Altering Substrate Specificity of Phosphatidylcholine-Preferring Phospholipase C of *Bacillus cereus* by Random Mutagenesis of the Headgroup Binding Site†

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ABSTRACT: PLC_{Bc} is a 28.5 kDa monomeric enzyme that catalyzes the hydrolysis of the phosphodiester bond of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine to provide a diacylglycerol and the corresponding phosphorylated headgroup. Because single replacements of Glu4, Tyr56, and Phe66 in the headgroup binding pocket led to changes in substrate specificity [Martin et al. (2000) *Biochemistry 39*, 3410−3415], a combinatorial library of approximately 6000 maltose binding protein−PLC_{Bc} fusion protein mutants containing random permutations of these three residues was generated to identify PLC_{Bc} mutants with altered specificity profiles and high catalytic activities. Members of this library were screened for hydrolytic activity toward the water soluble substrates C6PC, C6PE, and C6PS using a novel protocol that was conducted in a 96-well format and featured the in situ cleavage of the fusion protein to release the mutant PLC_{Bc}s. Ten mutant enzymes that exhibited significant preferences toward C6PE or C6PS were selected and analyzed by steady-state kinetics to determine their specificity constants, k_{cat}/K_M. The C6PS selective clones E4G, E4Q/Y56T/F66Y, and E4K/Y56V exhibited higher specificity constants toward C6PS than wt, whereas Y56T, F66Y, and Y56T/F66Y were C6PE selective and had comparable or higher specificity constants than wt for C6PE. The corresponding wt residues were singly reinserted back into the E4Q/Y56T/F66Y and E4K/Y56V mutants via site-directed mutagenesis, and the E4Q/F66Y mutant thus obtained exhibited a 10-fold higher specificity constant toward C6PS than wt, a value significantly higher than other PLC_{Bc} mutants. On the basis of available data, an aromatic residue at position 66 appears important for significant catalytic activity toward all three substrates, especially C6PC and C6PE. The charge of residue 4 also appears to be a determinant of enzyme specificity as a negatively charged residue at this position endows the enzyme with C6PC and C6PE preference, whereas a polar neutral or positively charged residue results in C6PS selectivity. Replacing Tyr56 with Val, Ala, Thr, or Ser greatly reduces activity toward C6PC. Thus, the substrate specificity of PLC_{Bc} can be modulated by varying three of the amino acid residues that constitute the headgroup binding pocket, and it is now apparent that this enzyme is not evolutionarily optimized to hydrolyze phospholipids with ethanolamine or serine headgroups.

A central goal of contemporary protein engineering is to generate useful enzymes with novel biological and chemical properties. For example, more stable and efficient biocatalysts that effect selective chemical transformations may be created by modifying existing enzymes (*2*). Modifying the substrate specificity of a particular enzyme (*3*) may also provide selective proteins that can be used to study fundamental biological processes such as cell signaling pathways (*4*) and mechanisms of substrate recognition (*5*). Structural and thermodynamic studies of these mutant enzymes and their complexes with substrate analogues can lead to a better understanding of the structural basis for function and activity (*6*, *7*). Hence, the design of new methodologies to create and identify enzymes with novel substrate selectivities is an important research area.

A number of tools have been developed to alter enzyme specificity and create new classes of enzymes (*8*). For example, site-directed mutagenesis is a popular method for altering enzyme selectivity that has been used to reprogram the substrate specificities of several enzymes (*9*−*11*). Whole gene randomization followed by iterative rounds of selection, often referred to as in vitro evolution, offers an attractive alternative to site-directed mutagenesis, and this procedure has been employed to modify enzyme specificities in remarkable fashion (*12*−*14*). However, technical difficulties associated with establishing suitable selection and/or screening protocols to evaluate large libraries of mutants may limit the applicability of this method. In a hybrid of these two approaches, peptide domains containing a few residues believed to be important for catalysis and/or binding are modified by random substitutions, and the resultant, relatively small library is then screened to identify mutants that exhibit the desired phenotype. This technique has been successfully used to engineer enzyme active sites to catalyze new reactions (*15*) and to design proteins with novel specificities (*16*−*20*), and in the present study we explore its use to alter substrate selectivity of PLC_{Bc}[1].

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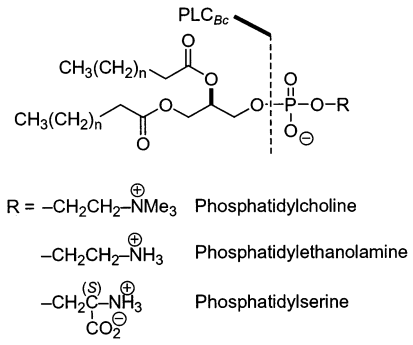

FIGURE 1: General structures of the three families of phospholipids: PC, PE, and PS. The dotted line shows the phosphodiester bond hydrolyzed by PLC$_{Bc}$.

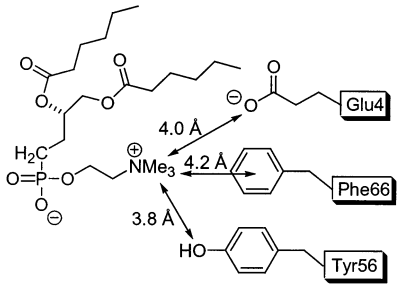

FIGURE 2: View of Glu4, Tyr56, and Phe66 interacting with a substrate analog inhibitor. The distances in angstroms between the methyl groups of the ammonium group of the choline moiety and the carboxyl group of Glu4, the phenolic hydroxyl of Tyr56, and the centroid of the aromatic ring of Phe66 are shown.

We have had a longstanding interest in enzymes of the phospholipase C family, particularly in PLC$_{Bc}$ (*1*, *21*−*28*), which is a 28.5 kDa monomeric enzyme containing three zinc ions in its active site. PLC$_{Bc}$ catalyzes the hydrolysis of the phosphodiester bond of PC, PE, and PS with the ratios of the corresponding specificity constants, k_{cat}/K_M, being about 10:7:1 to provide a DAG and the corresponding phosphorylated headgroup (Figure 1) (*21*, *22*). In mammalian systems, DAG acts as a secondary messenger in the signal transduction cascade by serving as an endogenous activator of protein kinase C (*29*). Although the precise biological function of PLC$_{Bc}$ remains uncertain, it may be involved in phosphate retrieval (*30*), and it may serve as a useful model for putative mammalian enzymes that hydrolyze phosphatidylcholines.

