General Base Catalysis by the Phosphatidylcholine-Preferring Phospholipase C from *Bacillus cereus*: The Role of Glu4 and Asp55[†]

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ABSTRACT: To assess what roles the active site residues Glu4 and Asp55 of the phosphatidylcholine-preferring phospholipase C of *Bacillus cereus* (PLC_{Bc}) might play in binding and catalysis, selected mutants were prepared through site-directed mutagenesis of the *plc* gene. The mutants were then expressed in *Escherichia coli* and purified as fusion proteins with the maltose binding protein (MBP). Kinetic analysis showed that mutations at Glu4 had only modest effects on the catalytic activity, whereas those at Asp55 led to proteins whose values for k_{cat}/K_M were 10^4-10^6 times less than that of the wild-type enzyme. The modest decrease in catalytic activity and the pH-dependent profile of the E4L mutant strongly suggest that glutamic acid at position 4 is not the general base in the PLC_{Bc}-catalyzed reaction. Rather, the results support the hypothesis that Glu4 is primarily involved in substrate binding, perhaps by electrostatic stabilization of the positive charge of the choline moiety of the phosphatidylcholine substrate. Examination of X-ray crystallographic data of PLC_{Bc} and its various complexes reveals that the carboxylate side chain of Asp55 is positioned such that it could activate a water for nucleophilic attack on the substrate or serve as a ligand for Zn1. However, the involvement of the side chain of Asp55 as an important Zn1 ligand is not consistent with the atomic absorption and thermostability data obtained for the D55L mutant, which are virtually identical with that of the wild-type enzyme. The large reduction in the measured k_{cat}/K_M of the D55E, D55N, and D55L mutants of PLC_{Bc} indicates that Asp55 plays a critical role in catalysis and likely serves as the general base in the hydrolysis of phosphatidylcholine by PLC_{Bc}.

The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc})[1] (EC 3.1.4.3) is a 28.5 kDa, monomeric zinc enzyme containing 245 amino acids that catalyzes the hydrolysis of phospholipids to produce diacylglycerol (DAG) and a phosphorylated headgroup. This enzyme is known to have its greatest activity on phosphatidylcholine phospholipids, although it processes phosphatidylethanolamine and phosphatidylserine at reduced rates (*1*). Like most phospholipases, PLC_{Bc} exhibits a marked increase in activity on micellular substrates (*2*). In mammalian systems, certain phospholipases are known to catalyze key reactions in the signal transduction cascade, and the antigenic similarity of PLC_{Bc} with a mammalian counterpart suggests that PLC_{Bc} may be a useful model for these higher order systems (*3*). However, the in vivo function of PLC_{Bc} itself appears more likely to be tied to a phosphate retrieval mechanism (*4*). X-ray crystallographic analysis of PLC_{Bc} (*5*), and of the enzyme complexed with several small ions (*6*, *7*) and a phosphonate inhibitor (*8*), revealed three zinc ions (Zn1, Zn2, Zn3) in the active site. Such trimetal centers are uncommon, with the alkaline phosphatase from *Escherichia coli* (APase) (*9*) and the P1 nuclease of *Penicillium citrinum* (*10*) being the only two other crystallographically characterized enzymes that have analogous metal constellations.

The recent cloning (*11*) and subsequent development of a system for the overexpression of PLC_{Bc} in *E. coli* (*12*) has set the stage for a detailed examination of the mechanism of phosphodiester hydrolysis by this enzyme. In analogy with other phosphodiesterases, the reaction is believed to proceed by an in-line attack of an activated water molecule on the phosphodiester, followed by collapse of the resulting pentacoordinate intermediate to give DAG and a phosphorylated headgroup with inversion of stereochemistry at phosphorus. However, it should be noted that this stereochemical outcome has yet to be proven experimentally.

