Catalytic Cycle of the Phosphatidylcholine-Preferring Phospholipase C from *Bacillus cereus*. Solvent Viscosity, Deuterium Isotope Effects, and Proton Inventory Studies†

Stephen F. Martin* and Paul J. Hergenrother

Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712

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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. Although much is known about the roles that various PLC_{Bc} active site amino acids play in binding and catalysis, there is little information about the rate-determining step of the PLC_{Bc}-catalyzed hydrolysis of phospholipids and the catalytic cycle of the enzyme. To gain insight into these aspects of the hydrolysis, solvent viscosity variation experiments were conducted to determine whether an external step (substrate binding or product release) or an internal step (hydrolysis) is rate-limiting. The data indicate that the PLC_{Bc}-catalyzed reaction is unaffected by changes in solvent viscosity. This observation is inconsistent with the notion of substrate binding or product release being rate-determining and supports the hypothesis that a chemical step is rate-limiting. Furthermore, a deuterium isotope effect of 1.9 and a linear proton inventory plot indicate one proton is transferred in the rate-determining step. These data may be used to formulate a comprehensive catalytic cycle that is for the first time based on experimental evidence. In this mechanism, Asp55 of PLC_{Bc} activates an active site water molecule for attack on the phosphodiester bond, the hydrolysis of which is rate-limiting. The phosphorylcholine product is the first to leave the active site, followed by diacylglycerol.

The phospholipase C class of enzymes catalyze the hydrolysis of phospholipids, yielding diacylglycerol (DAG)[1] and a phosphorylated headgroup. In mammalian systems, these two products act as secondary messengers in the signal transduction cascade, with the DAG serving as an endogenous activator of protein kinase C (*1*). The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PLC_{Bc}) (EC 3.1.4.3) is a monomeric, 28.5 kDa enzyme with three zinc ions in its active site (*2*). Because of this unusual trimetal center and its antigenic similarity with mammalian PLC (*3*), it has been the subject of extensive biochemical investigation (*4*−*7*).

Like other phosphodiesterases, PLC_{Bc} is believed to function by activating an active site water molecule for an in-line, $S_N2(P)$ attack on the phosphorus atom of the phosphodiester linkage. X-ray structures of the native enzyme (*2*) and its complexes with both inorganic phosphate (*8*) and a phosphonate substrate analogue inhibitor (*9*) have provided the first insights into the mechanism of action and the possible roles that selected amino acids and the three zinc ions might play in catalysis and binding. The cloning (*10*) and overexpression in *Escherichia coli* of PLC_{Bc} (*11*) then enabled the use of site-directed mutagenesis for preparing and characterizing mutants of selected active site residues. Those studies now support the role of Asp55 as the general base, although Glu4 and Glu146 had been previously suggested as possible candidates (*11*, *12*). The side chain carboxyl group of Glu146 appears to serve as a ligand for Zn2, whereas that of Glu4 is likely involved in substrate binding of the choline moiety.

Because there are three zinc ions at the active site, it was at first conceivable that one of these might activate a water molecule for nucleophilic attack in analogy with the phosphoryl transfer reactions catalyzed by alkaline phosphatase (*13*), *Eco*RV endonuclease (*14*), and purple acid phosphatase (*15*). However, the crystal structure of the complex of PLC_{Bc} bound to a phosphonate inhibitor reveals that there are no water molecules in the first coordination sphere of any of the zinc ions, so invocation of a zinc-bound water as the nucleophile is problematic. Rather, it seems more likely that the primary roles of the zinc ions are in binding substrate and activating the phosphate moiety toward nucleophilic attack through charge neutralization.