A number of X-ray crystal structures of PLC$_{Bc}$ in its native state and complexed with different ligands have been determined at high resolution (*31*−*34*), and the structure of the D55N mutant complexed with two ligands has also recently been solved (unpublished results). The structure of PLC$_{Bc}$ complexed with a PC-derived phosphonate inhibitor showed that the three residues Glu4, Tyr56, and Phe66 were each within 5 Å of the trimethylammonium cation of the substrate headgroup (*32*). For example, the side chain carboxylic acid group of Glu4 is positioned 4.0 Å from the methyl groups on the choline moiety, whereas the tyrosine hydroxyl group is 3.8 Å from these methyl groups. The centroids of the aromatic faces of Tyr56 and Phe66 lie approximately 4.7 and 4.2 Å, respectively, from the methyl groups on the choline moiety (Figure 2). In particular, the aromatic ring of Phe66 is positioned so that one π-face may be coordinated with the trimethylammonium ion, perhaps thereby stabilizing the positive charge through π-cation effects. Such interactions represent an important genre of protein−ligand recognition motifs that have been the focus of a number of studies (*35*−*40*).

The proximity of Glu4, Tyr56, and Phe66 to the choline headgroup in the PLC$_{Bc}$−inhibitor complex (Figure 2) suggested that these residues might play roles in modulating the substrate selectivity of PLC$_{Bc}$. To evaluate this hypothesis, these residues were varied via single-site mutagenesis. Mutating Glu4 and Phe66 had significant effects upon substrate specificity, whereas altering Tyr56 resulted in more modest changes (*1*). For example, replacing the negatively charged side chain of Glu4 with the isosteric but neutral side chain of Gln generated a mutant (E4Q) with a higher specificity constant, k_{cat}/K_M, for C6PS, whose headgroup has a net neutral charge, and significantly reduced specificity constants toward C6PC, and C6PE, which each have a net positive charge on their respective headgroups. An aromatic residue at position 66 was found to be important for high catalytic activity toward all substrates but especially C6PC and C6PE.

Because replacements of Glu4, Tyr56, and Phe66 influenced the substrate selectivity profile of PLC$_{Bc}$, we were intrigued by the possibility that random combinatorial substitutions of this constellation of residues might lead to the discovery of catalytically active mutants having altered specificity profiles. Changing these residues in a combinatorial fashion rather than singly by saturation mutagenesis would enable us to determine whether multiple changes, which could be either independent or synergistic, could lead to mutants having higher substrate preferences and specificity constants. Once mutants that preferentially hydrolyzed C6PE or C6PS relative to C6PC were identified, we envisioned that structural and thermodynamic studies of complexes of these mutants with non-hydrolyzable substrate analogues would elucidate how different amino acid side chains interact with ionic phospholipid headgroups.

Toward these objectives, we employed site-directed mutagenesis to randomize the residues at positions 4, 56, and 66 of PLC$_{Bc}$ in the MBP−PLC$_{Bc}$ fusion protein construct that had been previously developed for expressing mutant PLC$_{Bc}$s (*24*). The resulting recombinant library containing approximately 6000 mutants was screened in a 96-well format using a novel protocol that involved in situ cleavage of the MBP−PLC$_{Bc}$ fusion protein to release the mutant PLC$_{Bc}$s in the presence of one of the water soluble substrates, C6PC, C6PE, or C6PS. Wells containing active mutants with altered selectivity profiles were identified using a coupled enzyme assay for detecting inorganic phosphate (*21*). Ten mutants containing one to three amino acid replacements exhibited selectivity profiles significantly different from wt. To assess the impact of specific substitutions in the double and triple mutants, the corresponding wt residues were singly

[1] Abbreviations: PLC$_{Bc}$, phosphatidylcholine-preferring phospholipase C from *Bacillus cereus*; 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; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DAG, diacylglycerol; MBP, maltose binding protein; TBE, tris-boric acid, EDTA; LBamp^{100}, 100 μg/mL ampicillin; P_i, inorganic phosphate; CMC, critical micelle concentration; A_{700}, absorbance at 700 nm; k_{cat}/K_M, specificity constant.

reinserted back into the mutants E4K/Y56V and E4Q/Y56T/F66Y via site-directed mutagenesis, thereby generating two new families of mutant PLC_{Bc}s. All mutants were purified, and their specificity constants, k_{cat}/K_M, toward C6PC, C6PE, and C6PS were determined by steady-state kinetics. The results of these studies are presented herein.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, T4 DNA ligase, Vent polymerase, and amylose resin were purchased 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 immobilized on 4% beaded agarose (T-0637) were acquired from Sigma (St. Louis, MO). The phospholipid substrates C6PC, C6PE, and C6PS were purchased 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 at the Davis Sequencing Center in Davis, CA. QIAquick DNA gel purification kits and QIAprep plasmid purification kits were purchased from Qiagen (Chatsworth, CA). QuikChange Site-directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA).

Random Mutagenesis of Residues 4, 56, and 66. Random mutagenesis of the residues at 4, 56, and 66 of PLC_{Bc} was performed by a two-step PCR technique. The primers used were 4NNS (5′-GCTCGGTACCCGGCCGGGGATCCATCGAGGGTAGGTGGTCTGCTNNSGATAACATAAAGAAGGTG-3′), rev5666NNS (5′-CATAGAAATGTGAAGCSNNTGTGCTATTATCATAATAAGGATTTTCSNNGTCAGAGCATAAATACCG-3′), and revpMAL (5′-GGGTTTTCCCAGTCACGACGTTGTAAAACG-3′), where N represents an equimolar mixture of A, C, G, and T, and S represents that of G and C. The underlined portion of the oligonucleotide is the noncomplementary mutagenic region. In the first PCR reaction, 100 ng of DNA from the expression plasmid for wt pMal-plc was used as the template, and 4NNS and rev5666NNS primers were applied for amplification. The reaction was performed in a Peltier Thermal Cycler (MJ Research) under standard PCR conditions (94 °C, 2 min; 1 cycle; 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min; 30 cycles; indefinitely, 4 °C) to generate a random assortment of mutants at positions 4, 56, and 66. The product of the first PCR was analyzed on a 0.8% TBE agarose gel to verify that the expected 260 bp product had been formed. This product was isolated and purified using the QIAquick gel extraction kit. The second PCR was performed with the product of the first PCR reaction and revpMal primer with pMal-plc as the template using a lower annealing temperature (94 °C, 2 min; 1 cycle; 94 °C, 1 min; 42 °C, 2 min; 72 °C, 3 min; 5 cycles; 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min; 25 cycles; indefinitely, 4 °C). This reaction generated an approximately 960 base pair fragment of DNA containing the plc_{Bc} gene with random mutations at sites 4, 56, and 66. Both PCR reactions were carried out using hot-start protocol and Vent polymerase in a total volume of 100 μL. All primers were at a concentration of 1 pmol/μL, and all nucleotide triphosphates were at a concentration of 200 μM.