The mechanism by which PLC_{Bc} catalyzes the hydrolysis of phospholipids has been the subject of speculation for some time. The primary focus of these efforts has been upon identifying the functionality that serves as the general base to activate the nucleophilic water for attack on the phosphodiester (*8*, *12*−*15*). The pH dependence of PLC_{Bc} activity is characterized by a bell-shaped curve with a maximum between pH 7 and 8 (*12*, *16*). From the pH dependence curve, a pK_a of approximately 5.3 has been assigned to the general base (*12*, *13*). The side chain carboxyl groups of Glu4, Asp55, and Glu146 have been postulated as potential candidates for the general base, but a strong case was recently

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[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; kD, kilodalton(s); MBP, maltose binding protein; PC, phosphatidylcholine; PCR, polymerase chain reaction; PLC, phospholipase C; PLC_{Bc}, PC-preferring phospholipase C from *Bacillus cereus*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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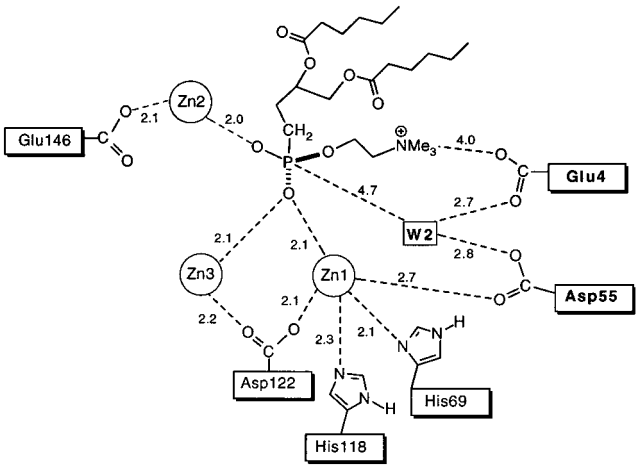

FIGURE 1: Distances (in angstroms) of selected active site residues of PLC_{Bc} from a phosphonate substrate analogue inhibitor (*8*). Both Glu4 and Asp55 are in a position to serve as the general base in the PLC_{Bc}-catalyzed reaction by activation of W2, an active site water molecule. However, they are also in a position to fulfill other roles, Glu4 in substrate binding and Asp55 as a Zn1 ligand.

made against Glu146; this residue instead appears to be an important ligand for Zn2 (*12*). In analogy with mechanisms invoked for APase (*17*) and carboxypeptidase A (*18*), a zinc-bound water or hydroxide ion could be the attacking nucleophile. However, inspection of the crystal structure of PLC_{Bc} with a phosphonate inhibitor reveals that there are no water molecules in the first coordination sphere of any of the zinc ions (*8*), so there is no available experimental evidence that supports the direct activation of a water molecule by one of the metal ions.

The present investigation was designed to address the question of whether Glu4 or Asp55 were likely to serve as the general base in the PLC_{Bc}-catalyzed hydrolysis of phospholipids. Examination of the X-ray structure of PLC_{Bc} with a bound phosphonate inhibitor reveals that the free carboxyl groups of each of these residues are in position to perform this critical function (Figure 1) (*8*). One carboxylate oxygen of Glu4 is 2.7 Å from an active site water that is identified as W2. The W2 molecule lies 4.7 Å from the phosphorus atom of the inhibitor, and has an attack angle of 126°, which is defined by W2, phosphorus, and the methylene carbon of the phosphonate. These values are consistent with those that have been suggested for the nucleophilic attack of water on phosphorus by an associative mechanism (*19*). Because a carboxylate oxygen of Asp55 is 2.8 Å from W2, this residue is also a candidate for the general base.

Although the spatial relationships of Glu4 and Asp55 side chain carboxyl groups with W2 are consistent with either being the general base, each of these carboxyl functions is also positioned to fulfill other roles. One carboxylate oxygen of Glu4 is 4.0 Å from the methyl groups of the quaternary amino group of the choline moiety, so it may help stabilize the local positive charge on the substrate. One oxygen of the side chain carbonyl group of Asp55 is 2.7 Å from Zn1, thereby placing it within the first coordination sphere so it may serve as a ligand for Zn1, even though this distance is considerably longer than the other Zn−ligand interactions, which are typically 2.1−2.3 Å. Because experimental evidence supporting the specific roles that Glu4 and Asp55 play in binding or catalysis by PLC_{Bc} was lacking, a series of studies was undertaken in which these residues were mutated, and the resulting mutant proteins were evaluated in kinetic and several structural assays. The results are presented herein.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes, T4 DNA ligase, Vent polymerase, and amylose resin were obtained from New England Biolabs (Beverly, MA). AmpliWax PCR Gems were obtained from Perkin-Elmer (Norwalk, CT). 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, PD-10 columns, and Chelating Sepharose were obtained from Pharmacia (Piscataway, NJ). All oligonucleotides were acquired from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed by the DNA sequencing center at the University of Texas at Austin with an Applied Biosystems Model 373A. 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