Despite the number of studies aimed at defining the mechanistic roles of different active site residues of PLC_{Bc}, there are no detailed kinetic studies that provide information regarding the catalytic cycle for the hydrolysis of phosphatidylcholine. The order of release of the two products, DAG and phosphorylcholine, and the rate-determining step of catalysis have not been established, although some experiments that touch on these questions have been reported. For example, the PLC_{Bc}-catalyzed hydrolysis of phosphatidylcholines exhibits product inhibition by DAG (*16*). PLC_{Bc} is also activated in the presence of certain detergents (*17*) that

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* To whom correspondence should be addressed. Telephone: (512) 471-3915. Fax: (512) 471-4180. E-mail: sfmartin@mail.utexas.edu.

[1] Abbreviations: DAG, diacylglycerol; DMG, dimethylglutaric acid; PC, phosphatidylcholine; PLC, phospholipase C; PLC_{Bc}, PC-preferring phospholipase C from *Bacillus cereus*; Tris, tris(hydroxymethyl)-aminomethane; C6PC, 1,2-*O*-di-*n*-hexanoyl-*sn*-glycero-3-phosphocholine.

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might interact with the hydrophobic DAG to accelerate its release from the enzyme. PLC_{Bc} is also activated in micellular systems (*18*), wherein the release of DAG from the enzyme into the hydrophobic environment of the aggregated lipid substrate should be more facile than its release into an aqueous medium. On the basis of these observations, it had been proposed that the release of DAG might be the rate-determining step (*17*, *19*). However, while these studies may suggest that DAG release is the rate-determining step, this indirect evidence was not conclusive. This investigation was thus initiated to resolve whether the rate-determining step in the PLC_{Bc}-catalyzed hydrolysis of phospholipids was an internal (chemical) or external (substrate binding or product release) event and to gain insights into the details of the catalytic cycle. These results are presented herein.

MATERIALS AND METHODS

Materials. Wild-type PLC_{Bc} was expressed and purified from *E. coli* as described previously (*11*). Sucrose, ficoll-400, choline oxidase, peroxidase, and alkaline phosphatase were obtained from Sigma. The 1,2-*O*-di-*n*-hexanoyl-*sn*-glycero-3-phosphocholine (C6PC) used as a substrate was purchased from Avanti Polar Lipids.

Methods. Assays for PLC_{Bc} activity were performed as described previously (*20*). Briefly, the phosphorylcholine product of the PLC_{Bc}-catalyzed hydrolysis of C6PC was converted into a red dye with an absorbance maximum at 490 nm through the action of alkaline phosphatase, choline oxidase, peroxidase, phenol, and 4-aminoantipyrine. Assays in solvents with increased viscosities were conducted in an identical fashion, except with the presence of the added viscogenic agent. Because control experiments indicated that glycerol gave a large background absorbance in the coupled chromogenic assay, it was not used as an agent to increase the microviscosity of the assay medium. Identical control experiments with sucrose and ficoll-400 exhibited no background absorbance increase. Viscosities were determined with a Cannon-Fenske viscometer at 37 °C and referenced to the buffer without the added viscogen. C6PC was used at substrate concentrations ranging from 0.7 to 5.0 mM, well below the critical micelle concentration of C6PC, which is 11.1 mM (*18*). This ensured that the substrate existed as a monomer in the assay medium.

Assays of PLC_{Bc} in the presence of increasing concentrations of sucrose were performed on both recombinant PLC_{Bc} (*11*) and commercially available PLC_{Bc} from Sigma. Each enzyme source was tested with the viscosity variation assay on three separate occasions, and the results were identical. To reduce the error inherent in enzymatic assays, controls in a buffer with a viscosity of 1 (no viscogen) were performed in parallel with those with increased viscosities. Assays in the presence of differing concentrations of D_2O were conducted in an analogous fashion. pD values were determined by adding 0.4 to the reading of the pH meter (pD = pH + 0.4) (*21*). To ensure that each reaction performed at differing concentrations of D_2O included equal quantities of enzyme, additions were made from the same PLC_{Bc} stock solution. Kinetic parameters were determined from a nonlinear least-squares fit of the data by KaleidaGraph, utilizing the equation

