MECHANISTIC STUDIES ON PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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ABSTRACT

This work combines the use of thio effects and site-directed mutagenesis (SDM) to characterize the catalytic mechanisms of phosphatidylinositol-specific phospholipase Cs (PI-PLCs).

Eukaryotic PI-PLCs utilize Ca\(^{2+}\) in catalysis, whereas *Bacillus thuringiensis* PI-PLC (btPLC) uses a spatially conserved guanidinium group. A Ca\(^{2+}\)-dependent mutant of btPLC was constructed and characterized to understand the functional differences between these two positively charged moieties. The following results indicate that a true catalytic metal site was created by a single mutation at position 69: (1) R69D was activated by Ca\(^{2+}\). (2) Titration of R69D with Ca\(^{2+}\), monitored by \(^{15}\)N-\(^1\)H HSQC NMR, showed that addition of Ca\(^{2+}\) to R69D restores the environment of the catalytic site analogous to that attained by WT. (3) Upon Ca\(^{2+}\) activation, the \(S_p\)-thio effect (\(k_O/k_{Sp} = 4.4 \times 10^5\)) approached a value similar to that of WT, suggesting a structural and functional similarity between Arg69 and Asp69-Ca\(^{2+}\). (4) Results from additional SDM suggest that the Ca\(^{2+}\) binding site is comprised of side chains from Asp33, Asp67, Asp69, and Glu117.

A natural occurring metal-dependent *Streptomyces antibioticus* PI-PLC (saPLC1) was cloned, expressed, purified, and characterized. The results enrich the knowledge in application of thio effect and provide useful mechanistic information for metal-dependent
PI-PLCs: (1) WT has an extraordinarily high $S_p$-thio effect ($k_{O}/k_{S_p} = 1.6 \times 10^8$) in the presence of Ca$^{2+}$. Substitution of Ca$^{2+}$ by Cd$^{2+}$ in WT enhances the activity toward $S_p$-DPPsI by a factor of 400, but does not affect the activity with PI or $R_p$-DPPsI, which are evident that the metal cofactor interacts with the pro-$S_p$ oxygen. (2) In the presence of Ca$^{2+}$, the $R_p$-thio effect of H16A ($k_{O}/k_{R_p} = 1.1$) is lowered from that of WT ($k_{O}/k_{R_p} = 47$). Ca$^{2+}$/Cd$^{2+}$ replacement in H16A dramatically increases the $R_p$-thio effect ($k_{O}/k_{R_p} = 7.9 \times 10^3$). Tentatively, these results suggest that both Ca$^{2+}$ and His16 are involved in the interaction with the pro-$S_p$ oxygen. (3) WT and most of its mutants demonstrated small bridging thio effects ($k_{O}/k_{S} = 0.5 \sim 2$), whereas H55A displays an inverse thio effect ($k_{O}/k_{S}$) of 0.0019, implicating that His55 functions as the general acid in the phosphotransferase reaction.
Dedication

To the memory of my grandmother
ACKNOWLEDGMENTS

I would like to first thank my adviser and mentor, Dr. Ming-Daw Tsai, for his encouragement, support, and patience during my graduate studies in The Ohio State University. His devotion to science, his principle in research, and his modesty yet ambitious character are of great influence and inspiration to me.

One of the theme works in my thesis is stereochemical studies. It owes to the kind collaboration with Professor Karol Bruzik. I thank Yinghui Liu, a graduate student of Dr. Bruzik, for providing the thio analog in my studies.

I could not have made it without my family and friends.

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in my mind and never ceases growing. I am grateful for them being such hardworking, optimistic, and understanding parents, who were able to cast my character and determine where I stood. My elder sister, Yan, has always been my best friend and positive influence. I want particularly thank her being such a tremendous support during my graduate life: for giving me encouragement and belief when I felt most incompetent, for giving me care and love when I needed them the most yet thousands of miles away from home. I thank my family for being my harbor during the storm and my light tower in the darkness. I own much more than I can well express.

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VITA

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PUBLICATIONS

Research Publication

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   Unprecedented Magnitudes of Thio Effect, Inverse Thio Effect, and

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   metal binding site into a calcium-independent phosphatidylinositol-specific
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   (2003).

3. Kravchuk, A. V., Zhao, L., Kubiak, R. J., Bruzik, K. S. & Tsai, M. D.
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<tr>
<td>1D</td>
<td>one-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
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<td>bPI-PLC</td>
<td>phosphatidylinositol-specific phospholipase C from <em>Bacillus cereus</em></td>
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<td>btPLC</td>
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<td>°C</td>
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<td>GPI</td>
<td>sn-glycero-3-(1-phospho-1D-my-o-inostiol)</td>
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<td>h</td>
<td>hour(s)</td>
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<td>HEPES</td>
<td>N- (2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid)</td>
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<td>HSQC</td>
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<td>inositol 1,2-cyclic phosphate</td>
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<td>IPTG</td>
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<td>phosphatidylinositol phosphate</td>
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<tr>
<td>PIP₃</td>
<td>phosphatidylinositol diphosphate</td>
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PI-PLC  phosphatidylinositol-specific phospholipase C
ppm  parts per million
RT  room temperature
saPLC1  PI-PLC1 from *Streptomyces antibioticus*
SDS  sodium dodecyl sulfate
SDS-PAGE  SDS polyacrylamide gel electrophoresis
TLC  thin layer chromatography
Tris  Tris (hydroxyethyl) aminomethane
WT  wild type
Phosphate esters and anhydrides dominate the living world. Frank H. Westheimer had raised a question – “Why nature chose phosphate?” and subsequently offered quite insightful explanations in a journal article published in Science 1987. One of the explanations is that phosphoric acid is multivalent, it can form an anhydride and still carries a negative charge: this negative charge repels nucleophiles, so such anhydrides are kinetically stable. However, a biochemical system must not be so stable that it cannot be taken apart. In fact, enzymes can readily cleave a phosphate diester, which undergoes spontaneous hydrolysis at a negligible rate, with a rate enhancement by a factor of $10^9$ to $10^{12}$.

How do enzymes achieve such astonishing rate enhancement in catalysis? From the early “Lock and Key Theory” suggested by Fisher in 1894 to the “Transition State Theory” proposed by Haldene in 1930, and a later “Induce-Fit Theory” by Koshland. People have never ceased trying to comprehend how nature works and where the hidden “truth” lies. With so many biochemical techniques and methods available – cloning, mutagenesis, high field nucleus magnetic resonance (NMR), mass spectroscopy (MS), pre-steady state kinetics techniques, etc., nowadays, we are at a much high stand in
understanding enzymes, particularly in the field of structure – function relationships. However, as we learn so much, there is so much more to learn.

My dissertation work focused on the catalytic mechanism of a metal-independent phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* and a metal-dependent PI-PLC from *Streptomyces antibioticus*, seeking to understand the more complicated counterparts in mammalian systems. In this chapter, I will summarize the current knowledge of this enzyme family, their structures, functions, regulations, and catalytic mechanisms; I will also illustrate the rational for choosing the specific subjects in my research.