Molecular Cloning of Randomized plc Genes. The product obtained from 10 PCR cycles was combined and purified via QIAquick DNA purification kit to give the insert in 50 μL of sterile water. The insert was subjected to a double restriction digest at 37 °C for 3 h using *Nsi*I and *Eag*I. The vector pMal-plc (6 ng/μL) was digested with the same restriction conditions. The restriction digested pMal-plc vector and the insert were purified by electrophoresis on a 0.8% TBE gel with the QIAquick gel purification kit to give the vector and the insert in 50 and 20 μL sterile water, respectively. The mutant product was subcloned into the large fragment of plasmid pMal-plc to generate a mutagenic piece with a random assortment of substitutions at positions 4, 56, and 66. Ligation of 24 ng insert to 40 ng vector was performed by T4 ligase at 37 °C for 16 h. The mutagenic pMal-plc (2 μL) was combined with 20 μL of electrocompetent *Escherichia coli* cells and transformed into *E. coli* cells using a Cell-Porator with a voltage booster at 400 amps with the following settings: voltage booster—4Ω; capacitance—330 μF; charge rate—fast; C volts—low. After transformation, the cells were suspended in 1 mL of terrific broth (Gibco BRL) and shaken (200 rpm) at 37 °C for 1 h. This suspension of cells (150 μL/plate) was plated onto agarose plates containing $LBamp^{100}$.

Screening for Clones Selectively Cleaving C6PE or C6PS. Preliminary screening of transformants was initiated by culturing colonies at 37 °C on $LBamp^{100}$ plates for 30 h. Each single colony was suspended in 300 μL of filtered buffer A (0.1 M DMG and 0.1 mM $ZnSO_4$, pH = 7.3) in one well in a sterile 96-well plate. A wt colony was included in each 96-well plate as a control. To ensure that each well contained approximately equal cell concentrations, the A_{600} was used to determine the optical density of the suspension. If the A_{600} was above 0.2, 50 μL of the cell suspension were replaced by buffer A. The A_{600} was measured again, and adjusted until all wells had an A_{600} between 0.1 and 0.2. Aliquots (50 μL) of cells suspended in buffer A were transferred to three empty 96-well plates to provide an original and three 96-well replica plates. The original plate containing 150 μL of suspended cells was stored at 4 °C to preserve each clone until the screen was complete.

The three replica plates were used to test the activity of the mutant enzymes toward C6PC, C6PE, and C6PS. A 50 μL volume of a solution containing alkaline phosphatase (60 units/mL), substrate (C6PC, C6PE, or C6PS) (0.4 mM), and trypsin (3 μg/mL) was added to each well in the three replica plates, and the plates were incubated at room temperature for 30 min. Enzyme activity in the wells was determined by a Pi quantitation assay (*21*). Briefly, solutions A (2% ammonium molybdate in H_2O) and B (10.5% ascorbic acid in 37.5% aqueous trichloroacetic acid) were combined in a 1:1.5 ratio and mixed immediately prior to use. A 50 μL aliquot of the A:B mixture was added to each of the wells, and the contents of the wells were mixed by drawing liquid up into a multichannel pipet and returning it into the wells in a repetitive fashion. Two min after the addition of the A:B solution, 50 μL of solution C (2% sodium metaarsenite and 2% trisodium citrate in 2% acetic acid in H_2O) was added to all wells. The blue color was allowed to develop for 20 min, and the A_{700} was determined using a microplate reader (Molecular Devices Spectra Max 340). The wells with $A_{700} > 0.2$ were identified, and the wells containing mutant enzymes that gave an $A_{700} > 0.2$ for either C6PE or C6PS were located on the mother-plate, and the cell suspensions

contained in these wells were grown up in 5 mL of LBamp^{100} for sequencing and preservation as glycerol stocks.

Single Site-Directed Mutagenesis of wt and Mutants. Site-directed mutagenesis was performed using QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) to generate the following mutants: E4K, Y56V, E4Q, Y56T, F66Y, E4Q/Y56T, Y56T/F66Y, and E4Q/F66Y. Primers containing the requisite mutations were obtained from Integrated DNA Technologies (Coralville, IA). A total of 100 ng of DNA from the expression plasmid for wt pMal-plc was used as the template, and the complementary oligonucleotides containing the desired mutation (2.5 ng/μL each) were applied for amplification. The reaction was performed in a Peltier Thermal Cycler (MJ Research) under standard PCR conditions (95 °C, 30 s; 1 cycle; 95 °C, 30 s; 55 °C, 1 min; 68 °C, 15 min; 16 cycles; indefinitely, 4 °C) to generate the desired PLC$_{Bc}$ mutants. To the PCR reaction mixture, *Dpn* I (1 μL) was added. The reaction mixture was mixed by pipeting the solution up and down several times. The reaction mixtures were spun down in a microcentrifuge for 1 min and incubated at 37 °C for 1 h to digest the parental supercoiled dsDNA. The product was analyzed on a 0.8% TBE agarose gel to verify that the expected 7.4 Kb product had been formed, and the mutant pMal-plc plasmid was transformed into *E. coli* cells via heat shock by transferring 1 μL of the *Dpn* I-treated pMal-plc DNA to 50 μL of the XL1-Blue super competent *E. coli* cells. The transformation reaction was gently mixed and incubated on ice for 30 min, then heat pulsed for 45 s at 42 °C, and placed on ice for 2 min. To this reaction, 0.5 mL SOC broth that had been preheated to 42 °C was added, and the transformation reaction was incubated at 37 °C for 1 h with shaking at 250 rpm. Transformed cells (150 μL) were then plated on LBamp^{100} plates.