$$v = V_{max}[S]/K_m + [S]$$

RESULTS AND DISCUSSION

Solvent Viscosity Effects. The rate at which small molecules diffuse through a solvent is known to be inversely proportional to the microviscosity of that solvent (*22*). Consequently, if a diffusion-controlled process is the rate-determining step of an enzymatic reaction, the reaction will be slower in solvents with higher viscosities, provided there are no unforeseen effects on the enzyme itself. Because the binding of a substrate to an enzyme and the release of products from its active site are both diffusion-controlled processes, the reaction rate should be sensitive to solvent viscosity if either one of these steps were rate-determining. Solvent viscosity studies have been classically used to demonstrate that a diffusion-controlled process, usually substrate binding, is the rate-determining step in an enzymatic reaction (*23*−*26*). For example, in seminal studies conducted with triosephospate isomerase (TIM), Knowles showed that the reaction rate decreased proportionally with an increase in solvent microviscosity (*23*), thus indicating that catalysis by TIM was limited by substrate binding or product release. The effects of changing viscosity on the rates of other enzymatic reactions have also been used as evidence to support a hypothesis that product release was the rate-determining step (*27*). In cases where an increase in viscosity does not affect the enzymatic activity, the rate-determining step is then usually ascribed to a chemical reaction, not a diffusion-controlled process (*28*).

Changes in macroviscosity (a property of the solvent that can be determined with the aid of a viscometer) do not affect the rate of diffusion of small molecules (*29*), whereas increases in the microviscosity of a solution do retard their rate of diffusion. Polyhydroxylated organic molecules such as glycerol and sucrose affect both the macroviscosity and microviscosity of the solution. To control for the effects of the macroviscosity on the enzyme, experiments must be performed that separate the effects of "macroviscosity" and "microviscosity", ensuring that any variation observed in the reaction rate is due only to increases in solvent microviscosity. Toward this end, control experiments were conducted in which polymeric viscogenic agents such as polyethylene glycol or ficoll (polysucrose), which increase the macroviscosity of the solution but have no effect on the microviscosity, were utilized as additives. These data are summarized in Figure 1, in which the ratios of k_{cat}/K_M for the PLC_{Bc}-catalyzed hydrolysis of C6PC are plotted versus the relative macroviscosity of the solution, which was increased by the addition of the polymeric additive ficoll. The results of this experiment clearly indicate that changes in the macroviscosity of the assay medium have an insignificant effect upon PLC_{Bc} activity.

The rate of an enzymatic reaction in which a chemical step is rate-limiting should not be affected by changes in the microviscosity of the solution (*26*). In such cases where the chemistry is rate-limiting and a change in enzymatic reactivity with viscosity is observed, the addition of the viscogenic agent is not merely retarding the rate of diffusion; it must also be exerting some direct effect on the enzyme. To eliminate this possibility, control experiments in which the microviscosity is varied under conditions where a chemical step is rate-determining are typically performed. This may involve use of either a poor substrate (*26*), the

