The Choline Binding Site of Phospholipase C (*Bacillus cereus*): Insights into Substrate Specificity†

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ABSTRACT: The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc}) is a 28.5 kDa enzyme with three zinc ions in its active site. The roles that a number of amino acid residues play as zinc ligands and in binding and catalysis have been elucidated. Recent mechanistic studies indicate that the rate of the reaction is limited by a proton-transfer step during chemical hydrolysis and not substrate binding or product release. An X-ray structure of PLC_{Bc} complexed with a phosphonate inhibitor related to phosphatidylcholine revealed that the three amino acid residues Glu4, Tyr56, and Phe66 comprise the choline binding pocket. However, because the contributions that these three residues make to substrate recognition and specificity were unknown, a series of site-specific mutants for Glu4, Tyr56, and Phe66 were constructed by PCR mutagenesis. On the basis of a comparison of their respective CD spectra and melting temperatures, it appears that the mutants adopt folded structures in solution that are virtually identical to that of wild-type PLC_{Bc}. The kinetic parameters k_{cat} and K_m for the hydrolysis of the three soluble substrates 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (C6PC), 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (C6PE), and 1,2-dihexanoyl-*sn*-glycero-3-phospho-L-serine (C6PS) at concentrations below their corresponding critical micelle concentration (cmc) values were determined for each mutant. Replacement of Phe66 with a nonaromatic residue dramatically decreased k_{cat} (∼200-fold) and reduced PLC_{Bc} activity toward C6PC, C6PE, and C6PS, whereas changes to Glu4 and Tyr56 typically led to much more modest losses in catalytic efficiencies. Mutations of Glu4 had relatively little effect upon k_{cat} and K_m for C6PS, but they significantly influenced K_m for C6PC and C6PE. Replacing Tyr56 with nonaromatic residues also affects catalytic efficiency, albeit to a much lesser degree than the corresponding changes at position 66. However, the presence of an aromatic residue at position 56 seems to confer some substrate selectivity for C6PC and C6PE, which bear a positive charge on the headgroup, relative to C6PS, which has no net charge on the headgroup; this increase in specificity arises largely from a reduced k_{cat} for C6PS.

The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc})[1] is a 28.5 kDa, monomeric enzyme containing three zinc ions in its active site (*1*). PLC_{Bc} catalyzes the hydrolysis of a phosphodiester bond in phospholipids to provide diacylglycerol and a phosphorylated headgroup as products (Figure 1). While this enzyme preferentially catalyzes the hydrolysis of phosphatidylcholines (PC), it also processes phosphatidylethanolamines (PE) and phosphatidylserines (PS), albeit with correspondingly decreased catalytic efficiencies (*2*). In mammalian systems, the products of PLC-mediated hydrolysis act as important secondary messengers in the signal transduction cascade (*3*).

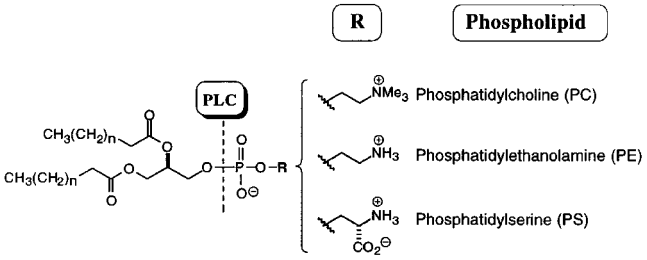

FIGURE 1: General structures of three families of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The dotted line shows the phosphodiester bond hydrolyzed by PLC_{Bc}.

Due to the immunological similarity of PLC_{Bc} with a mammalian counterpart, this bacterial enzyme may serve as a model for the poorly characterized mammalian PC-PLCs (*4*).

