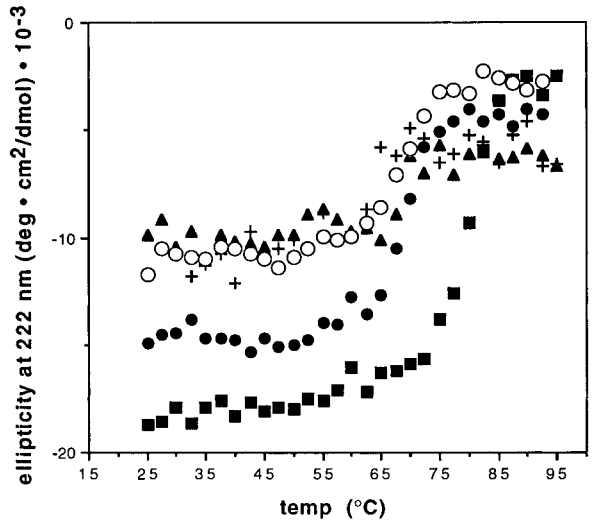

FIGURE 6: CD signal at 222 nm as a function of temperature. The spectra were recorded after a 2 min equilibration period at the desired temperature. Wild-type (closed squares), E146Q (closed circles), E146D (open circles), E146H (closed triangles), and E146L (crosses).

wild-type ≈ E146Q > E146D ≈ E146H ≈ E146L. The CD spectra of the recombinant proteins were also taken after they were dialyzed against a buffer containing no Zn^{2+} (1.0 mM DMG, pH 7.3), and these spectra were virtually identical to the spectra of the proteins in the Zn buffer (data not shown). The CD signal at 222 nm was measured as a function of temperature for all the recombinant proteins. The graph in Figure 6 indicates that the mutants begin to undergo significant denaturation at about 65 °C, whereas wild-type PLC_{Bc} is relatively stable up to 75 °C.

Atomic Absorption. Results from atomic absorption analysis are presented in Table 2. These data indicate that the wild-type recombinant PLC_{Bc} retains 3 mol of Zn^{2+}/mol of PLC_{Bc} after dialysis. The E146Q and E146D mutants, which retained 2.1 ± 0.3 mol of Zn^{2+}/mol of PLC_{Bc}, exhibited essentially the same stoichiometry of binding Zn^{2+} as the E146H and E146L mutants, which retained about 2.0 ± 0.4 mol of Zn^{2+}/mol of PLC_{Bc}. Mutations of the Glu residue at position 146 clearly have an adverse effect on the amount of Zn^{2+} that is present in these enzymes.

Table 2: Atomic Absorption Analysis on Recombinant PLC_{Bc} Proteins[a]

protein	mol of Zn/mol of protein
wild type	3.05 ± 0.15
E146Q	2.1 ± 0.3
E146D	2.1 ± 0.3
E146H	2.0 ± 0.4
E146L	2.0 ± 0.4

[a] Analysis performed after dialyzing samples 3 times (1:1000) vs 1.0 mM DMG, pH 7.3. Protein samples were between 40 and 100 μg/mL and moles of PLC_{Bc} were calculated using molecular mass = 28.5 kDa.

DISCUSSION

Elucidation of the specific function of individual amino acid residues at the active site of the phosphatidylcholine-preferring PLC from *B. cereus* represents a key step toward determining the catalytic mechanism for the enzyme. Examination of the three-dimensional structure of PLC_{Bc} complexed with an inhibitory substrate analogue (Hansen *et al.*, 1993) reveals that Glu146 might play a role in the hydrolysis of phospholipids by PLC_{Bc}. In this complex, one of the carboxylate oxygens in the side chain of Glu146 is positioned to serve as a ligand for Zn2, while the other carboxylate oxygen is within hydrogen bonding distance of a water molecule that has been suggested as attacking nucleophile. However, because the pK_a of a metalated carboxyl group is unsuited for a general base, the ability of Glu146 to serve simultaneously as a zinc ligand and a general base is problematic. In the present study, site-directed mutagenesis was used to replace Glu146 with Gln, Asp, His, and Leu to probe the function of Glu146 in the PLC_{Bc}-catalyzed hydrolysis of phospholipids.