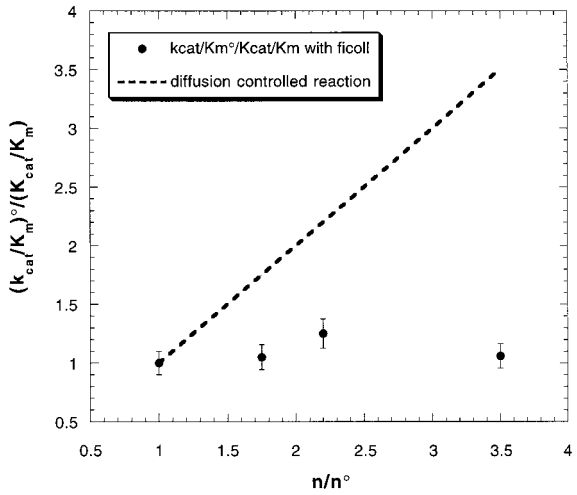

FIGURE 1: Plot of k_{cat}/K_M of PLC_{Bc} in the presence of the polymeric additive ficoll vs the relative viscosity of the assay medium. The solid circles represent the activity of PLC_{Bc} toward C6PC in assays conducted at pH 7.3. Points are obtained by dividing the value of the activity without added ficoll-100 by the activity in the presence of various concentrations of ficoll-100 (polysucrose). The relative viscosity of the assay medium is displayed on the *x*-axis. In this case, *n* represents the viscosity in the presence of added ficoll-100, while *n*° is the viscosity of the buffer under standard conditions, which is 1. The dashed line has a slope of 1 and represents the type of plot expected for a reaction that is diffusion-controlled. In a diffusion-controlled reaction, the activity decreases as the relative viscosity of the solution is increased.

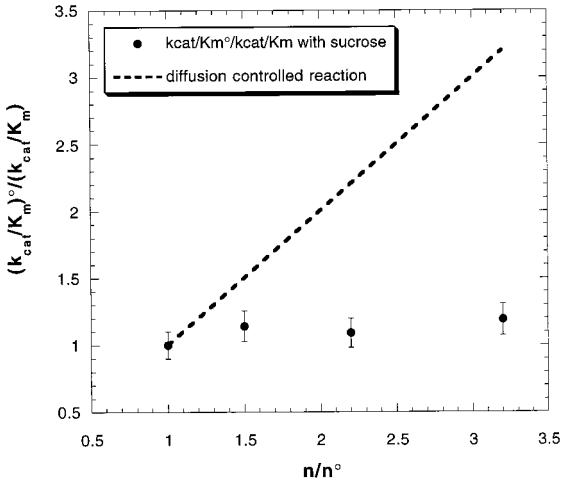

FIGURE 2: Plot of k_{cat}/K_M of PLC_{Bc} in the presence of sucrose vs the relative viscosity of the assay medium. The solid circles represent the activity of PLC_{Bc} toward C6PC in assays conducted at pH 7.3. Points are obtained by dividing the value of the activity without added sucrose by the activity in the presence of various concentrations of sucrose. The relative viscosity of the assay medium is displayed on the *x*-axis. In this case, *n* represents the viscosity in the presence of added sucrose, while *n*° is the viscosity of the buffer under standard conditions, which is 1. The dashed line has a slope of 1 and represents the type of plot expected for a reaction that is diffusion-controlled. In a diffusion-controlled reaction, the activity decreases as the relative viscosity of the solution is increased.

processing of which is known to be limited by a chemical step, or a crippled mutant (*23*). However, because PLC_{Bc} shows no change in rate with increasing viscosities (Figure 2), there was no need to conduct any control experiments.

The effect of varying the microviscosity of the solution on the rate of the PLC_{Bc}-catalyzed hydrolysis of C6PC at pH 7.3 was then examined. The data are displayed in Figure 2. The dashed line has a slope of 1 and shows the expected result for a system in which a diffusion-controlled process is rate-determining; namely, there should be a linear decrease in the catalytic efficiency as the amount of viscogen is increased. Examination of Figure 2 clearly indicates that the activity of PLC_{Bc} toward the water soluble substrate C6PC is not affected by sucrose-induced changes in the microviscosity of the medium.

Inasmuch as the catalytic efficiency of PLC_{Bc} (335 mM^{-1} s^{-1}) (*11*) does not approach the diffusion-controlled limit of 10^8-10^{10} M^{-1} s^{-1} (*23*), substrate binding was not expected to be rate-limiting, and product release is the only diffusive step that could be rate-limiting. Indeed, release of DAG from the active site had been suggested as the likely rate-determining step (*17*). However, because this process would be susceptible to changes in microviscosity, the results of these experiments make a persuasive case that release of neither DAG nor phosphorlycholine is rate-limiting. These results are consistent with an internal, chemical step being rate-determining for the PLC_{Bc}-catalyzed hydrolysis reactions of water soluble phospholipids.