### 1.1 Introduction

Phosphatidylinositol-specific phospholipase Cs (PI-PLCs) are ubiquitous enzymes that catalyze the specific cleavage of the phosphodiester bond of phosphatidylinositols (PI, PIP, PIP2) to generate water-soluble inositol phosphates (InsP, InsP2, InsP3) and membrane-bound diacylglycerol (DAG) ([Figure 1.1](#)).

In higher eukaryotes, PI-PLCs are key enzymes in most receptor-mediated signal transduction pathways. They catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, inositol 1,4,5-triphosphate (InsP3) and DAG. InsP3 is released into the cytoplasm and results in influx of calcium ion from internal stores, whereas DAG remains membrane resident and stimulates protein kinase C isozymes. Mammalian PI-PLCs have been classified into four families based on their primary structure and mode of activation ([Figure 1.2](#)): the β-family (150 kDa) is activated by association with heterotrimeric G-protein subunits, γ-family (145 kDa) is
activated by association with tyrosine kinases, δ-family (85 kDa) is regulated by a GTP binding protein – G\(\text{h} \)\(\text{c} \) and ε-family (230-260 kDa) is associated with Ras.

The mammalian PI-PLCs are strictly dependent on Ca\(^{2+}\) and show a clear substrate preference in the order PIP\(_2\) > PIP>> PI. Furthermore, they generate both cyclic and acyclic inositol phosphate simultaneously. The ratio of cyclic to acyclic products depends on the isozyme class (β > δ > γ), substrate (PI, PIP, PIP\(_2\)), pH, and calcium concentrations\(^2\).

In prokaryotes, PI-PLCs are secreted proteins about 30-35 kDa. They play a role as virulence factors in pathogenic bacteria; however, in most cases their precise physiological functions remain elusive. These single-domain proteins are metal-independent and specially hydrolyze glycosylphosphatidylinostiol and PI but not PIP or PIP\(_2\). In contrast to mammalian PI-PLCs, bacterial PI-PLCs produce primarily cyclic inositol phosphate.

A distinguish type of prokaryotic PI-PLC (saPLC1) was recently discovered in one of the highest prokaryotes, *Streptomyces antibioticus*. Unlike other prokaryotic PI-PLCs, this 34-kDa enzyme is, in many aspects, similar to eukaryotic PI-PLCs. It shows sequence homology to the catalytic domain of eukaryotic PI-PLCs, uses Ca\(^{2+}\) as cofactor, and produces acyclic inositol from PI hydrolysis\(^3\).

1.2 Structural Comparison between Mammalian and Bacterial PI-PLC.

The X-ray crystal structures have been determined for PI-PLC from *Bacillus cereus* (bPLC) and rat PI-PLC-δ1 (PLC-δ)\(^4\)\(^6\). PLC-δ1 consists of four domains (PH domain, EF-hand domain, catalytic domain, and C2 domain), whereas bPLC folds to a single
domain structure similar to the catalytic domain of PLC-δ1 (Figure 1.3). On the basis of the overall topologies, both bPLC and the catalytic domain of PLC-δ1 belong to the large structural superfamily of (βα)₈-barrels (TIM-barrels). However, the TIM-barrel folds in both PI-PLCs are distorted rather than ideal. This might dictate to the steric requirements for the enzyme to dock on the phospholipid membrane and to allow the entry of phospholipid head groups into the active site or the release of product following catalysis.

When the structures of bPLC and the catalytic domain of PLC-δ1 were superimposed, an excellent fit was found for their N-terminal halves (X regions, which correspond to the residues 1 to 163 and residues 299 to 440 in bPLC and PLC-δ1, respectively). Despite of the low sequence identity (11%) between these two X-regions, the r.m.s. deviation for 104 equivalent Cα-positions is close to 1.8 Å. The catalytic residues are also located within this region. Particularly, there is a striking similarity of the spatial arrangement between catalytic histidine residues (His311/His356 in PLC-δ1 and His32/His82 in bPLC) and a positive moiety (Ca²⁺ in PLC-δ1 and the arginine side chain in bPLC). Unlike the N-terminal half, no apparent conservation is found for the C-terminal half (Y-region) of the TIM-barrel. It is interesting to note that in PLC-δ1 most contacts of the catalytic domain with the neighboring C2 domain are made by the surface helices of the Y-region. This buried interface counts for 9% of the total surface area of the catalytic domain.

The substrate specificity shown by bacterial and mammalian PI-PLC can be well explained using the three dimensional structures. First, the structures of both enzymes show that the 2- and 3-hydroxyl groups of the inositol ring make several hydrogen bonds
with the enzyme. This rationalizes the stringent requirement for 1D-configuration of 
myo-PI substrate by both enzymes. Second, PLC-δ1 specifically recognizes the 4- and 5-
phosphoryl groups of PIP₂ via positively charged amino acids (Lys438, Lys440 and 
Arg549); by contrast, bPLC specifically interacts 4- and 5-hydroxyl groups of inositol 
ring, which leaves no space to accommodate the bulky phosphate groups of InsP₃. Third,
neither enzyme possesses a hydrophobic channel for the specific interaction with acyl 
chains, which was observed in phospholipase A2, but rather a broad and shallow cleft 
extending from the active site pocket. This explains why PI-PLCs could easily 
accommodate different DAG stereoisomers, consequently lack of stereospecificity 
toward the DAG moiety.

*Bacillus cereus* PI-PLC (bPLC) differs from *Bacillus thuringiensis* PI-PLC (btPLC) 
only by 8 non-conserved amino acids⁸. If not indicated otherwise, the *Bacillus* enzyme 
in our studies refers to btPLC.

### 1.3 Mechanistic Comparison between Mammalian and Bacterial PI-PLC

Accumulated information⁴,⁶,⁹-²² on PI-PLCs from X-ray crystal structures 
complexes, stereochemical analyses, enzyme kinetics, and site-directed mutagenesis, led 
to a sequential catalytic mechanism that involves general base/acid catalysis. The 
reaction proceeds in two steps. A phosphotransfer step generates a stable cyclic 
phosphodiester intermediate. This is followed by a phosphohydrolase step that generates 
the acyclic inositol phosphate. The second step of this two-step mechanism is 
sufficiently slow for bacterial enzymes that the principal product of the reaction is the
cyclic intermediate itself; in contrast, the principal product of eukaryotic PI-PLCs is the acyclic inositol phosphate.23

Both enzymes utilize a general acid and general base mechanism to catalyze the hydrolysis of phosphoinositides (Figure 1.4). However, they use different types of residues to carry out the roles of general acid and base in catalysis. In the case of btPLC, Asp274-His32 dyad acts as the general base to deprotonate the 2-OH group of the inositol ring, which subsequent attacks the phosphate center; a novel catalytic triad involving Arg69-Asp33-His82 functions to lower the $pK_a$ of the 2-OH group of the inositol ring, stabilizes the negatively charged transition state via interaction with the pro-$S$ oxygen of the phosphate moiety, and protonates the DAG leaving group. In the second step of the reaction, the general acid and general base roles of the histidine residues are reversed.