Predicting *a priori* the effects upon catalysis of mutating the Glu146 residue is complicated not only by the possible structural changes that might accompany the mutation but also by the uncertainty regarding whether this residue is the general base or a ligand for Zn2. In the absence of structural changes, the conserved E146D mutant should exhibit somewhat diminished catalytic activity if Glu146 were the general base. On the other hand, the E146Q and E146L mutants would be expected to be significantly less active because these side chains are incapable of activating the nucleophilic water; however, the histidine side chain of the E146H mutant has the potential to be the general base. If Glu146 stabilizes the structure of PLC_{Bc} by serving as a ligand for Zn2, the side chains of histidine, aspartic acid, and perhaps glutamine might be suitable functional replacements, whereas the side chain of leucine obviously is not. Glutamine has recently been incorporated into the coordination sphere of Zn1 in alkaline phosphatase (Ma & Kantrowitz, 1996), and histidine triads for a catalytic zinc are present in carboxypeptidase (Dideberg *et al.*, 1982) and carbonic anhydrases I and II (Kannan *et al.*, 1975; Liljas *et al.*, 1972).

Comparison of the catalytic efficiencies at pH 7.3 of the PLC_{Bc} mutants in which the Glu146 residue was replaced with glutamine, aspartic acid, histidine, and leucine (Table 1) indicates that this residue is essential for full catalytic activity. The E146Q mutant, which retained about 1.6% the activity of the wild type, was the most active, whereas the E146D, E146H, and E146L mutants were 0.2−0.3% as active as the wild type. The kinetic data indicate that the major effect on lowering the catalytic efficiency is in k_{cat} as the

K_m values of all mutants were similar to that of the wild type. Because the kinetic results obtained for wild-type and mutant PLC_{Bc}s at $ZnSO_4$ concentrations up to 1.0 mM were identical to those at 0.1 mM, the occupancies of the Zn2 sites of the mutants do not appear to increase at zinc ion concentrations greater than 0.1 mM.

The observation that E146L has roughly the same activity as E146D and E146H, even though the leucine side chain cannot possibly serve as a general base, suggests that in these mutants the 146 side chain has no role as a general base. Indeed, if Glu146 were the general base in the catalytic mechanism of PLC_{Bc}, one might expect larger decreases in the catalytic efficiencies for the mutants rather than the 60-fold (E146Q) to 300−500-fold (E146D, E146H, E146L) that was observed. For example, replacing the general base Glu165 of triosephosphate isomerase (TIM) with alanine or glycine resulted in an enzyme that has a catalytic activity 10^6 times less than the wild type (Knowles, 1991). In fructose-1,6-bisphosphatase, which is another phosphoryl transfer enzyme, the E98Q mutant had a k_{cat}/K_m 10^4 times less than the wild-type enzyme (Kelly *et al*., 1996). These examples clearly indicate the potential power of general base catalysis in well-documented systems.

The pH−rate profiles for the wild-type PLC_{Bc} and the E146Q mutant further support the hypothesis that Glu146 is not the general base. If Glu146 were acting as the general base, the ascending limb at acidic pH for the wild-type enzyme should not appear in the plot of k_{cat}/K_m vs pH for the E146Q mutant. However, examination of Figure 4 reveals that the shapes of the curves of k_{cat}/K_m vs pH for wild-type recombinant PLC_{Bc} and for E146Q are nearly identical from pH 5 to 8, and each has an initial slope of 1 in the acidic limb. For both the wild type enzyme and the E146Q mutant, a general base with a similar pK_a is being ionized as the pH is raised, thereby leading to a higher catalytic activity for each enzyme.

The CD data (Figures 5 and 6) and the atomic absorption data (Table 2) provide compelling evidence that Glu146 plays a critical role as a ligand for Zn2 to stabilize the secondary and tertiary structure of PLC_{Bc}. Even the conservative replacements of Glu146 with glutamine or aspartic acid result in a significant loss of structural stability and reduced affinity for zinc ion. Comparison of the CD spectra in Figure 5 reveals that the solution structure of E146Q is the most similar to wild type, whereas the structures of the E146L, E146D, and E146H mutants contain significantly less helical structure. When the CD spectra of the wild-type PLC_{Bc} and its mutants are measured at 222 nm as a function of temperature (Figure 6), the differences in the thermal stabilities of the enzymes are evident. The wild type PLC_{Bc} undergoes denaturation beginning at 75 °C and is fully denatured at 85 °C. Both the E146Q and E146D mutants begin to denature at about 67 °C and are fully denatured at about 75 °C, whereas the E146H and E146L mutants start to denature at 65 °C and are fully denatured at 70 °C.