Solvent Isotope Effects. Given the likelihood that the rate-determining step in the PLC_{Bc}-catalyzed hydrolysis now appears to be a chemical one, the deuterium isotope effects were measured to probe whether proton transfer might be occurring in this step. It is again important to perform proper controls, because D_2O has a higher viscosity that H_2O (*30*) and the pK_a's of protein residues can be slightly perturbed in D_2O (*31*). The results from the solvent viscosity experiments described above indicate that the rate of PLC_{Bc} processing is unaffected by solvent viscosity, so the increased viscosity of D_2O should not be a factor. However, to eliminate any effects that might arise from changes in the pK_a's of any amino acid residues, the PLC_{Bc}-catalyzed hydrolysis of C6PC in D_2O was determined at more than one pD (*21*). Thus, at pD 7.7, a deuterium isotope effect of 1.9 on the second-order rate constant k_{cat}/K_M [$k_{cat}/K_M(H_2O)/k_{cat}/K_M(D_2O)$] was observed, and when the assays were conducted at pH 8.7, an identical deuterium isotope effect of 1.9 was obtained, indicating that in this region the enzyme has the same pH (pD) profile. Solvent isotope effects in the range of 1.5−2.1 have been observed previously in situations where proton transfer was rate-limiting (*30*, *32*−*34*).

Proton Inventory. Having established that proton transfer is involved in the rate-determining step of the PLC_{Bc}-catalyzed reaction, we still needed to answer the question of how many protons were "in flight" in this step. This issue is most easily addressed with a proton inventory experiment in which the enzyme is assayed in buffers containing different mole fractions of D_2O (*35*). The shape of the resulting plot of the activity versus mole fraction of D_2O provides information regarding the number of protons that are being transferred during the rate-determining step of the enzymatic reaction. The most straightforward analysis occurs when a straight line is obtained in the graph, indicating that the activity decreases proportionally as the mole fraction of D_2O is increased. This indicates that the transfer of one proton is involved in the rate-determining step. Curved lines are obtained when two or more protons are in flight, and although the precise number of protons involved can sometimes be determined on the basis of the shape of the

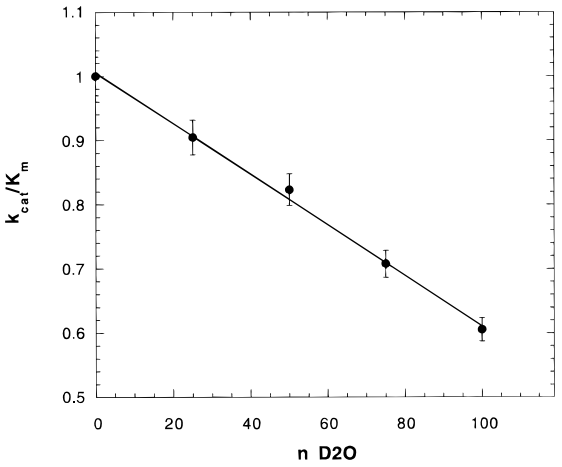

FIGURE 3: Proton inventory experiments for PLC_{Bc} toward C6PC, with the activity plotted against mole fraction of D_2O. Assays were conducted using a coupled chromogenic assay as described in the text. The slope of the line of -0.58 ($R = 0.998$).

graph, the interpretation of such plots is often subject to uncertainty.

The proton inventory data for PLC_{Bc} are displayed in Figure 3. The fact that these data are linear with a slope of -0.58 ($R = 0.998$) is consistent with the transfer of a single proton in the rate-determining step for the PLC_{Bc}-catalyzed hydrolysis of phospholipids.