Expression and Purification of Recombinant PLC$_{Bc}$ Proteins. Recombinant wt and mutant enzymes were expressed as fusion proteins with MBP in *E. coli* DH5α cells and purified as previously described (*24*). After growth (30 °C, 225 rpm) and induction (at $OD_{600} = 0.6$, 0.3 mM IPTG) of large cultures (1 L), the cells were centrifuged, suspended in buffer A, and lysed on ice using a Fisher Scientific 60 Sonic Dismembrator at a power setting of 15. The lysate was centrifuged, and passage of the supernatant through an amylose column yielded the wt or mutant MBP−PLC$_{Bc}$ fusion protein. The fusion protein was cleaved with trypsin and incubated with trypsin inhibitor immobilized on cross-linked 4% beaded agarose (Sigma), and the PLC$_{Bc}$ enzyme was separated from the MBP via chromatography on Q-Sepharose and chelating-Sepharose resins (Amersham Pharmacia Biotech A B, Uppsala, Sweden) (with Cu^{2+} as the metal). Protein quantitation was performed via a Bradford protocol and A_{280} measurements.

Kinetic Measurements of Specificity Constants. The specificity constants, k_{cat}/K_M, of the mutant enzymes were determined using a sensitive, enzyme-coupled assay that is based on the quantitation of P_i (*21*). In summary, the phosphorylated headgroup produced by the PLC-catalyzed hydrolysis of phospholipids was treated with alkaline phosphatase to free Pi, which formed a complex with ammonium molybdate. The complex was then reduced to a blue molybdenum state with ascorbic acid to yield a blue solution with a λ_{max} at 700 nm. The initial velocity versus substrate (C6PC, C6PE, and C6PS) concentration data were obtained in duplicate at substrate concentrations well below their CMCs in the assay buffer, and the specificity constants, k_{cat}/K_M, were obtained from the initial slopes of the initial velocity versus substrate concentration curves at low substrate concentrations. In these assays, the concentrations of mutant enzymes was varied from 0.02 to 8 nM to give adequate responses in the assay.

RESULTS AND DISCUSSION

We had previously determined that wild-type PLC$_{Bc}$ hydrolyzed the soluble phospholipid substrates C6PC, C6PE, and C6PS with the ratios of the corresponding specificity constants, k_{cat}/K_M, being about 10:7:1 (*21*). Because the residues Glu4, Tyr56, and Phe66 comprise the choline headgroup binding pocket of PLC$_{Bc}$, we employed site-directed mutagenesis to singly replace each of these residues. The kinetic parameters k_{cat} and K_M for selected mutants were determined and compared to ascertain the effects of individual mutations upon substrate specificity and catalytic activity (*1*). Mutations of Glu4 suggested that a side chain carboxyl group at position 4 was beneficial for catalyzing the hydrolysis of C6PC and C6PE, both of which have a positive charge on the headgroup, but not for C6PS, the headgroup of which is zwitterionic. The presence of an aromatic residue at position 56 appeared to confer a preference for the hydrolysis of substrates having a net positive charge on the headgroup, whereas replacing Phe66 with a residue lacking an aromatic ring in the side chain resulted in dramatically reduced catalytic activity toward all three substrates.

Although site-directed mutagenesis of wt PLC$_{Bc}$ revealed the impact of selected single substitutions of Glu4, Tyr56, and Phe66, we reasoned that combinatorial replacement of these three residues might lead to the identification of more selective mutants, structural studies of which might lead to some understandings of the basis of PLC$_{Bc}$ substrate specificity. Toward this end, residues 4, 56, and 66 were randomized in a combinatorial fashion by mutagenesis to generate a library of MBP−PLC$_{Bc}$ fusion proteins that were cleaved, and the resultant PLC$_{Bc}$ mutants were screened for activity toward the water soluble substrates C6PC, C6PE, and C6PS. The specificity constants, k_{cat}/K_M, of the active variants for the three substrates were then determined by steady-state kinetics.

Generating and Screening Library of MBP−PLC$_{Bc}$ Mutants. Having previously established the basic protocol for producing PLC$_{Bc}$ mutants containing replacements of Glu4, Tyr56, and Phe66 (*1*), the task of generating a library by randomizing these residues using a mega-primer strategy for PCR mutagenesis (see Experimental Procedures) was straightforward. The requisite primers were designed so that the first two nucleotides of the desired codons contained a statistical mixture of C, G, A, or T, while the third codon was either C or G. This common tactic removes some of the degeneracy of the genetic code and increases the probability that rarely encoded amino acids, such as tryptophan, will appear in the mutants. It also reduces the number of colonies that must be screened for a particular probability that all possible amino acid combinations at sites 4, 56, and 66 are examined. For example, using this protocol the number of possible unique

sequences n is 32 768 $(4 \cdot 4 \cdot 2)^3$, and the number of transformant colonies that constitute the mean of a normal distribution can be estimated as $n \ln(n)$, or 340 696 colonies. Hence, the number of colonies that would have to be screened to ensure with 90% probability that all possible combinations at sites 4, 56, and 66 were examined is given by the expression $n \ln(n) + 1.29\{n[1.65n - \ln(n)]\}^{1/2}$ or 394 988 colonies (*41*). Given the practical limitations of our 96-well plate based screen, a library consisting of 6000 mutant enzymes was prepared and screened to establish the utility of the protocol and identify at least some mutants having altered specificity profiles.

While generating the library of MBP−PLC_{Bc} mutants was relatively straightforward, the development of an effective method to screen the library for phospholipase C activity toward the three different phospholipid substrates was complicated by several factors. First, the pMAL-plc expression vector employed produces inactive MBP−PLC_{Bc} fusion proteins that must be cleaved to produce the PLC_{Bc} mutants. It was necessary to employ a fusion protein strategy to generate the library of mutants because the amino group of the N-terminal Trp residue of PLC_{Bc} is involved in zinc ion complexation and is essential for significant enzyme activity (*42*). Since a Met followed by Trp is not removed in vivo (*43*), it is not possible to express directly PLC_{Bc} with a N-terminal Trp residue. Second, the activity of the mutants cannot be determined directly on an agarose plate because the products of the enzymatic reaction are neither chromogenic nor indispensable for survival. Third, each mutant must be simultaneously screened for activity toward three different substrates.