A number of X-ray crystal structures of PLC_{Bc} in its native state and as complexes with several different ligands have been solved (*1*, *5*, *6*). However, the structure of PLC_{Bc} complexed with a phosphatidylcholine-derived phosphonate inhibitor has provided the best insights into the roles that active site residues play in catalysis and substrate recognition

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[1] Abbreviations: DAG, diacylglycerol; DMG, dimethylglutaric acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PLC, phospholipase C; PLC_{Bc}, phosphatidylcholine-preferring phospholipase C from *Bacillus cereus*; Tris, tris(hydroxymethyl)aminomethane; C6PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; C6PE, 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine; C6PS, 1,2-dihexanoyl-*sn*-glycero-3-phospho-L-serine; PCR, polymerase chain reaction; cmc, critical micelle concentration; CD, circular dichroism.

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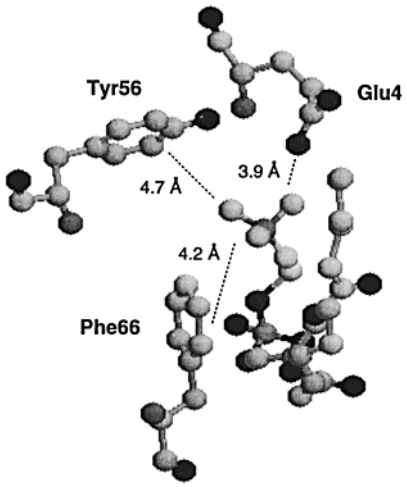

FIGURE 2: View of Glu4, Tyr56, and Phe66 interacting with a substrate analogue inhibitor; the distances between the methyl groups of the ammonium group of the choline moiety and the centroid of the aromatic rings of Tyr56 and Phe66 and the carboxyl group of Glu4 are shown. Adapted from ref *7*. (PDB access code 1AH7 for wild-type PLC_{Bc} structure.)

and binding (*7*). In analogy with other phosphodiesterases (*8*), PLC_{Bc} is believed to effect catalysis by activating a water molecule for an in-line attack on the phosphorus atom of phosphodiester moiety. Recent experiments indicate that the rate of the PLC_{Bc}-catalyzed reaction is limited by a proton-transfer step during the chemical hydrolysis and not by substrate binding or product release, but it is unclear whether deprotonation of the nucleophilic water or protonation of the leaving group is the rate-determining step (*9*). Inspection of the PLC_{Bc}−phosphonate inhibitor complex revealed that Glu4, Asp55, and Glu146 were properly positioned to activate the attacking water molecule, and mutagenesis of these residues provided evidence to support the role that each plays in the PLC_{Bc}-mediated hydrolysis of phospholipids. Asp55 appears to be the general base that activates the water for nucleophilic attack on the phosphodiester, because mutations at this position generate enzymes having structures very similar to that of the wild type but whose catalytic activities are reduced by 10^4-10^6 fold (*10*). Glu146 serves as a critical ligand for Zn2, as mutations at this position generate proteins that not only have reduced thermostabilities relative to wild-type PLC_{Bc} but also bind only two zinc ions rather than the usual three (*11*). Mutations at Glu4 were less detrimental to catalytic activity than mutations at Asp55 or Glu146, so this residue was suggested to be involved in substrate binding by stabilizing the positive charge of the choline group (*10*).

The structure of the PLC_{Bc}−inhibitor complex reveals that the three amino acid residues Glu4, Tyr56, and Phe66 comprise the site to which the charged choline moiety of the substrate binds (Figure 2) (*7*). The presence of an ammonium ion on the headgroup appears to be an important substrate requirement, as an isosteric phosphatidylcholine analogue in which a *tert*-butyl group replaced the trimethylammonium moiety was hydrolyzed by PLC_{Bc} approximately 1000 times less efficiently than the corresponding phosphatidylcholine (*12*). Thus, the choline binding pocket in PLC_{Bc} provides an interesting case study of how the side chains of amino acids may interact with ammonium ions in biological systems. For example, the carboxyl group on the side chain of Glu4 could help stabilize the choline positive charge via an electrostatic interaction. Both Tyr56 and Phe66 are then positioned so that one π-face of each of the respective aromatic rings, which have partial negative character because of the quadrupole moment of the rings (*13*), is coordinated with the trimethylammonium ion to stabilize the positive charge largely through electrostatic effects. Such π-cation interactions represent a genre of protein−ligand recognition motifs that was not widely recognized until about a decade ago (*14*), but there have since been a number of studies documenting their importance (*15*−*21*). However, despite their obvious significance, there are relatively few investigations that probe the contributions of these π-cation interactions to catalysis and binding for enzymes in which recognition of a cationic substrate involves aromatic residues.