Oligonucleotide-Directed Mutagenesis. Site-directed mutagenesis was performed via PCR methods as described (*12*). All PCR reactions were performed under standard conditions using the 'hot start' protocol and Vent polymerase. The E4L mutation was created by a two-primer method with the mutagenic primer E4QL (GCTCGGTACCCGGCCGGG-GATCCATCGAGGGTAGGTGGTCTGCTCWGGAT-AAACATAAAGAAGGTGTAAATTCTC, all primers listed 5′ to 3′) covering a unique *Kpn*I restriction site. The underlined portion of the oligonucleotide is the noncomplementary mutagenic region that codes for either glutamine or leucine (W = A, T). PCR with the primer E4QL and the reverse primer, primer B (CGACGGCCAGTGCCAAGCT-TGCC), on the pMAL-plc (*12*) template yielded a PCR fragment that was spin-purified (QIAquick) and sequentially restricted from *Kpn*I to *Nsi*I; the resulting fragment was gel-purified (Geneclean). In an analogous fashion, the plasmid pMAL-plc was restricted from *Kpn*I to *Nsi*I; after gel purification, it was ligated to the gel-purified PCR product using T4 DNA ligase. Culturing, plasmid preparation, and DNA sequencing of colonies obtained after electroporation into *E. coli* DH5α produced the E4L mutant. The entire gene of this clone was sequenced in each case to ensure that no undesired mutations were introduced during the mutagenesis.

The codon for aspartic acid at position 55 was replaced with the codons for asparagine, glutamic acid, glutamine, and leucine, utilizing a four-primer overlap extension method. Primer MalE (GGTCGTCAGACTGTCGATGAAGCC) and primer rev@54 (GTCAGCAGCATAAATACCGTTCTCT-AACTCCG) were used in a PCR reaction on the pMAL-plc template to provide product 1 for the overlap extension. Product 2 for the overlap extension was created with the reverse primer revpMalMCS (GGGTTTTCCCAGTCACG-

TACGTTGTAAAACG) and one of two mutagenic primers, D55EQ (GAGAACGGTATTTATGCTGCTSAGTATGAAAATCCTTATTATG) or D55HILN (GAGAACGGTATTTATGCTGCTMWCTATGAAAATCCTTATTATG). The underlined codon represents the mutagenic portion of the primer with S = C, G; M = A, C; W = A, T. A PCR reaction was performed using products 1 and 2 with primers MalE and rev@MCS. The resulting mutant fragments were then restricted from *Kpn*I to *Nsi*I and subcloned into pMAL-plc. DNA sequencing identified the D55E, D55Q, D55N, and D55L mutants, and the remainder of the gene was sequenced to ensure that no undesired mutations had occurred.

The codon for the histidine residue at position 118 of *plc* was mutated to leucine by a megaprimer method. The mutagenic primer H118L (GGATTATCTCTTCTGTATTTAGGAGATGTAAACCAACC) was used in a standard PCR reaction on the pMAL-plc template with primer revpMalMCS to provide PCR product 3. Product 3 was used as the 'megaprimer' with primer MalE in a second PCR on the pMAL-plc template. This PCR product was restricted from *Kpn*I to *Nsi*I, gel-purified, and cloned into pMAL-plc. DNA sequencing confirmed the H118L mutation.

Expression and Purification of Recombinant Proteins. Wild-type PLC_{Bc} and its mutants were expressed in DH5α cells and purified as previously described (*12*). After growth (at 30 °C, 225 rpm) and induction (with IPTG to 0.3 mM) of large cultures, the cells were spun down and lysed using a French pressure cell. Centrifugation of the lysate and passage of the soluble fraction over an amylose column yielded the wild-type and mutant MBP−PLC_{Bc} fusion proteins at a level of ~80 mg/L. After cleavage of this fusion protein with trypsin, chromatography on Q-Sepharose and Chelating Sepharose resins (with Cu^{2+} as the metal) afforded pure recombinant protein. Protein quantitation was performed via a modified Bradford protocol (*12*).