A novel strategy was therefore designed and developed whereby *E. coli* colonies were screened directly for enzymatic activity in a 96-well format using a modification of a chromogenic assay we had developed for determining the activity of PLC_{Bc} mutants (*21*). In the first step of the screen, the individual mutant colonies were resuspended in a low salt buffer in the wells of a 96-well plate. A cocktail of trypsin, alkaline phosphatase, and the appropriate phospholipid substrate (C6PC, C6PE, or C6PS) was added to each of the wells. The cells lysed releasing some MBP−PLC_{Bc}, perhaps from the periplasmic space, to the buffer solution. Control experiments were conducted to demonstrate that there was sufficient transcription of the MBP−PLC_{Bc} fusion protein in the absence of IPTG. The full-length PLC_{Bc} mutant was then released by cleaving the fusion protein with trypsin. If the resulting mutant enzyme was active, it would hydrolyze the C6PC, C6PE, or C6PS that was present, thereby producing diacylglycerol and the corresponding phosphorylated headgroup. The phosphorylated headgroup in turn was immediately hydrolyzed by alkaline phosphatase to give Pi and an alcohol. Solutions of ammonium molybdate and ascorbic acid were then added, and the wells containing active mutants for C6PE or C6PS were identified based upon their absorbance at 700 nm.

During a typical screen of a plate containing 95 mutants and a wt colony, less than 2% of the wells had an A_{700} greater than approximately 0.2 (background level) for only one of the three substrates. Thirty selective mutants were initially isolated from the recombinant library of about 6000 independent transformants. One single mutant, three double mutants, and six triple mutants were confirmed to hydrolyze C6PE or C6PS preferentially in a second screen in which eight colonies of each clone were retested in 96-well plates. The substitutions at sites 4, 56, and 66 were then determined by DNA sequencing, and the variants were confirmed to be free of extraneous mutations.

Table 1: Specificity Constants of Mutants and wt PLC_{Bc}[a]

	Specificity Constants (k_{cat}/K_M) ($mM^{-1}\,s^{-1}$)			Preference	Preference
mutant	C6PC	C6PE	C6PS	C6PE/C6PC[b]	C6PS/C6PC[b]
wt	113.0	80.2	11.5	0.7	0.1
E4G	12.5	1.7	40.4	0.2	3.2
E4K/Y56V	1.2	1.4	21.6	1.2	18
Y56L/F66L	0.1	2.9	0.1	29.0	1.0
Y56T/F66P	0.3	2.2	0.25	7.3	0.8
E4Q/Y56T/F66Y	7.1	15.8	30.9	2.2	4.4
E4P/Y56S/F66N	0.3	1.5	5.8	5.0	19.0
E4R/Y56S/F66N	0.2	1.4	4.2	7.0	21.0
E4D/Y56D/F66N	0.2	3.2	0.5	16.0	2.5
E4G/Y56T/F66N	0.9	3.1	2.3	3.4	2.6
E4G/Y56I/F66N	0.2	0.9	2.3	4.5	11.5

[a] Values represent the mean of at least two independent measurements. Estimated error of k_{cat}/K_M (±15%). [b] Values measured as ratios of ((k_{cat}/K_M) for C6PE or C6PS)/((k_{cat}/K_M) for C6PC).

Kinetic Measurements. The 10 mutant enzymes identified by the screening experiments were purified to homogeneity as determined by gel electrophoresis. The specificity constants, k_{cat}/K_M, toward C6PC, C6PE, and C6PS were then determined by steady-state kinetics within 5 days of purification to prevent loss of enzymatic activity. In previous work, we used the Michaelis−Menten equation (eq 1) to determine kinetic constants of wt PLC_{Bc} and selected mutants toward these substrates at concentrations below their respective CMCs (*1*). However, this technique could not be used to analyze the kinetic data and obtain accurate specificity constants for the mutants in this study because the CMCs of the substrates in the assay buffer are low (C6PC, CMC = 8.1 mM; C6PE, CMC = 7.0 mM; C6PS, CMC = 12.5 mM) (*21*), and initial rate data typically could not be collected at saturating substrate concentrations to determine V_{max}. Consequently, the initial velocity data for the various mutants was measured at low concentrations (ca. 0.02−1.0 mM) of the relevant substrate, and the specificity constants shown in Table 1 were obtained from eq 1 by the limiting slope of the concentration dependence of the initial rate where $[S] \ll K_M$. The specificity constants determined in this study for the hydrolytic cleavage of C6PC (113 $mM^{-1}\,s^{-1}$), C6PE (80 $mM^{-1}\,s^{-1}$), and C6PS (12 $mM^{-1}\,s^{-1}$) by wt were somewhat lower than those obtained previously [C6PC (417 $mM^{-1}\,s^{-1}$); C6PE (300 $mM^{-1}\,s^{-1}$); C6PS (47 $mM^{-1}\,s^{-1}$)] (*21*), but the ratios of the specificity constants obtained by the two methods were identical (C6PE/C6PC = 0.7 and C6PS/C6PC = 0.1).

$$v_{int} = \frac{V_{max}[S]}{k_M + [S]} + c \qquad (1)$$

C6PE Selective Mutants. The four mutants Y56L/F66L, Y56T/F66P, E4D/Y56D/F66N, and E4G/Y56T/ F66N all exhibit C6PE selectivity, although each of these mutants has a lower specificity constant toward C6PE than wt. This observation is perhaps not surprising because the headgroups of C6PC and C6PE are similar, and mutant proteins that

Table 2: Specificity Constants of Combinations of E4K/Y56F and wt[a]

mutant	Specificity Constants (k_{cat}/K_M) ($mM^{-1}\,s^{-1}$) C6PC	C6PE	C6PS	Preference C6PE/C6PC[b]	Preference C6PS/C6PC[b]
wt	113.0	80.2	11.5	0.7	0.1
E4K	4.7	5.3	34.3	1.1	7.3
Y56V	38.4	115.0	17.3	3.0	0.5
E4K/Y56V	1.2	1.4	21.6	1.2	18.0

[a] Values represent E4Q/Y56T/F66Y the mean of at least two independent measurements. Estimated error of k_{cat}/K_M (±15%). [b] Values are measured as the ratios of the specificity constants ((k_{cat}/K_M) for C6PE or C6PS)/((k_{cat}/K_M) for C6PC).