It seems reasonable to assume that the headgroups of PE and PS would bind to the active site of PLC_{Bc} in a fashion similar to that observed in the complex of the enzyme with the phosphatidylcholine-derived inhibitor. Because Glu4, Tyr56, and Phe66 would be expected to contribute differently to stabilizing the positive charge on the headgroups of these phospholipids, these residues could play a role in dictating the substrate specificity of PLC_{Bc}. Thus, to begin to elucidate the underlying structural basis for the observed substrate selectivity of PLC_{Bc}, we constructed a series of single site-specific mutants at the three choline binding residues Glu4, Tyr56, and Phe66. The kinetic parameters of each of these mutants toward a water-soluble PC, PE, and PS were then determined. The results of these studies 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). The phospholipid substrates 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (C6PC), 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (C6PE), and 1,2-dihexanoyl-*sn*-glycero-3-phospho-L-serine (C6PS) (see Figure 1, $n = 3$) were procured 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 polymerase chain reaction (PCR) methods as described (*11*). All PCR reactions were performed under standard conditions with the "hot start" protocol and Vent polymerase. The F66A, Glu4, and F66W

mutations were created by a four-primer strategy that consisted of creating two PCR fragments (one mutagenic, one nonmutagenic) followed by an overlap/extension PCR reaction utilizing these two fragments. PCR with the primer F66AW and the reverse primer, primer B (CGACGGCCAGTGCCAAGCTTGCC), on the pMAL-plc (*11*) template yielded a PCR fragment that was spin-purified (Qiagen). This provided the mutagenic fragment for the overlap/extension. The nonmutagenic region was created with primers MalE and rev56−44, which were used in a simple two-primer PCR reaction to obtain a ~250 bp fragment. This fragment, together with the mutagenic fragment, was used in an overlap extension reaction (along with primers MalE and revpmalMCS) to create the large piece (~900 bp) suitable for restriction (*Kpn*I and *Nsi*I) and cloning. 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 overlap/extension, restricted PCR product with T4 DNA ligase. Culturing, plasmid preparation, and DNA sequencing of colonies obtained after electroporation into *Escherichia coli* DH5α produced the F66W and F66A mutants.

The other mutants at positions 55 and 66 of the *plc* gene were produced in analogous fashion utilizing the following mutagenic primers (all primers listed 5′ to 3′): F66ASWE, GAACGGTATTTATGCTGCTGACTATGAAAATCCTTATTATGATAATAGCACAKVGGCTTCACATTT; F66RKTM, GAACGGTATTTATGCTGCTGACTATGAAAATCCTTATTATGATAATAGCACAANGGCTTCACATTT; Y56ASWE, GAACGGTATTTATGCTGCTGACKVGGAAAATCCTTATTATG; and Y56RKTM GAACGGTATTTATGCTGCTGACANGGAAAATCCTTATTATG.

The underlined portion of the oligonucleotide is the noncomplementary mutagenic region, where N = AGTC, K = GT, and V = GCA.

Expression and Purification of Recombinant Proteins. Recombinant wild-type PLC_{Bc} and its mutants were expressed as fusion proteins with maltose binding protein (MBP) in DH5α cells and purified as previously described (*11*). 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 in 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 about 150 mg/L. After cleavage of this fusion protein with trypsin and incubation with trypsin inhibitor beads, 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 as described (*11*).