Two active site histidine residues, namely His311 and His356 in PLC-δ1, are strictly conserved in all eukaryotic PI-PLCs. Based on the X-ray crystal structure of PLC-δ1 in complex with IP$_3$, it is seems that His356 functions similar to His82 of btPLC, which protonates the leaving DAG group in the first step of the reaction. However, the function of His311 is not clear. Mutation of His311 to leucine leads to a more than 1000-fold decrease in activity. This could be attributed to His311 acting as the general base (analogous to His32) or stabilizing the transition state (indicated by X-ray structures). The counterpart of Arg69 in PLC-δ1 is Ca$^{2+}$, which coordinates to the side-chain of Asn312, Asp343, Glu341, Glu390, as well as a water molecule and the 2-OH group of InsP$_3$. On the basis of the structural and mutagenesis analyses, Glu341 was suggested to be the general base that protons the 2-OH group of the inositol ring.10
However, whether a carboxyl group can accept a proton while coordinating to a metal ion is still under debating\textsuperscript{24,25}.

### 1.4. Perspectives

Due to the large size of mammalian PI-PLCs, bacterial PI-PLCs have been the molecule of choice in detailed mechanistic studies during the past decade. The accumulated knowledge has revealed distinguish catalytic differences between the metal-independent bacterial PI-PLCs and the Ca\textsuperscript{2+}-dependent eukaryotic PI-PLCs. One major difference that might have profound applications is: the bacterial PI-PLC that utilizes an arginine residue evolved to catalyze only the first step of the two-step reaction, whereas the eukaryotic PI-PLC that uses a Ca\textsuperscript{2+} cofactor evolved to catalyze a sequential two-step reaction (Figure 1.1).

In most mammalian PI-PLC catalyzed reactions, formation of small amounts of the cyclic phosphate accompanies generation of IP\textsubscript{3} can be detected in cells with vigorous phosphoinositides turnover\textsuperscript{2,26-29}. In contrast to IP\textsubscript{3}, however, this cyclic intermediate has not received much attention, presumably because it has been considered merely a by-product of the second-messenger generation and its cellular levels are usually too low to activate the IP\textsubscript{3} receptors. However, in certain cells, e.g., human breast cancer cell lines, cyclic phosphates are the major products from phosphoinositides cleavage\textsuperscript{26}. In addition, several of the putative inositol-containing second messengers, namely inositolphosphoglycans\textsuperscript{30} and prostaglandin-inositol conjugate\textsuperscript{31}, contain the 1,2-cyclic phosphate residue. These lead us to speculate that the functional significance of the
cyclic inositol phosphate could be much greater than what the current state of knowledge might have indicated.

Seeking to understand how the two-step reaction was controlled by mammalian PI-PLCs, I (i) engineered a metal dependent PI-PLC from a metal-independent bacterial PI-PLC and examined how an arginine to metal switch could possibly affect the catalytic mechanism of a metal-independent PI-PLC; (ii) performed mechanistic studies on the 34 kDa Ca\(^{2+}\)-dependent bacterial PI-PLC (saPLC1) – a model system of mammalian PI-PLCs.
Figure 1.1 The two-step reaction catalyzed by PI-PLCs. Metal-independent bacterial PI-PLCs evolved to catalyze the first step of the reaction (circled by the dash line), whereas metal dependent PI-PLCs evolved to catalyze the sequential two-step reaction.

$R' = PO_3^{2-}$ (PIP$_2$), $R' = H$ (PI, GPI)

$R'' =$ glucosaminoglycan (GPI), $R'' = H$ (PI, PIP$_2$)
Figure 1.2  Domain architectures of the four types of mammalian PLC isozymes in comparison to two bacterial PLC isozymes. The X and Y catalytic domain as well as the PH, EF-hand, C2, SH2, SH3, RasGEF, and RA domains are indicated.
Figure 1.3  Ribbon plots of the crystal structures of bacterial PI-PLC (left, pdb entry code 1PTG) and mammalian PI-PLC-δ1 (right, pdb entry code 1DJX). The view is into the active site pocket with Ins (color: grayish blue) bound to bacterial PI-PLC and InsP₃ (color: grayish blue) bound to the catalytic domain of mammalian PI-PLC-δ1. α-helices are shown in red, β-strands in green and loops in gray. The Ca²⁺ located in the active site of PI-PLC-δ1 is indicated by a blue sphere. This figure was prepared using WebLab ViewerPro.
Figure 1.4  Proposed general acid/general base catalytic mechanisms for btPLC and PLC-δ1. The identity of the general base (B') in PLC-δ1 is not yet clear. DAG, sn-1,2-diacylglycerol. At physiological condition, btPLC catalyzes only the first step of reaction (phosphotransferase step) and releases cyclic inositol phosphate as the final product, whereas PLC-δ1 produces IcP and inositol phosphate (IP) simultaneously, with IP as the principle product.
**Figure 1.5** A schematic diagram of the network of InsP₃/Ca²⁺ interactions in the active site of rat PI-PLCδ1. (Adapted from reference 5 with minor modification) Hydrogen bonds are shown by dashed lines.
**Figure 1.6** A schematic diagram of the network of inositol interactions in the active site of the bacterial PI-PLC from *Bacillus cereus*. (Adapted from reference 4 with minor modification) Hydrogen bonds are shown by dashed lines.
CHAPTER 2

STEREOSELECTIVITY OF AN ENGINEERED METAL DEPENDENT \textit{B. thuringiensis} PI-PLC (R69D) AND THE ROLE OF CALCIUM COFACTOR IN CATALYSIS


2.1 INTRODUCTION

Engineering of metal binding sites in proteins (reviewed in\textsuperscript{32-34}) has been used to address a variety of problems such as: protein-protein interactions and topology of transmembrane domains\textsuperscript{35,36}, regulation of enzyme activity and specificity\textsuperscript{37,38}, and modification of enzyme redox chemistry\textsuperscript{39}. Surprisingly, the interchange between positively charged amino acid side chains and metal ions was successfully achieved only in the metal to amino acid direction\textsuperscript{40}. To the best of our knowledge, there are no examples in the literature describing a catalytic metal site that would replace, completely or partially, the function of a lysine or an arginine side chain.