Table 3: Specificity Constants of Combinations of E4Q/Y56T/F66Y and wt[a]

mutant	Specificity Constants (k_{cat}/K_M) ($mM^{-1}\,s^{-1}$) C6PC	C6PE	C6PS	Preference C6PE/C6PC[b]	Preference C6PS/C6PC[b]
wt	113.0	80.2	11.5	0.7	0.1
F66Y	126.1	157.9	15.9	1.3	0.1
Y56T	55.3	151.8	28.1	2.7	0.5
E4Q	57.3	26.8	66.6	0.5	1.1
E4Q/F66Y	78.5	44.3	109.1	0.6	1.4
Y56T/F66Y	19.6	88.1	18.0	4.5	0.9
E4Q/Y56T	12.0	17.0	45.4	1.4	3.8
E4Q/Y56T/F66Y	7.1	15.8	30.9	2.2	4.4

[a] Values represent E4Q/Y56T/F66Y the mean of at least two independent measurements. Estimated error of k_{cat}/K_M (±15%). [b] Values are measured as the ratios of the specificity constants ((k_{cat}/K_M) for C6PE or C6PS)/((k_{cat}/K_M) for C6PC).

efficiently cleave C6PE may also readily cleave C6PC. Hence, the more active PE mutants might have been discarded as nonselective in the screening process. None of the C6PE selective variants isolated in this study have an aromatic residue at position 66, a fact that may help explain their low catalytic activities (*1*). Indeed, the low catalytic activities of all of the C6PE selective mutants renders making conclusions regarding the basis for the observed selectivity somewhat problematic. However, it may be significant that three C6PE selective clones have a negatively charged residue at position 4, and this may help stabilize the positive charge on the ammonium ion of the ethanolamine headgroup.

C6PS Selective Mutant Enzymes. Six C6PS selective clones were isolated and identified as E4G, E4K/Y56V, E4Q/Y56T/F66Y, E4P/Y56S/F66N, E4R/Y56S/F66N, and E4G/Y56I/F66N. It is noteworthy that E4G, E4K/Y56V, and E4Q/Y56T/F66Y are superior to wt for the hydrolysis of C6PS as they have specificity constants that are 4-, 2-, and 3-fold higher, respectively, toward C6PS than wt. Perhaps even more impressive is that the ratios of the relevant specificity constants of these mutants indicate they exhibit correspondingly 32-, 180-, and 44-fold higher C6PS/C6PC preferences than wt. These three mutants are like wt in that they each possess an aromatic residue at position 66; however, unlike wt, which has a negatively charged Glu at position 4, the C6PS selective variants have a neutral or positively charged residue at this site. Thus, based upon the available data, it appears that a neutral or positively charged binding pocket favors hydrolysis of C6PS with its zwitterionic headgroup over hydrolysis of C6PC and C6PE, each of which have positively charged ammonium ions in their headgroups. Hydrolysis of C6PC and C6PE then seems to be favored by the presence of a negatively charged residue at position 4, but the validity of this hypothesis must be confirmed by further studies.

In the single-site mutant E4G, it is apparent that replacing Glu4 with Gly results in the observed increased activity toward C6PS and decreased activities toward C6PC and C6PE (Table 1). However, it is not clear whether any one amino acid replacement is primarily responsible for the altered selectivities of the double and triple-site mutants E4K/Y56V and E4Q/Y56T/F66Y. To probe the effect of each individual mutation upon substrate specificity, the corresponding wt residues were singly reinserted back into the E4K/Y56V and E4Q/Y56T/F66Y mutants via site-directed mutagenesis. These enzymes were purified, and their specificity constants toward C6PC, C6PE, and C6PS were determined as described previously (Tables 2 and 3).

The specificity constants of mutants obtained by reinsertion of wt residues into the C6PS selective double mutant E4K/Y56V are presented in Table 2. Examination of these data reveals that replacing Glu4 by Lys yields a mutant, which relative to wt, has significantly lower specificity constants toward C6PC and C6PE but an increased specificity constant toward C6PS. Hence, this replacement alone results in a C6PS selective enzyme. Because the activity profile of E4K is very similar to E4K/Y56V, the altered selectivity of PLC_{Bc} mutant E4K/Y56V appears to arise largely from replacing the negatively charged Glu4 with a positively charged Lys. This observation lends some support to the hypothesis that C6PS selective enzymes may be favored by the presence of a positively charged residue in the headgroup binding pocket. Furthermore, the replacement of Tyr56 of wt with Val gives a C6PE preferring enzyme (Y56V) that exhibits 3-fold lower activity toward C6PC than wt with little corresponding change in activity toward C6PE and C6PS. Y56A has a similar activity profile as Y56V (unpublished results), demonstrating that a residue bearing an aromatic or an alcohol moiety at position 56 in the PLC_{Bc} substrate binding pocket is not an absolute requirement for activity toward C6PE or C6PS.

The specificity constants of mutants obtained by reinsertion of wt residues into the triple mutant E4Q/Y56T/F66Y are summarized in Table 3. The data show that replacing Phe66 with Tyr in mutants F66Y, Y56T/F66Y, E4Q/F66Y, and E4Q/Y56T/F66Y has only a small impact on substrate specificity and activity. Hence, the F66Y replacement does not appear to contribute substantially to the altered selectivity pattern of the C6PS selective mutant E4Q/Y56T/F66Y (Table 3). Conversely, replacement of the negatively charged Glu4 by neutral Gln in the mutants E4Q, E4Q/Y56T, E4Q/F66Y, and E4Q/Y56T/F66Y produced a significant change in selectivity. For example, the specificity constants of E4Q are about 6-fold higher than wt toward C6PS and 2−3-fold lower than wt toward C6PC and C6PE, thereby resulting in a noticeably altered selectivity pattern. Upon replacement of Glu4 by Gln in each mutant E4Q/Y56T, E4Q/F66Y, and E4Q/Y56T/F66Y, the specificity constants toward C6PS, C6PE, and C6PC change in a similar manner to give new mutants that are selective for C6PS. Substituting Tyr56 with Thr in wt and in the F66Y, E4Q, and E4Q/F66Y mutants to give the mutants Y56T, Y56T/F66Y, E4Q/Y56T, and E4Q/Y56T/F66Y results in lowering the specificity constants

toward C6PC by 2−11-fold and increasing the C6PE/C6PC preference by at least 3-fold.