Catalytic Mechanism. A catalytic cycle for the PLC_{Bc}-catalyzed hydrolysis of phospholipids that is consistent with all the available data can now be formulated as shown in Figure 4. The phosphatidylcholine substrate binds at the enzyme active site to give the E·S complex, which is stabilized by an ionic attraction between the nonbridging oxygens of the phosphodiester moiety and the three zinc ions, the interaction of the choline moiety with the choline binding region comprised of Glu4, Tyr56, and Phe66, and the formation of a hydrogen bond between the amide backbone of Asn134 and the *sn*-2 carbonyl group of the substrate (Figure 5).

Catalysis commences when a proton from an active site water molecule is abstracted by a base. The pH dependence curve of wild-type PLC_{Bc} shows an ascending acidic limb with a slope of 1, consistent with one residue being ionized. From the acidic limb of this curve, the side chain undergoing ionization is estimated to have a pK_a of about 5.3 (*6*, *11*). Site-directed mutagenesis studies suggest that Asp55 is the general base for this step, as its replacement with asparagine provides a protein that is structurally similar to the wild type but 4 orders of magnitude less active (*12*). The normal isotope effect of 1.9 determined in this study is also consistent with general base catalysis by an amino acid side chain. An alternate possibility is that the water is activated by coordination to one of the three zinc ions. Enzymes that utilize a zinc-bound water as a nucleophile include AMP deaminase (*36*), thermolysin (*37*), stromelysin (*38*), and a desuccinylase (*39*); the first three enzymes possess one active site zinc, while the last contains two. However, these enzymatic reactions exhibit an inverse isotope effect that results from a dominant equilibrium solvent isotope effect for proton transfer at the metal-bound water. If a zinc-bound water were utilized by PLC_{Bc}, a normal isotope effect would be observed only if the proton transfer step were not at equilibrium, but such a proton transfer would be different from those of the four enzymes cited above. Thus, although the available evidence supports the hypothesis that the side chain of Asp55 of PLC_{Bc} serves as the general base in activating the nucleophilic water to catalyze phospholipid hydrolysis, further scrutiny of this issue is necessary as these data do not exclude some involvement of a zinc ion.

Nucleophilic attack by the activated water molecule or hydroxide ion on phosphorus is then believed to occur by an in-line, associative mechanism that proceeds via a trigonal bipyramidal transition state (*40*), but there are at present no stereochemical studies to support this hypothesis. Collapse of the pentacoordinate intermediate then leads to the two products, phosphorylcholine and diacylglycerol. Cleavage of the phosphodiester bond requires the activation of the diacylglycerol leaving group with a general acid, which might be a zinc ion, a zinc-bound water molecule, or an amino acid residue.

Elucidating the details associated with the second proton transfer step to the diacylglycerol moiety remains a challenge, and there are no experimental data that explicitly address this issue. Molecular modeling studies have led to a proposal that Asp55 is a likely candidate for the general acid (*41*), but convincing experimental evidence strongly implicates Asp55 as the general base (*12*). The plot of the activity of PLC_{Bc} versus pH is bell-shaped (*4*, *6*), and if it is assumed that pH changes do not induce unforeseen effects on the enzyme, this curve indicates that the general base and general acid are being titrated on the corresponding acidic and basic limbs. Under such circumstances, it is kinetically impossible for a single residue to function as both a general acid and general base. Rather, if Asp55 served in this dual capacity, the pH dependence curve would be expected to have an ascending acidic limb with no descending basic limb because the general acid would be formed continuously regardless of pH. This clearly is not the case. No other residues emerge as likely candidates for the general acid, and the possibility that a zinc ion or a zinc-bound water serves in this capacity must be considered.