Both mammalian and bacterial PI-PLCs have been proposed to utilize the general acid and general base mechanisms to catalyze the hydrolysis of PI, yet their catalytic sites
are quite different, as shown in shown in Chapter 1 (Figure 1.5 and Figure 1.6). However, there is a striking similarity of the spatial arrangement between the calcium ion in rat PI-PLC-δ1 and the arginine side chain in *Bacillus cereus* PI-PLC (Figure 2.1)\(^{11,41,42}\). *Bacillus cereus* PI-PLC (bPI-PLC) differs from *Bacillus thuringiensis* PI-PLC (btPLC) only by 8 non-conserved amino acids\(^8\). If not indicated otherwise, the *Bacillus* enzyme in our studies refers to btPLC. Both structural information and the results from stereochemical and mutagenesis studies have indicated that these two similarly positioned positively charged moieties play similar catalytic roles: both stabilize the transition state of the phosphoryl transfer reaction *via* interaction with pro-\(S\) oxygen of the phosphodiester moiety, and facilitate deprotonation of the 2-OH of the inositol ring\(^{11,43-46}\). However, it is not clear whether, and how, the use of Ca\(^{2+}\) vs. Arg could be responsible for the differences in the substrate specificity and the product profile (ratio of the cyclic to acyclic product) between mammalian and bacterial enzymes. In order to address these questions, efforts from other laboratories have been devoted to engineering the Ca\(^{2+}\)-independent PI-PLC into a Ca\(^{2+}\)-dependent enzymes, and *vice versa*, but so far without success. Specifically, the double mutant bPI-PLC (R69D/K115E) displayed no detectable catalytic activity in either the absence or presence of Ca\(^{2+}\) although it was shown to be folded correctly\(^{42}\). The authors suggested that the Ca\(^{2+}\)-binding site in mPI-PLC probably has very stringent steric and electronic requirements\(^{42}\). Conversely, an attempt to eliminate the Ca\(^{2+}\)-dependence of mPI-PLC also failed as the calcium affinity was not mitigated upon mutating the negatively charged calcium binding residues (E390K, E343R, or E390K/E343R)\(^{47}\). It was suggested that the role of calcium is not
restricted to providing a positive charge but is also involved in sterically accelerating
catalysis via additional interactions with the transition state 47.

This chapter describes successful conversion of btPLC into a metal-dependent
enzyme by R69D mutation. The kinetic data demonstrate that the presence of Ca$^{2+}$
activates the catalytic activity of this mutant substantially, while the structural data
obtained by NMR spectroscopy indicate that presence of Ca$^{2+}$ is essential for restoring
the microenvironment of the catalytic site of the mutant to that attained by the wild type
(WT) enzyme. We also used $R_P$- and $S_P$-isomers of the phosphorothioate analogues of
phosphatidylinositol to probe the role of Ca$^{2+}$ in catalysis. The results of the
stereochemical study indicate that in the presence of Ca$^{2+}$ R69D is highly specific for the
$R_P$-isomer of DPPsI, and its $R_P/S_P$ stereoselectivity is 5-fold higher than the $R_P/S_P$ ratio of
the WT btPLC. In addition, this constructed Ca$^{2+}$ binding site was further characterized
by site-directed mutagenesis studies and kinetic analyses.

A note of clarity is appropriate for the readers of this chapter. This project was
initiated by Dr. Alexander V. Kravchuk, conducted under the effort of both of us, and
completed by me. This is noted because the results from the HSQC experiments and the
metal ion dependency studies for mutants R69D and D33N/R69D appeared in his
dissertation.
2.2 MATERIALS AND METHODS

Materials. DPPsI was synthesized as reported previously 44. PI from bovine liver and 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC) were purchased from Avanti Polar Lipids. L-α-[myo-inositol-2-3H(N)] Phosphatidylinositol ([3H]-PI) was purchased from Dupont NEN. 15NH4Cl (at 99% 15N) was from Isotech Inc. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. The E. coli strains XL1 Blue and BL21 (DE3) LysE used for gene manipulation and protein overexpression were from Stratagene. All DNA-modifying enzymes were from New England Biolabs. 99.9% atom D2O and "100%" D2O were purchased from Cambridge Isotope Laboratories.

Construction and Purification of Metal Dependent Variants. Mutations were introduced by the double-stranded, site-directed mutagenesis method (Stratagene). The mutagenic primers used are listed 5’ to 3’ with base substitutions underlined and codons of interest in italics. Only forward primer sequences are shown for each mutant oligonucleotide, and the mutation produced is in parentheses: 5’-CGC ATT TTT GAT ATC GAT GGA CGT TTA ACA GAT G-3’ (R69D); 5’-CGC ATT TTT GAT ATA GAG GGA CGT TTA ACA GAT G-3’ (R69E); 5’-CGC ATT TTT GAT ATC AAC GGA CGT TTA ACA GAT G-3’ (R69N); 5’-CGC ATT TTT GAT ATC GCA GGA CGA CGT TTA ACA G-3’ (R69A); 5’-CGC ATT TTT GAT ATC GGA GGA CGA CGT TTA ACA G-3’ (R69G); 5’-GGA GCT CGC ATT TTT AAT ATT GAT GGA CG-3’ (D67N/R69D); 5’- GGA GCT CGC ATT TTT GAA ATT GAT GGA CG-3’ (D67E/R69D); 5’- CCA ATT ATT ATG TCT TTA AAA AAA CAG TAT GAG GAT
ATG-3’ (E117Q); 5’-CAA TTC CAG GAA CAC ACC A4A GTG GGA CG-3’ (D33E). Mutant D33N/R69D was constructed by using the R69D primer on the existing plasmid carrying the mutant D33N gene. All mutations were verified by sequencing. Mutant R69C was constructed previously 45. All mutant proteins were purified as described previously 48,49, except that all but the final dialysis buffer included 1 mM EDTA in order to prevent metal contamination.

Activity Assay of PI-PLC with \(^3\mathrm{H}\)-PI Substrate. The specific activities of mutants were measured according to the procedure reported earlier 45 with minor modifications. \(^3\mathrm{H}\)-PI was mixed with unlabeled PI from bovine liver to obtain an overall PI concentration of 5 mM and a specific activity of ca. \(1.25 \times 10^6\) cpm/mol. In the divalent metal ion activation study, the reaction mixture contained 2 mM PI, 8 mM DHPC, 0-2 mM MeCl\(_2\), 1 mM EDTA, 40 mM HEPES, pH 7.5 (NaOH). In the monovalent metal ion activation study, the reaction mixture contained 2 mM PI, 8 mM DHPC, 0-50 mM MeCl, 40 mM HEPES, pH 7.5, where the pH of the buffer was adjusted by Tris instead of NaOH to avoid introduction of sodium ions. In the monovalent metal ion inhibition study, the mixture included 2 mM PI, 8 mM DHPC, 1 mM CaCl\(_2\), 0-50 mM MeCl, 40 mM HEPES, pH 7.5 (Tris Base). An aliquot of 20 \(\mu\)L of PI-PLC solution was added to the reaction mixture and incubated at 37 °C for 10 min. The concentrations of enzymes were adjusted so that the substrate conversion does not exceed 10-30%. The reaction was stopped by the addition of 0.5 mL CHCl\(_3\):CH\(_3\)OH:HCl (66:33:1). Phases were separated by a brief centrifugation and radioactivity of 50 \(\mu\)L of the aqueous phase
was measured by scintillation counting (Beckman). Enzymatic activity was expressed in 
µmol×min⁻¹×(mg of protein)⁻¹ or U/mg.