On the basis of these data, the C6PS selectivity of E4Q/Y56T/F66Y appears to derive primarily from the two replacements of Glu4 with Gln and Tyr56 with Thr. The E4Q mutation decreases activity toward C6PC and C6PE, while increasing activity toward C6PS. The Y56T mutation further decreases activity toward C6PC, resulting in increased C6PE/C6PC and C6PS/C6PC preferences. Replacing Tyr56 with Ser in wt and several mutants has a similar impact (unpublished results). These observations further demonstrate that the presence of an aromatic side chain at position 56 is not essential to maintain high specificity constants toward C6PE or C6PS. The effect of substituting Glu4 with Gln suggests that a neutral, polar residue at the 4 position of the headgroup binding pocket of PLC_{Bc} leads to decreased activity toward substrates having positively charged headgroups of choline and ethanolamine and increased activity toward the zwitterionic serine headgroup.

The advantage of mutating the three residues at positions 4, 56, and 66 of PLC_{Bc} combinatorially rather than singly to generate selective mutants is evidenced by the identification of the C6PS-preferring double and triple mutants E4K/Y56V and E4Q/Y56T/F66Y. Not only did each of these mutants exhibit a high preference for C6PS, but the specificity constant for C6PS of each was significantly larger than wt. These mutants were thus highly efficient catalysts. The subsequent step of using site directed mutagenesis to reinsert wt residues back into the multiple mutants produced by the combinatorial replacement of these residues was obviously meritorious. For example, reinserting Glu4 and Tyr56 into the C6PS selective double mutant E4K/Y56V gave the C6PS selective mutant E4K and the C6PE selective mutant Y56V. Similarly, reinserting Glu4, Tyr56, and Phe66 into the C6PS selective triple mutant E4Q/Y56T/F66Y gave the C6PS selective double mutants E4Q/Y56T and E4Q/F66Y, the C6PE double mutant Y56T/F66Y, the C6PE selective single mutants F66Y and Y56T, and the C6PS selective single mutant E4Q. These mutants generally exhibited higher specificity constants toward their preferred substrates than wt and thus maintained high catalytic efficiencies.

It is significant that the multiple mutants invariably exhibited higher substrate selectivities toward their preferred substrates than the single mutants, which could also have been produced by saturation mutagenesis. Indeed, single amino acid replacements have not thus far produced mutants having comparably large differences in substrate specificity profiles and high specificity constants for a single substrate. Comparing the specificity constants for the various mutants also suggests, perhaps not surprisingly, that specificity profiles of mutants having multiple amino acid substitutions cannot be accurately predicted from the specificity profiles of the respective single mutants. Rather, the effects of individual mutations at sites 4, 56, and 66 on substrate selectivity are dependent upon the identity of the other residues at these sites.

Summary. Modifying the substrate specificity and catalytic activity of enzymes to produce novel catalysts is one of the central goals of protein engineering. In this context, we developed a novel protocol to generate and screen mutants of PLC_{Bc}. Specifically, a library of approximately 6000 maltose binding protein−PLC_{Bc} fusion protein mutants containing random permutations of the Glu4, Tyr56, and Phe66 residues was generated. The members of this library were screened for hydrolytic activity toward the water-soluble substrates C6PC, C6PE, and C6PS in a 96-well format that featured in situ cleavage of the fusion protein to release the mutant PLC_{Bc}s; the active mutants were identified using a coupled enzyme assay in which inorganic phosphate was detected. Significantly, this process should be readily adaptable to other enzymes that are normally expressed as fusion proteins, although further improvements will be required to screen larger libraries.

Ten PLC_{Bc} variants, six of which were triple mutants, were thus identified and found to have significantly altered selectivity profiles relative to wt as evidenced by comparison of their respective specificity constants, k_{cat}/K_M, (Table 1). Indeed, the C6PS selective mutants E4G, E4K/Y56V, and E4Q/Y56T/F66Y exhibited specificity constants greater than wt. A second round of studies was then performed in which the corresponding wt residues were singly reinserted back into E4K/Y56V and E4Q/Y56T/F66Y using site-directed mutagenesis. The resultant C6PS selective (E4K, E4Q, E4Q/Y56T, and E4Q/F66Y) and C6PE selective (Y56V, Y56T, F66Y, and Y56T/F66Y) mutants thus produced exhibited specificity constants (Tables 2 and 3) for their preferred substrates that were higher than wt. Significantly, the double and triple mutants uniformly had higher substrate preferences than the single mutants, thereby illustrating the advantage of randomly mutating all three binding pocket residues in a combinatorial fashion rather than singly mutating these residues by saturation mutagenesis. Comparing the substrate preferences of single mutants with those of multiple mutants reveals that the effects of individual mutations may depend on the nature of the other residues in the binding pocket.

Because of the relatively small size of libraries in this study, one must exert extreme caution in making general observations. Given this important caveat, only a few comments are appropriate. All C6PE selective enzymes with higher specificity constants toward C6PE than wt have a negatively charged Glu at position 4 as found in wt, so it seems likely that the hydrolysis of both C6PC and C6PE, each of which bears a cationic headgroup, are favored by the presence of a negatively charged residue at position 4. On the other hand, the isolated mutants that selectively hydrolyze C6PS with its zwitterionic headgroup have a neutral (Gln or Gly) or a positively charged (Lys) residue at position 4, and several of these variants exhibited specificity constants for C6PS greater than wt. These replacements significantly decrease enzyme activity toward C6PC and C6PE. All PLC_{Bc} variants isolated thus far exhibiting >1 wt % activity toward C6PC or C6PE have an aromatic residue in position 66. This observation supports previous findings (*1*) and reinforces the prevailing hypothesis that there is an important interaction between the aromatic ring of Phe66 and the substrate headgroups, especially choline and ethanolamine. Replacement of Tyr56 with a nonaromatic Val, Thr, or Ser residue lowers the specificity constant for C6PC, thereby resulting in increased C6PE/C6PC and C6PS/C6PC preferences for the mutants studied. This observation is consistent with previous results (*1*) and shows that an aromatic or a hydroxyl moiety is not required at position 56 for activity toward C6PE or C6PS.