Analysis of Recombinant Proteins. Enzymatic assays of PLC_{Bc} and mutants were performed via a coupled chromogenic assay in which the phosphorylcholine product of the PLC_{Bc}-catalyzed hydrolysis of 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine was converted to a red dye through the action of alkaline phosphatase, choline oxidase, and peroxidase (*20*). For the E4L mutant, protein concentrations of 600 nM were used from pH 5.0 to 6.0, and protein concentrations of 100 nM were used from pH 6.5 to 8.0. The pH-dependent assays, the circular dichroism experiments, and the atomic absorption analyses were performed as described (*12*). Circular dichroism experiments were performed at enzyme concentrations of 20 μg/mL. Samples were prepared for atomic absorption by passage over a PD-10 column previously equilibrated with 1.0 mM DMG, pH 7.3, and eluted with this buffer.

RESULTS AND DISCUSSION

Elucidating the mechanistic details for the enzymatic processing of a substrate is a challenging task. A classical approach to solving this problem is to establish the roles that specific amino acid residues of the enzyme play in binding and catalysis by structural and/or kinetic studies of the wild-type enzyme and appropriate mutants. The general base that activates a water molecule for nucleophilic attack on the phosphorus atom of the phosphodiester group in the PLC_{Bc}-catalyzed hydrolysis of phospholipids has not yet been identified, although Glu146, Glu4, and Asp55 have all been proposed as candidates (*12*−*15*). However, structural evidence suggests that the side chain carboxyl group in each of these residues is positioned such that two possible roles might be assigned to each (*8*). That Glu146 is a ligand for Zn2 rather than the general base is supported by recent studies involving analysis of site-directed mutants of that residue (*12*). The side chain of Glu4 is oriented so that either it could act as the general base by activating a water such as W2 or it could stabilize the positive charge on the choline moiety through electrostatic effects. The side chain of Asp55 is positioned so it could serve as a general base, or it could be a ligand for Zn1. The results of the present investigation support the hypothesis that Asp55, not Glu4, activates a water molecule for nucleophilic attack on the phosphodiester linkage of phospholipids.

Table 1: Kinetic Parameters for Recombinant PLC_{Bc} and Mutants[a]

	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1}·mM)	relative activity (%)
wild-type	570 ± 75	1.7 ± 0.2	335	100
E4D	285 ± 50	5.5 ± 1.0	52	15
E4L	215 ± 50	10 ± 3.0	21.5	6.4
D55E	0.19 ± 0.1	2.1 ± 1.5	0.090	0.027
D55N	0.09 ± 0.1	3.0 ± 2.0	0.032	0.0096
D55L	0.0006 ± 0.0005	3.0 ± 2.0	0.0002	0.00009
H118L	0.5 ± 0.2	3.5 ± 2.0	0.14	0.04

[a] Kinetic parameters for the substrate 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine were measured at pH 7.3 in the coupled chromogenic assay (*20*),with k_{cat} values calculated using a molecular mass of 28.5 kDa. All assays were conducted in the presence of 33 μM $ZnSO_4$.

The question of whether a specific residue is the general base can be examined by creating a mutant whose side chain is not ionizable. If the residue is the general base, a dramatic loss of catalytic activity and a flattening in the acidic limb of the pH-dependent curve would be anticipated. Likewise, the effect of a residue upon choline binding can be gauged by examining mutants at the choline binding residue. Although kinetic results will vary depending on the degree to which the side chain of a residue is involved in binding, an increase in the K_M for the enzymatic reaction of a mutant suggests that the residue plays a role in binding of substrate or release of product. The importance of a residue in zinc binding can be evaluated by quantitating the amount of zinc present in mutants lacking the putative zinc ligand compared to the wild type by atomic absorption spectroscopy. For example, mutations of Glu146 of PLC_{Bc}, which is a ligand for Zn2, led to proteins that no longer bound three zinc ions; these mutants also exhibited significantly reduced thermostablility relative to wild-type PLC_{Bc} (*12*). This latter finding is consistent with other observations that proteins containing only zinc (*21*) or a combination of zinc and other metal ions (*22*) have decreased thermal stability when one of the zinc ions is lost.