The weight of the available evidence suggests that Glu146 plays a key role as a ligand for Zn2. Replacing Glu146 with glutamine is less deleterious to the structure and function of PLC_{Bc} than replacing this residue with aspartic acid, histidine, or leucine. It thus seems reasonable to conclude that the E146Q mutant is the most active because its three dimensional structure more closely resembles that of the wild type than the other mutants, so substrate binding and catalysis are not as adversely affected. Indeed, as measured by circular dichroism, the solution structure of the E146Q mutant is more similar than the other three Glu146 mutants to the wild type. One major consequence of losing a ligand for Zn2 in each of the four mutants is that the Zn2 site does not appear to be occupied by a zinc ion. This necessarily results in a decrease in catalytic activity because one of the roles of Zn2 is to neutralize the charge on the phospholipid phosphate group, thereby facilitating nucleophilic attack by a water molecule. The additional loss of three-dimensional structure exacerbates the situation and reduces activity further, so the relative activities of the mutants correlate roughly with their solution structure as measured by CD.

The observed reductions in activities and zinc affinities for the Glu146 mutants of PLC_{Bc} are similar to those observed upon mutations of the Zn ligands in other zinc-dependent enzymes. For example, mutagenesis of the Glu408 in aminopeptidase A to an aspartic acid residue gave a protein that exhibited a k_{cat} of 5% of the wild type with a similar K_m (Vazeux *et al.*, 1996). The Zn content of the E408D mutant was 60% of the wild type. Similarly, when measured in the presence of a phosphate acceptor, the k_{cat} for the H142Q mutant of the alkaline phosphatase from *E. coli* was approximately 3% of the wild type (Ma & Kantrowitz, 1996); this mutant contained roughly 50% of the zinc that the wild type possessed. In addition, a series of single mutants at His94, His96, or His119 of human carbonic anhydrase II have been kinetically characterized, and the catalytic efficiencies of these enzymes ranged from approximately 14% to 0.1%, depending on the amino acid substituted and its position (Kiefer & Fierke, 1994).

Since Glu146 is not the general base, another active site residue, one of the zinc ions, or a combination of a carboxylate group and a zinc ion must activate water for attack on the phosphorus atom. Asp55 (Sundell *et al.*, 1994) and Glu4 (Byberg *et al.*, 1992) have been proposed as potential candidates for the general base. However, these residues are also positioned to fulfill other roles, with Asp55 serving as a ligand for Zn1 and Glu4 stabilizing the positive charge on the choline moiety. The alternate possibility that a Zn ion-activated water is the attacking nucleophile is based on ample precedent for zinc-containing enzymes in which one or more zinc ions play an active role in catalysis (Vallee & Auld, 1990, 1993). For example, metal ions activate nucleophiles in phosphoryl transfer processes as illustrated by the zinc ion activation of Ser102 and a water molecule during two steps of the hydrolysis of phosphate monoesters by the alkaline phosphatase (Kim & Wyckoff, 1991). The active site that is associated with the 3′-5′ exonuclease activity of DNA polymerase I from *E. coli* contains two divalent metals (Derbyshire *et al.*, 1988). After several residues were excluded as candidates for the general base by mutagenesis studies (Derbyshire *et al.*, 1991), a two metal ion mechanism was proposed in which one of the active site metal ions generates the attacking hydroxide ion (Beese & Steitz, 1991), which is hydrogen bonded to the amino acid side chain of Glu357.

Examination of the X-ray structure of the complex of PLC_{Bc} with a bound phosphonate inhibitor reveals two water molecules that are plausible candidates for the attacking nucleophile (Figure 1). The W1 is 4.40 Å from the phosphorus atom and 2.49 Å from one of the oxygens on the side chain carboxyl group of Glu146, whereas W2 is

located 4.73 Å from the phosphorus atom, 2.82 Å from an oxygen on the side chain of Asp55, and 2.69 Å from a Glu4 oxygen. The attack angles are 135° and 126° for W1 and W2, respectively. Because these waters are removed from the zinc ions by 4.6−7.1 Å, making a compelling case for activating either of these by a zinc ion is presently problematic. However, it must be recognized that the inhibitor complex is nonproductive, and the positions of water molecules and the substrate in the active site during catalysis will likely be different.

In summary, the present results suggest that Glu146 serves as a key ligand for Zn2 in the active site of PLC_{Bc}, but it does not appear to function as the general base during the enzymatic hydrolysis of the phosphodiester bonds of phospholipids by PLC_{Bc}. That role must be played by another residue and/or one of the three Zn ions at the active site. The emergence of new insights of the catalytic mechanism of PLC_{Bc} must await further kinetic and structural studies of other site-specific mutants.

ACKNOWLEDGMENT

We are grateful to Dr. Terje Johansen (University of Tromsø, Norway) for providing the construct pMALc-plc(Bst) and Professor W. W. Cleland (University of Wisconsin at Madison) for helpful discussions. We also thank Dr. Karen Browning (University of Texas at Austin) for discussions on experimental protocols and Mr. Andrew Breksa (University of Texas at Austin) for blotting of the PLC_{Bc} degradation product for N-terminal analysis.

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BI961316F