Analysis of Recombinant Proteins. Enzymatic assays of PLC_{Bc} and mutants were performed via a sensitive assay based on the quantitation of inorganic phosphate (P_i) (*2*). Briefly, the phosphorylated headgroup produced by the PLC_{Bc}-catalyzed hydrolysis of phospholipids is treated with alkaline phosphatase to liberate P_i, which then forms a complex with ammonium molybdate. This complex is reduced to the molybdenum blue state with ascorbic acid to give a blue solution with a λ_{max} at 700 nm. Each of the three substrates was assayed at concentrations below their respective cmc values. Circular dichroism experiments were performed at enzyme concentrations of 20 μg/mL on a Jasco-6000 as described (*11*).

Concentrations of Mutant Enzymes Used in Assays. For the assays of Y56R with C6PS, the protein concentration was 125 nM; for assays with C6PC, the protein concentration was 25 nM; and for assays with C6PE; the protein concentration was 10 nM. Similarly concentrations for other mutants with respective phospholipids were as follows: Y56W, PE = 15 nM, PS = 30 nM; PC = 15 nM; Y56A, PC = 15 nM, PS = 10 nM, PE = 5 nM; E4D, PC = 40 nM, PE = 15 nM, PS = 40 nM; E4L, PS = 450 nM, PC = 150 nM, PE = 325 nM; E4Q, PC = 20 nM, PS = 20 nM, PE = 20 nM; F66R, PS = 100 nM (1.5 min time points), PC = 75 nM (1 min time points); F66A, PC = 75 nM (30 s time points); F66W, PC = 5 nM, PE = 5 nM, PS = 5 nM.

RESULTS AND DISCUSSION

Wild-type PLC_{Bc} preferentially processes the soluble substrates C6PC over C6PE and C6PS with corresponding catalytic efficiencies (k_{cat}/K_m) of 417/(s·mM) (C6PC), 300/(s·mM) (C6PE), and 47/(s·mM) (C6PS) (*2*). The question thus naturally arises: What is the structural basis for this difference in substrate specificity? The structure of PLC_{Bc} complexed with a phosphatidylcholine-derived, phosphonate inhibitor reveals that the choline binding pocket is formed by the side chains of Glu4, Tyr56, and Phe66 (*7*). Since these residues should occupy approximately the same regions of space when the choline headgroup of a phospholipid substrate is replaced with the ethanolamine or serine moiety, these three residues would seem likely to play a role in substrate recognition and enzyme specificity.

Thus, to probe the function of these three residues, a series of single mutants were constructed in which Glu4, Tyr56, and Phe66 were replaced by site-directed PCR mutagenesis with pMAL-plc as the template. The mutant proteins were expressed and purified, and a comparison of their CD spectra and relative thermostabilities leads to the reasonable assumption that each mutant adopts a folded structure that is essentially the same as that of wild-type PLC_{Bc}. The kinetic parameters k_{cat} and K_m for each mutant toward C6PC, C6PE, and C6PS were then determined at concentrations below the respective cmc of each substrate (Table 1). It has been generally assumed that classic Michaelis−Menten kinetics are followed for the PLC_{Bc}-catalyzed hydrolysis of phospholipid analogues under such conditions (*2*, *22*, *23*). However, the possibility that there are contributions to the observed enzymatic activity from either premicellular aggregates or reaction on the vessel walls cannot be rigorously excluded (*24*). Thus, the interpretation of the results is subject to the caveats that the mutants are correctly folded and that the Michaelis−Menten formalism applies to the assay conditions

Mutations at Glu4. In the phosphonate inhibitor complex of PLC_{Bc}, the side chain carboxylic acid function of Glu4 is about 3.9 Å from the methyl groups of the trimethylammonium moiety of choline (Figure 2), suggesting the possibility of charge stabilization via an electrostatic interaction (*7*). The mutants E4Q, E4D, and E4L were thus created in order to probe the effects upon substrate selectivity and catalytic efficiency of replacing Glu4 with amino acids having different side chains. The E4D mutant was selected to examine the consequence of withdrawing the carboxyl group approximately 1 Å away from the choline binding pocket.