**Ca²⁺ Titration of R69D Monitored by NMR.** Uniformly ¹⁵N-labeled WT and 
R69D were expressed in M9 minimal media using ¹⁵NH₄Cl as a single nitrogen source 
and purified as described above. Two-dimensional ¹⁵N-¹H HSQC spectra of the 
uniformly ¹⁵N-labeled protein at Ca²⁺ concentration ranging from 0 to 5 mM were 
obtained on a Bruker DRX-800 spectrometer at 37 °C. Each sample contained 0.4 mM 
enzyme and 50 mM HEPES buffer in 90% H₂O/10% D₂O, pH 7.5.

**³¹P NMR Assays with DPPsI.** The assays were performed according to the 
procedure reported earlier ⁴⁵. ³¹P NMR spectra were recorded on a Bruker DRX-600 
spectrometer at 242.88 MHz. The reactions were carried out at 25 °C in 50 mM HEPES 
buffer (pH 7.5). A mixture of Sₚ- and Rₚ-isomers (~ 6:4 ratio) of DPPsI at a total 
concentration of 10 mM and a 4-fold excess of DHPC were dispersed as micelles in a 
bath sonicator. For the reaction carried out in the absence of Ca²⁺, 0.5 mM EDTA was 
added to chelate any contaminating metal. For the reaction carried out in the presence of 
Ca²⁺, 1.5 mM CaCl₂ and 0.5 mM EDTA was added. Reactions were initiated by adding 
an appropriate amount of the enzyme diluted in the reaction buffer, and a second aliquot 
of the enzyme was added after the reaction with the preferred (Rₚ) isomer had been 
completed. The rates of the reactions were calculated from the linear portions of the plot 
of the inositol 1,2-cyclic phosphate (IcP) concentration versus time.
2.3 RESULTS AND DISCUSSIONS

2.3.1 Construction of a Mutant Showing Activation by Ca$^{2+}$. The design of the Ca$^{2+}$ binding site was based on the similarity of the spatial arrangement between the calcium ion in mammalian PI-PLC-δ1 (mPI-PLC) and its counterpart arginine side chain in *Bacillus* PI-PLC (bPI-PLC). In addition, crystallographic structural data show that Arg69 of bPI-PLC is hydrogen bonded to three acidic residues (Asp33, Asp67, and Glu117) (Figure 2B), which are located in positions similar to those of Asn312, Glu341, Asp343, and Glu390 that form the coordination sphere of the calcium ion in mPI-PLC (Figure 2A)\textsuperscript{11,41,42}. Therefore, eliminating the positive charge of Arg69 while maintaining the negative charges of Asp33, Asp67, and Glu117 as shown in Figure 2C provides a rough blueprint for the future metal-binding site proposed in our studies.

Our approach was first to construct a number of mutants (R69D, R69E, R69G, R69A, R69C, and R69N) and screen them for activation by Ca$^{2+}$ under steady-state conditions, by monitoring the formation of IcP. **Table 2.1** shows the activities of WT and various mutants in the conversion of PI to IcP, in the absence and presence of Ca$^{2+}$ ions (0.1 mM and 1 mM). It is interesting that two mutants with carboxyl side chain substitutions at position 69, R69D and R69E, behave very differently. The R69E mutant is inactive (0.044 U/mg, compared to 3560 U/mg for WT enzyme) and is not activated by any divalent metal ions tested, including Ca$^{2+}$. R69D, on the other hand, is relatively active (0.29 U/mg), and can be further activated ca. 30-fold by Ca$^{2+}$ addition. Mutants R69G, R69A, R69C, and R69N were also constructed and their metal dependencies examined. None of these constructs was activated by Ca$^{2+}$. These initial results suggested that the
presence of an aspartate residue at position 69 creates a catalytic metal binding site. Thus, further studies were carried out and focused on this mutant.

It is important to indicate that all of the assays described in this paper deal with the conversion of PI to IcP (the phosphotransfer reaction), not the hydrolysis of IcP which occurs 1000 times slower. On the basis of $^{31}$P NMR analysis, we found that like WT bPI-PLC, IcP is also the only detectable product for R69D-Ca$^{2+}$ under our experimental conditions.

2.3.2 Metal Ion Specificity of the R69D Mutant. After showing the unique Ca$^{2+}$ activation property of R69D, we examined the metal ion specificity of this mutant. The activation of R69D by alkaline earth metal ions was studied first. This mutant was activated to different extents by Mg$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$ as shown in Figure 2.2A. The enhanced activity of R69D by these metal ions obeyed saturation kinetics with respect to the metal ion concentration. Another alkaline earth metal ion, Ba$^{2+}$, neither activated nor inhibited R69D bPI-PLC at concentrations up to 1 mM. The magnitude of R69D activation by different alkaline earth metal ions is summarized in Table 2.2. The maximal velocities of R69D complexes with Mg$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$ at saturating substrate concentration (2 mM) were $10.2 \pm 0.2$, $12.0 \pm 0.3$, and $2.7 \pm 0.3$ U/mg, which give a 35-fold, 41-fold, and 9-fold activation, respectively. The results described above can be considered successful for a de novo metal binding site. For comparison, mPI-PLC was activated by Ca$^{2+}$ at lower concentrations (apparent dissociation constant for calcium: $K_{d}^{app} = 1.3$ µM) but not by Mg$^{2+}$ at concentrations up to 5 mM. Further analyses of the data show that the extent of activation correlates roughly with $K_{d}^{app}$ of the metal ion:
Ca\textsuperscript{2+} and Mg\textsuperscript{2+} bind to the mutant well and provide good activation, Sr\textsuperscript{2+} is intermediate in these properties, while Ba\textsuperscript{2+} binds to the mutant but does not activate it (Table 2). In addition, the $K_d^{\text{app}}$ (and $K_i^{\text{app}}$) values correlate approximately with the ionic radii: Mg\textsuperscript{2+} (0.66 Å) < Ca\textsuperscript{2+} (0.99 Å) < Sr\textsuperscript{2+} (1.12 Å) < Ba\textsuperscript{2+} (1.35 Å) \textsuperscript{53} with the Ca\textsuperscript{2+} ion best fitting the binding site. Although the exact cause for the different catalytic effects of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} is not clear without structural information, we suspect that upon arginine to aspartate mutation at position 69, the ligand donor atoms may not readily form a well-defined octahedral geometry that favors Mg\textsuperscript{2+}. On the other hand, this coordination sphere may favor Ca\textsuperscript{2+}, which forms looser complexes of higher and variable coordination numbers, without directionality, and with variable bond lengths \textsuperscript{53}.