Because of the high effective molarity of water, the hydrolysis reaction producing diacylglycerol and phosphorylcholine is most likely to be irreversible as shown in Figure 4. The products are then released from the active site in two separate steps, neither of which is rate-determining. Because Lineweaver−Burk plots show that diacylglycerol is a competitive inhibitor of PLC_{Bc} with a K_i of 10 mM, this product must bind to the same form of the enzyme that binds the substrate. The nature of a catalytic cycle dictates that a product that is a competitive inhibitor must be the last one released from the enzyme active site. It then follows that phosphorylcholine is released first. Supporting this hypothesis, we have found phosphorylcholine to be an extremely weak, noncompetitive inhibitor of PLC_{Bc}. This proposed order of product release corresponds to that predicted by Sundell et al. (*42*) based upon molecular modeling calculations on the PLC_{Bc} active site.

It now appears that the increased activity of PLC_{Bc} in micelles is not due to a more facile release of DAG, so the question remains about why interfacial activation, a phenomenon common among lipases, is observed. There are several potential reasons. The large increase in the effective molar concentration of the substrate when it is present as a

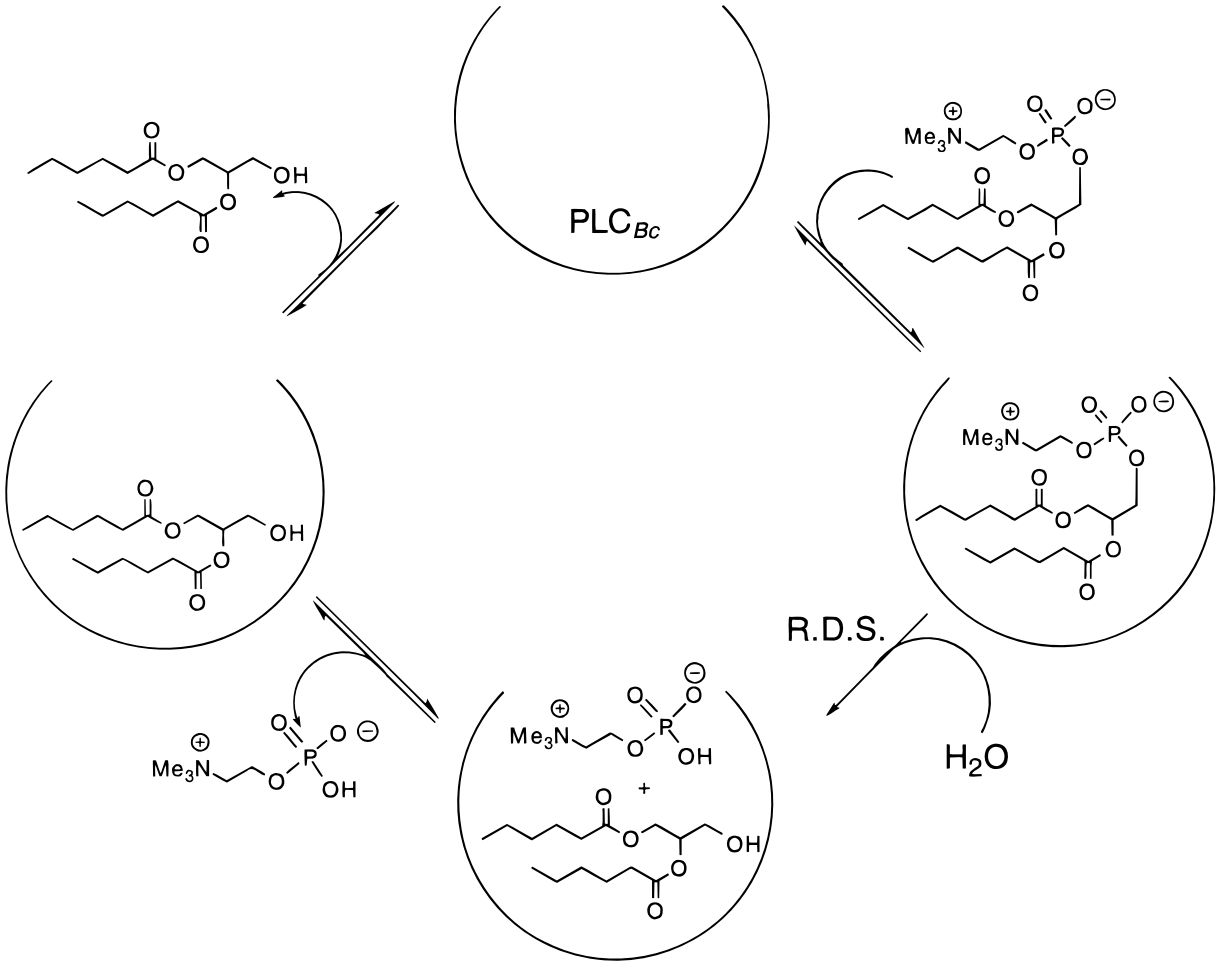

FIGURE 4: Postulated catalytic cycle for PLC_{Bc}. In the first step, the enzyme active site (represented by the partial circle) binds to the phosphatidylcholine substrate. This substrate is then hydrolyzed in the R.D.S. to give phosphorylcholine and diacylglycerol. Phosphorylcholine leaves the active site first, followed by DAG. This cycle is consistent with the data presented here, suggesting that a chemical step involving a single proton transfer is rate-limiting.

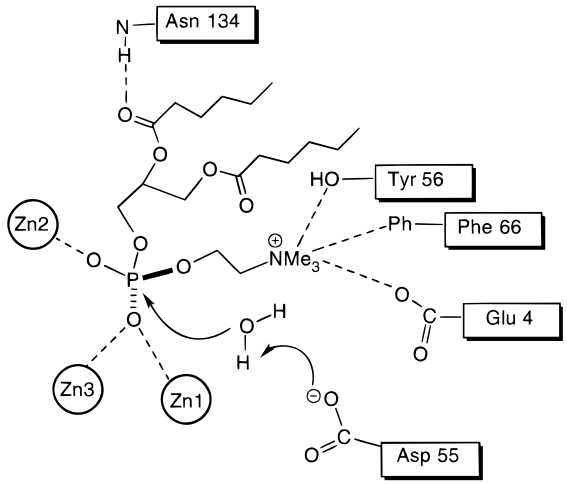