Table 1: Kinetic Parameters for Glu4, Tyr56, and Phe66 Mutants and Wild-type PLC_{Bc}[a]

	C6PC			C6PE			C6PS		
mutant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m $(s\cdot mM)^{-1}$	k_{cat} (s^{-1}	K_m (mM)	k_{cat}/K_m $(s\cdot mM)^{-1}$	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m $(s\cdot mM)^{-1}$
E4L	*b*	*b*	*b*	31	10	3	378	15	26
E4D	253	3	90	364	10	38	149	4	40
E4Q	*b*	*b*	*b*	132	4	34	413	5	88
Y56A	136	3	44	283	5	59	31	3	12
Y56R	171	7	25	383	9	43	35	3	15
Y56W	454	5	101	272	5	51	26	13	2
F66A	5	2.5	2	9	1	9	3	3	1
F66R	2	4	0.6	6	4	2	1	3	1
F66W	2932	9	319	339	1	308	533	10	54
wt PLC	1000	2.4	417	540	1.8	300	210	4.5	47

[a] Kinetic parameters for the substrates were measured at pH 7.3 by the inorganic phosphate assay (*2*). Estimated errors: k_{cat} (±14%); K_m (±27%). [b] K_m, which was estimated to be >15 mM, was too high to obtain accurate data for either k_{cat} or K_m.

In both the E4L and E4Q mutants, the carboxyl function is replaced with a group that is incapable of bearing the negative charge that might be involved in stabilizing interactions with the positively charged substrate. In the case of the E4L mutant the side chain is hydrophobic, whereas the side chain of E4Q is polar and isosteric with Glu.

The kinetic parameters for the Glu4 mutants were determined with the short-chain, water-soluble phospholipids C6PC, C6PE, and C6PS, and the results are summarized in Table 1. Examination of these data reveal that mutations at Glu4 led to significant decreases in the catalytic efficiencies for the hydrolysis of C6PC and C6PE; this loss of efficiency arose both from decreases in k_{cat} and from increases in K_m. The k_{cat} and K_m values for the hydrolyses of C6PC by E4L and E4Q could be estimated as being greater than about 100/s and 15 mM, which is above the critical micelle concentration of C6PC, and the rate curve remained linear so accurate data could not be obtained. However, the k_{cat}/K_m of E4D for C6PC was approximately 20% that of wild-type. The k_{cat}/K_m values for the E4L, E4D, and E4Q mutants with C6PE were about 1%, 13%, and 11%, respectively, that of wild type. Increased K_ms were the primary cause for the loss of efficiency in these mutants. In contrast, when these Glu4 mutants were assayed with C6PS as the substrate, the catalytic efficiencies were comparable or even slightly better than that of wild-type. For example, the nonconservative substitution in the E4L mutant only lowers k_{cat}/K_m for C6PS by about 50%, whereas the E4Q mutant actually processed C6PS somewhat *better* than the wild-type. With respect to the issue of enzyme specificity, it is noteworthy that the E4L and E4Q mutants hydrolyze C6PS, which lacks a net charge on the headgroup, more efficiently than C6PE or C6PC, each of which has a positively charged headgroup. The conserved E4D mutant retains substrate specificity resembling that of wild type, as it processes C6PC roughly 2 times better than C6PE or C6PS. Thus, Glu4 seems critical for optimal catalytic efficiency and recognition of PLC_{Bc} with C6PC and C6PE, but it appears to play a negligible role in the recognition and turnover of C6PS.

Mutations at Tyr56. Inspection of the X-ray data for the complex of PLC_{Bc} with a phosphonate inhibitor reveals that the centroid of the aromatic face of Tyr56 lies approximately 4.7 Å away from the ammonium methyl carbons on the choline moiety (Figure 2) (*7*); this orientation suggests possible stabilization by a π-cation interaction. The Y56A mutant was created to examine the consequence of removing any possibility for π-cation stabilization by replacing Tyr with a small, nonaromatic residue, whereas Y56W was selected to probe the consequence of introducing a more potent π-cation donor in the choline binding pocket. The Y56R mutant was designed to probe the effect upon substrate specificity of placing a positive charge in the choline binding pocket, as such a charge might interact favorably with the negatively charged carboxyl function of the serine headgroup in C6PS.