In contrast to the alkaline earth metal ions, Co\textsuperscript{2+}, Mn\textsuperscript{2+}, and Cd\textsuperscript{2+} provide different activity-versus-metal ion concentration profiles (Figure 2.2B). These transition metal ions increase the activity of the R69D mutant at low concentrations, reaching maxima at the concentrations of 25 μM, 50 μM, and 100 μM for Cd\textsuperscript{2+}, Mn\textsuperscript{2+}, and Co\textsuperscript{2+}, respectively. The maximal activation is less than 7-fold for Cd\textsuperscript{2+}, ca. 14-fold for Mn\textsuperscript{2+}, and 18-fold for Co\textsuperscript{2+}. Further increases in metal concentration inhibit the enzyme activity, although it stays above the background level even at 1 mM concentration for both Mn\textsuperscript{2+}, and Co\textsuperscript{2+}. The inhibitory effect of Cd\textsuperscript{2+} is stronger, and Zn\textsuperscript{2+} totally abolishes the activity of the enzyme at <1 mM concentration.

In addition, the effects of alkaline (monovalent) metal ions on the activity of R69D were tested. Na\textsuperscript{+}, K\textsuperscript{+}, and Cs\textsuperscript{+} showed no activation, whereas Li\textsuperscript{+} gave a 5-fold activation at saturating concentrations. None of these monovalent metal ions inhibits the Ca\textsuperscript{2+}-induced activity of R69D. Therefore, the sodium ions in the assay system, which were
introduced through EDTA and HEPES buffer, should not contribute to the activation and inhibition effects from divalent metal ions described above. Since the activation from Li$^+$ is mild and is not likely due to any specific binding to the active site of R69D, the exact cause for this activation was not further pursued.

As a control, the activity of WT bPI-PLC was also examined in the presence of various metal ions. Li$^+$, Na$^+$, K$^+$, Cs$^+$, Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, and Mn$^{2+}$ all showed slight activation effects ($\leq 30\%$), whereas Co$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ brought about severe inhibition. The mechanism of the slight activation effect of some metal ions is not likely due to any specific binding to the WT enzyme as indicated by the $^{15}$N-$^1$H HSQC experiments (described in a later section). The mechanism of the inhibitory effect of some other divalent ions, which has been observed previously$^{54}$, could be due to the non-specific binding of the ions at or near the active site. Therefore, the observed activation of R69D bPI-PLC by various metal ions is a specific property of this mutant, most likely resulting from the metal ion binding at the active site.

2.3.3 Structural Evidence for the Catalytic Metal Site in R69D. Heteronuclear NMR spectroscopy was used to examine the possible changes introduced by R69D mutation. We found that the mutation alters signal positions and intensities in both the backbone amide region and arginine side chain ($\epsilon$NH) region of the $^{15}$N-$^1$H HSQC spectra. Although the changes in the backbone amide region cannot be rationalized without a complete NMR assignment, the changes in the arginine region are informative for the following reasons: (i) The WT enzyme (35 kDa) possesses 9 arginine residues, each giving a separate signal in the arginine region of the $^{15}$N-$^1$H HSQC spectrum. (ii)
Three arginine signals (Arg69, Arg71, and Arg163) have been assigned based on the comparison between the spectrum of WT and those of the corresponding alanine mutants (unpublished results). (iii) Arg71 and Arg163 are adjacent to the active site, while other arginine residues are distant from the active site.

The comparison between the $^{15}$N-$^1$H HSQC spectra of WT and R69D in their arginine side chains ($^{8}$NH) region (Figure 2.3A and Figure 2.3B) shows that the signals of Arg69 and Arg71 disappeared, while the signal of Arg163 is shifted slightly upfield in the R69D mutant. In the absence of global conformational differences between WT and R69D, as indicated by 2-D NOESY spectra (data not shown), these signal changes/disappearances should be expected for those residues that are either mutated or adjacent to the mutation site. Upon Ca$^{2+}$ titration of the R69D mutant (Figure 4C and Figure 4D), the signal from Arg71 gradually reappears at the same position as WT, while the signal of Arg163 shifts back to its “original” position. As a control, the WT enzyme was also titrated with Ca$^{2+}$ while the $^{15}$N-$^1$H HSQC spectra were monitored. No noticeable change was observed in either the backbone amide region or arginine region of the $^{15}$N-$^1$H HSQC spectrum (data not shown). These results suggest that the R69D mutation triggers local conformational changes and/or perturbed dynamic properties at or near the active site. In addition, binding of Ca$^{2+}$ to R69D restores the environment of the catalytic site analogous to that attained by the WT enzyme. Taken together, these results strongly support the hypothesis that Ca$^{2+}$ binds to the active site of the R69D mutant, occupies the position of the Arg69 side chain in the WT enzyme, and thereby activates R69D.
2.3.4 Stereochemical Evidence for the Catalytic Metal Site. As has been shown earlier \(^45,48,49\), Arg69 is the key residue responsible for the WT’s unusually large thio effect toward \(S_p\)-DPPsI \((k_O/k_{Sp} = 3.1 \times 10^5)\) and very high stereoselectivity \((k_{Rp}/k_{Sp} = 7600)\). Results from these studies not only provided evidence for a direct interaction between the guanidinium group of Arg69 and \(pro-S\) oxygen of the substrate, but also suggested that the bidentate interactions of Arg69 during the transition state (hydrogen bonds to both \(2-OH\) and \(pro-S\) oxygen of the substrate) could be responsible for these observations. In this previous study, a systematic analysis of the origin of the unusually high nonbridging thio effect was conducted through a site-directed chemical modification method – the arginine residue at position 69 was replace by a cysteine residue and then chemically attached to acetamidine (AA), thioacetamidine (TAA), propylamine (PA), and ethylamine (EA). The mutant enzymes featuring bidentate side chains (R69C-AA and R69C-TAA) at position 69 display significantly higher activity, higher nonbridging thio effect, and greater stereoselectivity than do those with monodentate side chains (R69C-PA, and R69C-EA) (Table 2.3).

The magnitude of the \(S_p\)-thio effect was expected to be decreased in the R69D mutant resulting in a relaxed \(R_p/S_p\) stereoselectivity, as has been demonstrated by other Arg69 mutants (e.g., R69K, R69A, R69C-PA, and R69C-EA) \(^45\). If \(Ca^{2+}\) indeed substitutes functionally for the side chain of Arg69, as has been demonstrated in the conservative R69 mutants (R69C-AA and R69C-TAA) \(^45\), the presence of \(Ca^{2+}\) should restore some of the stereoselectivity lost in R69D. In order to test this hypothesis, we determined the stereoselectivity of R69D in the presence and absence of \(Ca^{2+}\) by \(^{31}P\)
NMR (Figure 2.4). The data, along with those from previous studies \(^{45}\), are also summarized in Table 2.3.