FIGURE 5: Principal active site interactions between PLC_{Bc} and a phosphatidylcholine substrate. The substrate is drawn into the PLC_{Bc} active site by electrostatic interactions between the nonbridging phosphate oxygens and the zinc ions, and the choline moiety with Tyr56, Phe66, and Glu4. Asp55 is the putative general base that activates a water molecule for nucleophilic attack on the phosphodiester.

micelle as opposed to its solvated monomeric form likely plays a role. In addition, there may be a certain entropic advantage in that the reaction incurs when the phospholipid is present in its micellular form. In such a state, the orientation of the substrate would be greatly restricted, and recognition of the substrate by the enzyme may be thereby facilitated. Finally, it has been suggested that an enzyme which is interfacially activated may undergo a subtle conformational change in the presence of micelles, leading to the observed increase in activity (*43*).

Conclusions. The data reported herein provide the first clear indication that the hydrolysis of monomeric phospholipids by PLC_{Bc} is limited by a chemical process, rather than by a substrate binding or product release step as had been previously speculated. Moreover, a significant deuterium isotope effect and the proton inventory data indicate that a single proton is being transferred in the rate-determining step. A cohesive picture of the PLC_{Bc}-catalyzed reaction of phosphatidylcholine may now be formulated in which the hydrolysis of the phosphodiester bond of the substrate is the irreversible and rate-determining step. During this process, Asp55 of PLC_{Bc} appears to serve as the general base to activate a water molecule for nucleophilic attack on the phosphodiester moiety; the identity of the general acid remains unknown. The phosphorylcholine product is then released from the active site, followed by diacylglycerol. Further work on elucidating mechanistic aspects of this reaction are in progress as are experiments aimed at

developing mutants with altered specificity and reactivity profiles. Progress in these areas will be reported in due course.

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