Effect of Mutations at Glu4 and Asp55. To probe the roles of Glu4 and Asp55, the E4D, E4L, D55E, D55N, and D55L mutants were prepared, and the kinetic data for these mutants toward 1,2-dihexanoylphosphatidylcholine as substrate are presented in Table 1. Although all mutations result in reduced catalytic activity, those mutations at Glu4 are much

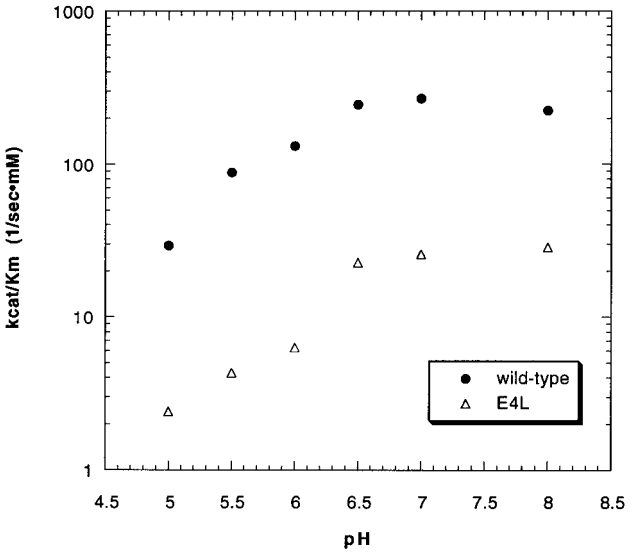

FIGURE 2: Dependence of rate on pH for wild-type PLC_{Bc} (closed circles) and the E4L mutant (open triangles). Experiments were conducted using a coupled chromogenic assay (*20*). The nearly identical ascending acidic limbs of these curves indicates that E4 is not the residue being ionized as the pH is increased.

less deleterious to the catalytic activity than those at Asp55.

Of the two mutants E4D and E4L, the less conserved E4L mutant is the least active on the dihexanoylphosphatidylcholine substrate with a k_{cat}/K_M that is 6.4% of the wild-type. These data reveal that while a glutamic acid residue at position 4 is required for optimal activity of PLC_{Bc} toward phosphatidylcholine substrates, the enzyme can clearly function at a reasonable rate without it. The k_{cat}'s for the E4D and E4L mutants are each about 50% that of the wild-type, whereas the corresponding K_M's are about 3- and 5-fold greater than that of wild-type. It should be noted, however, that although the observed trends in the values of K_M for the E4D and E4L mutants are valid, these K_M's could not be accurately determined over the entire range of 0.5 to 5 K_M due to the change in kinetics that is known to occur above the CMC (14 mM) (*2*) of dihexanoylphosphatidylcholine.

The value determined for k_{cat}/K_M of the E4L mutant is inconsistent with the 10^4-10^6 rate reduction that would be expected upon mutating a general base (vide infra). Further evidence against the role of Glu4 as the general base is found in the data showing the dependence of activity on pH for the unconserved E4L mutant and wild-type PLC_{Bc} (Figure 2). Both the wild-type and the E4L mutant have nearly identical ascending acidic limbs, indicating that the enzymatic activity increases as a side chain is being ionized with increasing pH. Because the leucine side chain of the E4L mutant cannot ionize, the ionization of the side chain of Glu4 in PLC_{Bc} cannot be responsible for the specific ionization that leads to increased activity at higher pH. On the other hand, the observed increases in the K_M's of the E4D and E4L mutants are consistent with Glu4 being involved in binding the choline moiety.

In contrast to the minor changes in k_{cat}/K_M that were observed with the Glu4 mutants, modification of the Asp55 residue resulted in dramatic changes in catalytic activity (Table 1). Even the conserved substitution of aspartic acid at position 55 by glutamic acid reduced k_{cat}/K_M by about 4000-fold. When aspartic acid is replaced with a nonionizable residue such as asparagine, glutamine, and leucine, catalysis occurs at a rate 10^4-10^6 times less than the wild-type enzyme. The K_M for each of the D55E, D55N, and D55L mutants was approximately the same, so the primary effect of mutating Asp55 is upon k_{cat}. This trend in catalytic activity is consistent with the removal of the general base from the active site and is similar to observations in other systems, including several phosphoryl transfer enzymes such as triosephosphate isomerase (*23*), fructose-1,6-bisphosphatase (*24*), ribonuclease A (*25*), and a phosphatidylinositol-specific phospholipase C (*26*). In each of these systems, mutation of the general base results in a dramatic drop in activity ranging from 4 to 6 orders of magnitude. Because the D55N and D55L mutants do not retain sufficient activity to obtain accurate kinetic data with the colorimetric assay used, it was not possible to obtain a meaningful pH dependence curve for either; similar difficulties in determining the pH dependence of mutants having extremely low activities have been reported (*27*). Nevertheless, within the large experimental error that is inherent in the present assay for mutants that are 4 orders of magnitude less active than wild-type, the D55N mutant exhibited virtually identical activity at pH 5.0 and 7.3.