The results in Table 3 raise several important points. (i) In the absence of Ca\(^{2+}\), R69D demonstrated a \(S_p\)-thio effect \((k_O/k_{S_p})\) of \(7.8 \times 10^2\), which is ca. 3 orders of magnitude lower than that of WT \((k_O/k_{S_p} = 3.1 \times 10^5\). Upon Ca\(^{2+}\) activation, the \(S_p\)-thio effect \((k_O/k_{S_p} = 4.4 \times 10^5\) was restored to the WT-level. These results suggest that the bound Ca\(^{2+}\) is likely to mimic the bidentate nature of Arg69 and is responsible for the very large \(S_p\)-thio effect. (ii) In the absence of Ca\(^{2+}\), the \(R_P\)-thio effect \((k_O/k_{R_p}) = 0.6\) of R69D is close to unity; this \(R_P\)-thio effect increases to 9.4 upon Ca\(^{2+}\) addition. The latter value is modestly lower than that of WT \((k_O/k_{R_p} = 42\). Together with the structural data obtained in the previous section, we postulate that the small \(R_P\)-thio effect demonstrated by R69D originates from increased flexibility in the active site, which allows the enzyme to accommodate a sulfur atom at the \(pro-R\) position. Binding of Ca\(^{2+}\) to R69D likely restores the network of active site interactions, causing this mutant to more closely resemble WT enzyme structurally. This would explain why the \(R_P\)-thio effect for R69D increases upon Ca\(^{2+}\) activation. (iii) The observed stereoselectivity \((k_{R_p}/k_{S_p})\) can be dissected into the ratio of the thio effects for both \(R_P\) and \(S_p\)-isomers, \((k_O/k_{S_p})/(k_O/k_{R_p})\). In the presence of Ca\(^{2+}\), the R69D mutant (R69D-Ca\(^{2+}\)) shows a similar \(S_p\)-thio effect but a decreased \(R_P\)-thio effect, as compared to the WT enzyme. Consequently, R69D-Ca\(^{2+}\) displays higher stereoselectivity \((k_{R_p}/k_{S_p} = 47,000\) than WT \((k_{R_p}/k_{S_p} = 7,600\). However, this increased stereoselectivity does not originate from a more stringent discrimination of Ca\(^{2+}\) against the sulfur atom (in forming the coordination sphere) compared to that of arginine side chain (in forming hydrogen bonding interaction). Rather, this increase in
stereoselectivity is derived from the greater flexibility of R69D-Ca\(^{2+}\) in accommodating a sulfur atom at the pro-\(R\) position of the substrate thus resulting in a relatively small \(R\)-thio effect.

**2.3.5 Further Characterization of the Metal Ion Binding Site Using Multiple Mutants.** Since the results in the two preceding sections suggest that the Ca\(^{2+}\) ion should bind to R69D bPI-PLC at the position occupied by the guanidinium group in the WT enzyme, its coordination sphere is expected to include the side chains of Asp33, Asp67, and Glu117 [i.e. the same residues which are hydrogen-bonded to the Arg69 side chain in the WT enzyme\(^{41,55,56}\)] as well as the newly introduced Asp69 (Figure 2.1C). To verify the participation of these amino acids in the metal coordination, all three carboxylic acid residues potentially participating in metal ion coordination were mutated (one at a time) to create the following double mutants: D33N/R69D, D33E/R69D, D67N/R69D, D67E/R69D, R69D/E117Q, and a triple mutant D33N/D67E/R69D.

As shown in Table 1, in the absence of metal ions the activities of the three double mutants D67N/R69D, D67E/R69D, and R69D/E117Q are of the same magnitude as of R69D. However, none of the three can be activated by Ca\(^{2+}\). These results suggest that Asp67 and Glu117 are involved in the binding of Ca\(^{2+}\).

Asp33, which forms a hydrogen bond with Arg69 in the WT enzyme, should also be involved in Ca\(^{2+}\) binding in R69D on the basis of the following results. The activities of all three mutants involving this residue, D33N/R69D, D33E/R69D, and D33N/D67E/R69D, are all lower than that of the R69D single mutant by two orders of magnitude. Noticeably, the D33N/R69D mutant is activated by 1 mM Ca\(^{2+}\) ca. 50-fold.
This prompted us to further examine the behavior of this mutant. As shown in Figure 2.6, its activation by Mg\(^{2+}\) and Ca\(^{2+}\) has a somewhat sigmoidal form and the maximal activations are 560-fold and 150-fold, respectively. Furthermore, the apparent dissociation constant of Mg\(^{2+}\) is almost 3-fold lower than that of Ca\(^{2+}\) as given in the legend of Figure 5. This modest preference of Mg\(^{2+}\) over Ca\(^{2+}\) is most likely due to the fact that Ca\(^{2+}\) favors ligands without nitrogen donors, while Mg\(^{2+}\) is more flexible in this regard. This discrimination comes at a price of lower maximal activity (1.35 U/mg vs. 10.2 U/mg with Mg\(^{2+}\) and 0.37 U/mg vs. 11.9 U/mg with Ca\(^{2+}\)) and lower affinity toward metal ions (0.73 mM vs. 0.18 mM for Mg\(^{2+}\) and 1.9 mM vs. 0.16 mM for Ca\(^{2+}\)) for this mutant in comparison to R69D.

The analyses presented above provide experimental support for the hypothesis that metal coordination at the active site involves the side chains from Asp33, Asp67, Asp69, and Glu117. On the basis of the results in the preceding section, the pro-\(S\) oxygen of the phosphate group and possibly the 2-OH group from the substrate should further complement the coordination sphere.

2.3.6 Product Profiles of R69D-Ca\(^{2+}\). One of the goals of this study has been to find out how Ca\(^{2+}\) vs Arg could be responsible for the differences in the product profile between mammalian and bacterial enzymes. The product profiles of R69D in the presence and absence of Ca\(^{2+}\) were examined via \(^{31}\)P-NMR. It was found that substitution of a catalytic calcium ion at the active site created by the R69D mutation is not sufficient to alter the product profile similar to that of mammalian enzymes (spectra not shown). This hints that some other factors besides Ca\(^{2+}\) in the active site of
mammalian PI-PLCs might also contribute to the slow release of cyclic inositol phosphate from the active site. Alternatively, the reduced IcP off-rate ($k_{off}$) due to the Ca$^{2+}$ substitution might still be several orders of magnitude higher than the reduced $k_2$ (IcP hydrolysis rate).