The catalytic efficiencies of the Y56A, Y56R, and Y56W mutants toward C6PC, C6PE, and C6PS were determined, and in all cases the mutant enzymes retained significant activity relative to the wild-type PLC_{Bc} (Table 1). The major effects upon the overall catalytic efficiencies of the Tyr56 mutants were in k_{cat} for C6PC and C6PS and in K_m for C6PE. Thus, the Y56A and Y56R mutants hydrolyzed C6PC with values of k_{cat}/K_m that were 5−10% that of the wild type, whereas in the Y56A- and Y56R-catalyzed hydrolyses of C6PE and C6PS, the k_{cat}/K_m values of the mutants were 15−20% and 25−30%, respectively, that found for the wild type. The Y56A and Y56R mutants exhibited little selectivity and hydrolyzed C6PC, C6PE, and C6PS with comparable efficiencies; however, Y56A and Y56R hydrolyzed C6PS with k_{cat}s 5−10-fold lower than for C6PC or C6PE. Although the aromatic ring of tryptophan is believed to form stronger π-cation interactions than either tyrosine or phenylalanine (*13*), mutation of Tyr56 to the bulkier tryptophan had a detrimental effect on the rate of hydrolysis of each of the short-chain phospholipid substrates. The Y56W mutant hydrolyzed C6PC and C6PE with k_{cat}/K_m values that were 17−21% that of wild type, whereas the k_{cat}/K_m for C6PS was only about 4% that of wild type with the effects being manifested in both k_{cat} and K_m. The Y56W mutant thus exhibits a somewhat higher specificity for C6PC (50-fold) and C6PE (25-fold) versus C6PS than wild-type PLC_{Bc}.

Mutations at Phe66. The centroid of the aromatic face of Phe66 lies approximately 4.2 Å from the ammonium methyl groups on the choline subunit in the PLC_{Bc}−phosphonate inhibitor complex (Figure 2). As in the case of Tyr56, this orientation suggested that the positive charge on the choline residue might be stabilized by a π-cation interaction, and a series of mutants of Phe66 were targeted to evaluate this possibility and what role the aromatic ring might play in determining substrate specificity. The F66A, F66R, and

Table 2: Qualitative Assessment of the Contributions of Glu4, Tyr56, and Phe66 to the Hydrolysis of Phospholipid Substrates by PLC_{Bc}

PLC_{Bc} residue	importance for PC processing	importance for PE processing	importance for PS processing
Glu 4	++	++	−
Tyr 56	++	+	+
Phe 66	+++	+++	+++

[a] Importance of contributions is presented as follows: +++, mutants retain <5% wild-type activity; ++, most mutants retain <15% wild-type activity; +, most mutants retain <30% wild-type activity; −, mutants retain >50% wild-type activity.

F66W mutants were selected on the basis of the same considerations previously used to identify the Tyr56 mutants. Namely, replacing Phe66 with alanine would exclude any possibility for π-cation stabilization, and substituting the aromatic ring of phenylalanine with the indole ring of tryptophan in the F66W mutant would be expected to result in an enhanced π-cation interaction. The effect upon substrate specificity of introducing a positive charge in the choline binding pocket would then be evaluated with the F66R mutant.