Table 2: Atomic Absorption Analysis[a] and Temperature Stability Studies on Recombinant PLC_{Bc} and Mutants

protein	mol of Zn/mol of protein	melting temperature (°C)
wild-type	3.05 ± 0.15	79
D55L	2.7 ± 0.30	80
H118L	1.6 ± 0.25	68

[a] Analysis performed after passage of protein over PD-10 columns equilibrated with 1.0 mM DMG, pH 7.3. Protein samples were between 40 and 100 μg/mL, and moles of PLC_{Bc} were calculated using a molecular mass of 28.5 kDa.

Because the carboxyl group of Asp55 lies within the first coordination sphere of Zn1 (*8*), the loss of this ligand was also considered as a possible cause for the large reduction in catalytic activity of the Asp55 mutants. Loss of an important ligand for Zn1 could lead to facile loss of this zinc ion from the active site with resultant destabilization of the tertiary structure. Loss of this zinc ion would also reduce charge neutralization on the phosphodiester group during hydrolysis, thereby resulting in a substantial reduction in activity. Several experiments were performed to explore the question of whether Asp55 played an important role as a ligand for Zn1. Atomic absorption studies indicated that the Zn1 site is fully occupied in the D55L mutant, which is the least conserved of the three mutants (Table 2). Moreover, the thermostability of D55L was determined by measuring the CD signal at 222 nm as a function of temperature. As is shown in Table 2, D55L and wild-type PLC_{Bc} have virtually identical melting temperatures, both beginning to denature at 75 °C, with a midpoint at 79−80 °C. In addition, the CD curves of the E4 and D55 mutants are very similar to that of the wild-type enzyme (data not shown). These experiments all suggest that the mutations did not cause a large change in structure that could account for the great decrease in k_{cat}/K_M.

The observations that the D55L mutant binds three zinc ions and exhibits the same melting temperature as wild-type PLC_{Bc} do not seem to be consistent with the side chain of Asp55 serving a critical function as a ligand for Zn1. To

gain support for this hypothesis, the putative Zn1 ligand His118 was replaced with leucine to afford the H118L mutant. While this mutant was fairly inactive, the effect of the mutation on activity was not as deleterious as mutations at D55 (Table 1). Moreover, it bound fewer zinc ions per active site and denatured at a lower temperature than the wild-type enzyme (Table 2), and the ellipticity and shape of the CD curve were distorted relative to the wild-type (data not shown). Such behavior is more characteristic of what we have observed upon mutating zinc ligands at the PLC_{Bc} active site. For example, we found that mutagenesis of Glu146, a Zn2 ligand, produced mutants that exhibited 60−400-fold reduced catalytic activity, lower zinc occupancy levels, and significantly lower thermostablility than wild-type (*12*). Similar trends have been observed upon mutagenesis of the Zn2 and Zn3 binding ligands His128, His142, and His14 of PLC_{Bc} (Martin *et al*., unpublished data). Thus, if Asp55 were a critical ligand for Zn1, it seems reasonable to conclude that mutants lacking a ligating side chain protein at this position would bind fewer zinc ions and have reduced thermostability compared to the wild-type; this is not observed.

The pH vs activity profile for PLC_{Bc} indicates that an acid with a $pK_a = 5.3$ must be ionized for activity. Although conventional wisdom would suggest that this residue is the general base in the enzymatic reaction, some care must be exercised in drawing this conclusion. For example, ionization of Glu290 is required for catalysis by *Yersinia* protein tyrosine phosphatase (*28*), but there is now evidence that this residue is not the general base (*29*). Despite this caveat, the results presented herein suggest that Asp55 is a likely candidate for the general base in the PLC_{Bc}-catalyzed hydrolysis of phospholipids. A recent molecular mechanics and molecular dynamics study supports this hypothesis (*30*).