The side chains of the residues at positions 4, 56, and 66 of PLC_{Bc} are not directly involved in catalysis but rather comprise the headgroup binding pocket (*25*). The results obtained herein suggest that these residues act in concert as a module in which one or more can be changed to give a modified binding pocket and a mutant enzyme that exhibits a specificity profile significantly different from wt. Obviously, PLC_{Bc} itself has not been evolutionary optimized to hydrolyze the phosphodiester bond of PE and PS derivatives. A better understanding of the structural basis for the observed trends in substrate specificity of PLC_{Bc} and its mutants must await determination of the three-dimensional structure of selected mutants and their complexes with the appropriate substrate analogues. Such studies are in progress, and the results will be reported in due course.

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REFERENCES

1. Martin, S. F., Follows, B. C., Hergenrother, P. J., and Trotter, B. K. (2000) *Biochemistry 39*, 3410−3415.
2. Arnold, F. H. (2001) *Nature 409*, 253−257.
3. Harris, J. L., and Craik, C. S. (1998) *Curr. Opin. Chem. Biol. 2*, 127−132.
4. Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3565−3570.
5. Park, Y., Luo, J., Schultz, P. G., and Kirsch, J. F. (1997) *Biochemistry 36*, 10517−10525.
6. Srivastava, D. K., and Peterson, K. L. (1998) *Biochemistry 37*, 8446−8456.
7. Czjzek, M., Cicek, M., Zamboni, V., Bevan, D. R., Henrissat, B., and Esen, A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13555−13560.
8. Penning, T. M., and Jez, J. M. (2001) *Chem. Rev. 101*, 3027−3046.
9. Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992) *Science 225*, 1249−1253.
10. Onuffer, J. J., and Kirsch, J. F. (1995) *Protein Sci. 4*, 1750−1757.
11. Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R., and Holbrook, J. J. (1988) *Science 242*, 1541−1544.
12. Crameri, A., Dawes, G., Rodriguez, E., Silver, S., and Stemmer, W. P. C. (1997) *Nature Biotech. 15*, 436−438.
13. Moore, J. C., and Arnold, F. H. (1996) *Nature Biotech. 14*, 458−467.
14. Yano, T., Oue, S., and Kagamiyama, H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 5511−5515.
15. Altamarino, M. M., Blackburn, J. M., Aguayo, C., Fersht, A. R. (2000) *Nature 403*, 617−622.
16. Hubner, B., Haensler, M., and Hahn, U. (1999) *Biochemistry 38*, 1371−1376.
17. Choo, Y., and Klug, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11163−11167.
18. Huang, W., Petrosino, J., Hirsch, M., Shenkin, P. S., and Palzkill, T. (1996) *J. Mol. Biol. 258*, 688−703.
19. Graham, L. D., Hagget, K. D., Jennings, P. A., Le Brocque, D. S., and Whittaker, R. G. (1993) *Biochemistry 32*, 6250−6258.
20. Viadiu, H., Osuna, J., Fink, A. L., and Soberon, X. (1995) *J. Biol. Chem. 270*, 781−787.
21. Hergenrother, P. J., and Martin, S. F. (1997) *Anal. Biochem. 251*, 45−49.
22. Hergenrother, P. J., and Martin, S. F. (2001) *Top. Curr. Chem. 211*, 131−167.
23. Martin, S. F., Wong, Y.-L., and Wagman, A. S. (1994) *J. Org. Chem. 59*, 4821−4831.
24. Martin, S. F., Spaller, M. R., and Hergenrother, P. J. (1996) *Biochemistry 35*, 12970−12977.
25. Martin, S. F., and Hergenrother, P. J. (1998) *Biochemistry 37*, 5755−5760.
26. Martin, S. F., and Hergenrother, P. J. (1998) *Bioorg. Med. Chem. Lett. 8*, 593−596.
27. Martin, S. F., and Hergenrother, P. J. (1999) *Biochemistry 38*, 4403−4408.
28. Martin, S. F., Follows, B. C., Hergenrother, P. J., and Franklin, C. L. (2000) *J. Org. Chem. 65*, 4509−4514.
29. Exton, J. H. (1997) *Eur. J. Biochem. 243*, 10−20.
30. Guddal, P. H., Johansen, T., Schulstad, K., and Little, C. (1989) *J. Bacteriol. 171*, 5702−5706.
31. 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.
32. Hansen, S., Hough, E., Svensson, L. A., Wong, Y. L., and Martin, S. F. (1993) *J. Mol. Biol. 234*, 179−187.
33. Hansen, S., Hansen, L. K., and Hough, E. (1993) *J. Mol. Biol. 231*, 870−876.
34. Hansen, S., Hansen, L. K., and Hough, E. (1992) *J. Mol. Biol. 225*, 543−549.
35. Kearney, P. C., Mizoue, L. S., Kumpf, R. A., Forman, J. E., McCurdy, A., and Dougherty, D. A. (1993) *J. Am. Chem. Soc. 115*, 9907−9919.
36. Ma, J. C., and Dougherty, D. A. (1997) *Chem. Rev. 97*, 1303−1324.
37. Wouters, J. (1998) *Protein Sci. 7*, 2472−2475.
38. Basran, J., Mewies, M., Mathews, F. S., and Scrutton, N. S. (1997) *Biochemistry 36*, 1989−1998.
39. Dougherty, D. A. (1996) *Science 271*, 163−168.
40. Ting, A., Shin, I., Lucero, C., and Schultz, P. G. (1998) *J. Am. Chem. Soc. 120*, 7135−7136.
41. We thank Professor Barbara S. Plake (Department of Educational Psychology, University of Nebraska at Lincoln) for this analysis.
42. Hergenrother, P. J. (1999) The Catalytic Mechanism of Phospholipase C and the Total Synthesis of Erythromycin B, Ph.D. Dissertation, University of Texas at Austin.
43. Hirel, P. H., Schmitter, J. M., Dessen, P., Fayat, G., and Balanquet, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8247−8251.

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