Replacement of Phe66 with Ala and Arg universally resulted in dramatic decreases in the catalytic efficiencies, which was primarily manifested in a marked reduction in turnover, for the hydrolysis of each of the short-chain phospholipid substrates (Table 1). The k_{cat}/K_m values for F66A and F66R ranged from 0.1% to 3% that of wild type, with the primary cause for the decrease being manifested in reduced k_{cat}s. Interestingly, the k_{cat}s for the hydrolysis of both C6PC and C6PS by F66W were 2−3 times greater than those found for wild-type PLC_{Bc}; however, in each case the K_m also increased, so there was a negligible change in k_{cat}/K_m for the enzymatic processing. The C6PE was hydrolyzed only slightly more slowly by F66W than by the wild type, but because of a slight decrease in K_m, k_{cat}/K_m was approximately the same for F66W and wild type. Thus, because of balancing compensations in the k_{cat} and K_m values, the efficiencies in the F66W-catalyzed hydrolysis of C6PC, C6PE, and C6PS were comparable to those of wild type.

Summary. The relative importance of the three residues Glu4, Tyr56, and Phe66 for the PLC_{Bc}-catalyzed hydrolyses of C6PC, C6PE, and C6PS are qualitatively summarized in Table 2, and some general comments and comparisons are instructive. The contributions of Glu4 and Tyr56 to substrate specificity and catalysis by PLC_{Bc} differ significantly from those of Phe66. Namely, mutations of Glu4 had relatively little effect upon k_{cat} and K_m for C6PS, but they did influence these kinetic parameters, especially K_m, for C6PC and C6PE. An amino acid having a carboxyl group in the side chain at position 4 thus appears beneficial for hydrolysis of C6PC and C6PE, which have a positive charge on the headgroups, but not C6PS, whose headgroup has a net neutral charge. Substituting an aromatic residue at position 56 with a nonaromatic group also reduces catalytic efficiency for each substrate, albeit to a much lesser degree than the corresponding change at position 66. The presence of an aromatic residue at position 56 appears to confer a preference for substrates with a net positive charge on the headgroup. Indeed, there are larger differences in the catalytic efficiencies with which wild-type PLC_{Bc} and Y56W process C6PC and C6PE relative to C6PS than is observed for the hydrolyses of these substrates by Y56A and Y56R.

Whereas changes to Glu4 and Tyr56 typically led to modest losses in catalytic efficiencies, replacing Phe66 with a nonaromatic residue dramatically decreased PLC_{Bc} activity, especially k_{cat}, toward all of the short-chain phospholipids C6PC, C6PE, and C6PS. Indeed, the single most important structural feature in the choline binding pocket to ensure efficient hydrolysis of each of these substrates by PLC_{Bc} is the presence of an aromatic residue at position 66. Because each phospholipid possesses a cationic ammonium group, the loss in catalytic activity is seemingly consistent with the hypothesis that Phe66 plays an important role in stabilizing the positive charge on the ammonium ion of the headgroup via a putative π-cation interaction. However, in addition to the >200-fold decrease seen in k_{cat}, one would expect to observe dramatically higher K_m values for nonaromatic mutants at Phe66 as has been reported for the several enzymes where π-cation interactions are purported to be important for catalysis and binding (*17*, *18*, *25*). Since this increase in K_m is not seen, other structural effects might account for the observed loss in catalytic activity for mutants lacking an aromatic residue at position 66.

The available structural data for PLC_{Bc} coupled with the kinetic parameters k_{cat} and K_m that were determined for the selected mutants presented herein tentatively support some involvement of π-cation interactions in catalysis by PLC_{Bc}. However, because the expected increases in K_m for mutants in which Phe66 is replaced with nonaromatic side chains are not observed, further studies are necessary to determine more precisely how Phe66 affects catalysis and binding. Nevertheless, the results of the present investigations have provided some new insights into the molecular basis for substrate specificity by PLC_{Bc}, and several mutants were identified that exhibited substrate specificities significantly different from wild type. For example, E4Q hydrolyzed C6PS faster and more efficiently than PLC_{Bc}, and Y56W was significantly more selective than wild type for substrate with headgroups bearing a net positive charge. Efforts to further modify the substrate specificity of PLC_{Bc} via random mutagenesis of Glu4, Tyr56, and Phe66 in the choline binding pocket as well as to quantify the interactions between these residues and substrates with different headgroups are in progress and will be reported in due course.

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