If Asp55 is the general base as proposed, the question arises of why its pK_a is raised to 5.3 from a value of about 3.9, which is observed for the side chain carboxyl group of aspartic acid itself. Although an increase in the pK_a of a Glu or Asp in an enzyme is not uncommon, the structural and electronic factors responsible for the increase are not always easy to identify. In hen egg white lysozyme, the elevated pK_a (∼6.2) determined for Glu35 has been attributed to its hydrophobic environment and an electrostatic repulsion from Asp52 (*31*, *32*). The pK_a's of Glu78 and Glu172 of the xylanase from *Bacillus circulans* were determined by ^{13}C NMR studies to be 4.6 and 6.7, respectively, but these values could not be directly correlated with any specific structural features (*33*).

In the case of PLC_{Bc}, both hydrophobic and electrostatic effects could modulate the pK_a of Asp55. Inspection of the X-ray structure of PLC_{Bc} reveals that hydrophobic residues in the vicinity of the side chain of Asp55 may cause an increase in the pK_a of Asp55. For example, the phenyl ring of Phe66 is 3.6 Å away from one oxygen of Asp55, whereas the methyl side chain of Ala3 is 3.7 Å removed from the other carboxyl oxygen. Electrostatic repulsion between the carboxyl groups of Asp55 and Asp122 may also be partially responsible for the raised pK_a of Asp55 as an oxygen of the Asp122 side chain is 4.8 Å away from an Asp55 oxygen. Another factor that may perturb the pK_a of Asp55 is the macrodipole of the α-helix of which it is a part. Asp55 is located at the extreme C-terminal end of helix B of PLC_{Bc}, and it is well-known that the permanent dipole in α-helical structures can alter the pK_a's of functional groups on the ends of the helix. This phenomenon, which has been demonstrated for both peptides (*34*) and proteins (*35*), is found to raise the pK_a of side chains at the helical *C*-terminus and lower the pK_a of side chains at the *N*-terminus of the helix.

Mechanistic studies of the PLC_{Bc}-catalyzed hydrolysis of phospholipids may be relevant to the mechanism of the P1 nuclease from *Penicillium citrinum*, because the three-dimensional arrangement of the zinc ligands and the three zinc ions in the active sites of these two enzymes are virtually superimposable (*10*). The only difference is that Zn2 of PLC_{Bc} possesses two histidines and a glutamic acid as ligands, whereas the Zn2 of P1 nuclease is coordinated by two histidines and an aspartic acid. With such similar active sites, it is possible that these two enzymes share a common mechanism. If this is true, then Asp45 of P1 nuclease, which is analogous to Asp55 in PLC_{Bc} and has a weak interaction with Zn1, would be a good candidate for the general base in the P1 nuclease-catalyzed reaction. It should be noted that the bell-shaped, pH dependence curve for P1 nuclease has its maximum at pH 5.0−8.0 (*36*). From this graph, a pK_a of approximately 3.5, which is close to the value of aspartic acid itself, would be expected for the general base.

In summary, it is clear from the data presented herein that Glu4 is not the general base in the PLC_{Bc}-catalyzed hydrolysis of phosphatidylcholine substrates, but rather this residue appears to be involved in choline binding. Microcalorimetric studies are underway to probe in a more quantitative sense the contribution of this residue to stabilization of the positive charge on the choline moiety. The available kinetic, thermostability, and atomic absorption data for the mutants at Asp55 are consistent with this residue serving as the general base in the PLC_{Bc}-catalyzed reaction, and NMR studies to determine its pK_a are in progress. Thus, for the first time there is experimental evidence to support the hypothesis that Asp55 is the general base in the PLC_{Bc}-catalyzed hydrolysis of phospholipids.

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REFERENCES

1. Hergenrother, P. J., and Martin, S. F. (1997) *Anal. Biochem. 251*, 45−49.
2. El-Sayed, M. Y., and Roberts, M. F. (1985) *Biochim. Biophys. Acta 831*, 133−141.
3. Clark, M. A., Shorr, R. G. L., and Bomalaski, J. S. (1986) *Biochem. Biophys. Res. Commun. 140*, 114−119.
4. Guddal, P. H., Johansen, T., Schulstad, K., and Little, C. (1989) *J. Bacteriol. 171*, 5702−5706.
5. Hough, E., Hansen, L. K., Birkness, B., Jynge, K., Hansen, S., Hordvik, A., Little, C., Dodson, E., and Derewenda, Z. (1989) *Nature 338*, 357−360.
6. Hansen, S., Hansen, L. K., and Hough, E. (1992) *J. Mol. Biol. 225*, 543−549.
7. Hansen, S., Hansen, L. K., and Hough, E. (1993) *J. Mol. Biol. 231*, 870−876.
8. Hansen, S., Hough, E., Svensson, L. A., Wong, Y.-L., and Martin, S. F. (1993) *J. Mol. Biol. 234*, 179−187.

9. Kim, E. E., and Wyckoff, H. W. (1991) *J. Mol. Biol. 218*, 449−464.
10. Volbeda, A., Lahm, A., Sakiyama, F., and Suck, D. (1991) *EMBO J. 10*, 1607−1618.
11. Johansen, T., Holm, T., Guddal, P. H., Sletten, K., Haugli, F. B., and Little, C. (1988) *Gene 65*, 293−304.
12. Martin, S. F., Spaller, M. R., and Hergenrother, P. J. (1996) *Biochemistry 35*, 12970−12977.
13. Ikeda, K., Inoue, S., Amasaki, C., Teshima, K., and Ikezawa, H. (1991) *J. Biochem. 110*, 88−95.
14. Byberg, J. R., JØrgensen, F. S., Hansen, S., and Hough, E. (1992) *Proteins: Struct., Funct., Genet. 12*, 331−338.
15. Sundell, S., Hansen, S., and Hough, E. (1994) *Protein Eng. 7*, 571−577.
16. Little, C. (1977) *Acta Chem. Scand. B31*, 267−272.
17. Butler-Ransohoff, J. E., Rokita, S. E., Kendall, D. A., Banzon, J. A., Carano, K. S., Kaiser, E. T., and Matlin, A. R. (1992) *J. Org. Chem. 57*, 142−145.
18. Christianson, D. W., and Lipscomb, W. N. (1989) *Acc. Chem. Res. 22*, 62−69.
19. Milvan, A. S. (1981) *Philos. Trans. R. Soc. London B 293*, 65−74.
20. Hergenrother, P. J., Spaller, M. R., Haas, M. K., and Martin, S. F. (1995) *Anal. Biochem. 229*, 313−316.
21. Keating, K. M., Ghosaini, L. R., Giedroc, D. P., Williams, K. R., Coleman, J. E., and Sturtevant, J. M. (1988) *Biochemistry 27*, 5240−5245.
22. Scolnick, L. R., Kanyo, Z. F., Cavalli, R. C., Ash, D. E., and Christianson, D. W. (1997) *Biochemistry 36*, 10558−10565.
23. Knowles, J. R. (1991) *Nature 350*, 121−124.
24. Kelly, N., Giroux, E. L., Guqiang, L., and Kantrowitz, E. R. (1996) *Biochem. Biophys. Res. Commun. 219*, 848−852.
25. Thompson, J. E., and Raines, R. T. (1994) *J. Am. Chem. Soc. 116*, 5467−5468.
26. Hondal, R. J., Zhao, Z., Riddle, S. R., Kravchuk, A. V., Liao, H., Bruzik, K. S., and Tsai, M.-D. (1997) *J. Am. Chem. Soc. 119*, 9933−9934.
27. Jones, S. J., Worrall, A. F., and Connolly, B. A. (1996) *J. Mol. Biol. 264*, 1154−1163.
28. Zhang, Z.-Y., Wang, Y., and Dixon, J. E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1624−1627.
29. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) *Nature 370*, 571−575.
30. Thrige, D., Buur, J. R., and Jorgensen, F. S. (1997) *Biopolymers 42*, 319−336.
31. Inoue, M., Yamada, H., Yasukochi, T., Kuroki, R., Miki, T., Horiuchi, T., and Imoto, T. (1992) *Biochemistry 31*, 5545−5553.
32. Bartik, K., Redfield, C., and Dobson, C. M. (1994) *Biophs. J. 66*, 1180−1184.
33. McIntosh, L. P., Hand, G., Johnson, P. E., Joshi, M. D., Korner, M., Plesniak, L. A., Ziser, L., Wakarchuk, W. W., and Withers, S. G. (1996) *Biochemistry 35*, 9958−9966.
34. Joshi, H. W., and Meier, M. S. (1996) *J. Am. Chem. Soc. 118*, 12038−12044.
35. Sancho, J., Serrano, L., and Fersht, A. R. (1992) *Biochemistry 31*, 2253−2258.
36. Fujimoto, M., Kuninaka, A., and Yoshino, H. (1974) *Agric. Biol. Chem. 38*, 785−790.

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