### 2.4 CONCLUSION

The results described here represent the first example of successful engineering of a catalytically functional metal binding site of an enzyme based on mimicry of a positively charged amino acid side chain (arginine) by a divalent metal-binding carboxylate residue. Knowledge of the precise positioning of active site residues and the reaction mechanisms involved were the key factors facilitating our design. The engineered enzyme is characterized by strong metal ion binding and high $R_P/S_P$ stereoselectivity, reminiscent of other Ca$^{2+}$-dependent PI-PLCs. Based on the results obtained, it is concluded that the functional switch of Arg69 into Asp-Ca$^{2+}$ has been achieved. Further investigation of this engineered metal-dependent bPI-PLC in the context of other naturally occurring calcium-dependent PI-PLCs could provide insight into how enzymes have evolved from metal-independence to metal-dependence, as well as how the metal binding site is fine-tuned for optimal specificity and catalytic function through the evolutionary process.
### Table 2.1 Activation of Various btPLC Mutants by Ca\textsuperscript{2+}.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (µmol min\textsuperscript{-1} mg\textsuperscript{-1})</th>
<th>Specific Activity (µmol min\textsuperscript{-1} mg\textsuperscript{-1})</th>
<th>Specific Activity (µmol min\textsuperscript{-1} mg\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 mM [Ca\textsuperscript{2+}]\textsubscript{free}</td>
<td>1 mM [Ca\textsuperscript{2+}]\textsubscript{free}</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>3560 ± 80</td>
<td>3450 ± 98</td>
<td>4240 ± 13</td>
</tr>
<tr>
<td>R69D</td>
<td>0.293 ± 0.009</td>
<td>4.18 ± 0.20</td>
<td>8.67 ± 0.01</td>
</tr>
<tr>
<td>R69E</td>
<td>0.044 ± 0.001</td>
<td>0.047 ± 0.001</td>
<td>0.047 ± 0.000</td>
</tr>
<tr>
<td>R69G</td>
<td>0.023 ± 0.0004</td>
<td>0.021 ± 0.001</td>
<td>0.021 ± 0.001</td>
</tr>
<tr>
<td>R69A</td>
<td>0.068 ± 0.0031</td>
<td>0.064 ± 0.001</td>
<td>0.065 ± 0.001</td>
</tr>
<tr>
<td>R69C</td>
<td>0.168 ± 0.0087</td>
<td>0.149 ± 0.003</td>
<td>0.142 ± 0.008</td>
</tr>
<tr>
<td>R69N</td>
<td>0.126 ± 0.000</td>
<td>0.116 ± 0.002</td>
<td>0.113 ± 0.010</td>
</tr>
<tr>
<td>D33N/R69D</td>
<td>0.0024 ± 0.0002</td>
<td>0.0029 ± 0.0001</td>
<td>0.107 ± 0.002</td>
</tr>
<tr>
<td>D33E/R69D</td>
<td>0.0052 ± 0.0003</td>
<td>0.0049 ± 0.0002</td>
<td>0.0054 ± 0.0003</td>
</tr>
<tr>
<td>D67N/R69D</td>
<td>0.305 ± 0.001</td>
<td>0.289 ± 0.005</td>
<td>0.260 ± 0.002</td>
</tr>
<tr>
<td>D67E/R69D</td>
<td>0.300 ± 0.010</td>
<td>0.278 ± 0.010</td>
<td>0.349 ± 0.011</td>
</tr>
<tr>
<td>R69D/E117Q</td>
<td>0.414 ± 0.004</td>
<td>0.423 ± 0.004</td>
<td>0.367 ± 0.005</td>
</tr>
<tr>
<td>D33N/D67E/R69D</td>
<td>0.0020 ± 0.0001</td>
<td>0.0019 ± 0.0001</td>
<td>0.0019 ± 0.0001</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Measured at 37 °C, 1 mM EDTA, 2.0 mM PI, and 8 mM DHPC in 40 mM HEPES buffer (pH 7.5).
<table>
<thead>
<tr>
<th>Metal ion</th>
<th>$V_{\text{max}}$ (µmole min$^{-1}$ mg$^{-1}$)</th>
<th>$K_d^{\text{app}}$ of M$^{2+}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>0.29 ± 0.01</td>
<td>NA$^b$</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>10.19 ± 0.19</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>11.93 ± 0.33</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Sr$^{2+}$</td>
<td>2.67 ± 0.28</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>0.30 ± 0.02</td>
<td>0.64 ± 0.11 $^b$</td>
</tr>
</tbody>
</table>

$^a$Measured at 37 °C, 1 mM EDTA, 0-1 mM free metal ion, 2.0 mM PI, and 8 mM DHPC in 40 mM HEPES buffer (pH 7.5). $^b$The $K_i^{\text{app}}$ value for Ba$^{2+}$ is extrapolated from Lineweaver-Burk plot using Ba$^{2+}$ as an inhibitor of Ca$^{2+}$. $K_d^{\text{app}}$ is calculated by using the equation: $K_d^{\text{app}} = K_d^{\text{app}} (1 + [I_0]/K_i^{\text{app}})$, where $K_d^{\text{app}}$ is the $K_d^{\text{app}}$ in presence of 0.5 or 1.0 mM Ba$^{2+}([I_0])$.

Table 2.2 Kinetic Parameters of R69D btPLC with Alkaline Earth Metal Ions. $^a$
<table>
<thead>
<tr>
<th>Enz.</th>
<th>Functional Group</th>
<th>$k_O^{c}$</th>
<th>$k_Rp^{c}$</th>
<th>$k_O/k_Rp$</th>
<th>$k_O/k_{Sp}$</th>
<th>$k_{Rp}/k_{Sp}$</th>
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<tr>
<td>WT$^d$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>2200</td>
<td>53</td>
<td>42</td>
<td>0.007</td>
<td>$3.1 \times 10^5$</td>
</tr>
<tr>
<td>R69D</td>
<td><img src="image" alt="Functional Group" /></td>
<td>0.29</td>
<td>0.48</td>
<td>0.6</td>
<td>0.00037</td>
<td>$7.8 \times 10^2$</td>
</tr>
<tr>
<td>R69D-$\text{Ca}^{2+}$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>12</td>
<td>1.3</td>
<td>9.4</td>
<td>0.000027</td>
<td>$4.4 \times 10^5$</td>
</tr>
<tr>
<td>R69C-$\text{AA}^d$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>160</td>
<td>4.0</td>
<td>40</td>
<td>0.001</td>
<td>$1.6 \times 10^5$</td>
</tr>
<tr>
<td>R69C-$\text{TAA}^d$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>50</td>
<td>1.3</td>
<td>38</td>
<td>0.0004</td>
<td>$1.2 \times 10^5$</td>
</tr>
<tr>
<td>R69C-$\text{PA}^d$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>1.5</td>
<td>0.043</td>
<td>35</td>
<td>0.0038</td>
<td>$3.9 \times 10^2$</td>
</tr>
<tr>
<td>R69C-$\text{EA}^d$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>0.50</td>
<td>0.014</td>
<td>36</td>
<td>0.0046</td>
<td>$1.1 \times 10^2$</td>
</tr>
<tr>
<td>R69K$^d$</td>
<td><img src="image" alt="Functional Group" /></td>
<td>0.1</td>
<td>0.0029</td>
<td>34</td>
<td>0.003</td>
<td>$2.7 \times 10$</td>
</tr>
</tbody>
</table>

$^a$Measured by radioactive assay. $^b$Measured by $^{31}$P-NMR. $^c$In µmole min$^{-1}$ mg$^{-1}$. $^d$R69C-AA, R69C-TAA, R69C-PA, and R69C-EA are chemically modified R69C mutants$^{45}$. AA, TAA, PA, and EA stand for the covalently linked functional groups: acetamidine, thioacetamidine, propylamine, and ethylamine, respectively.

**Table 2.3** Specific Activity, Thio Effects, and Stereoselectivities of WT and R69D btPLC.
Figure 2.1 A side-by-side comparison of the active sites (stereo view) of the mammalian PI-PLC-δ1 (A), WT Bacillus PI-PLC (B), and the R69D mutant Bacillus PI-PLC (C). A, B, and C are adapted from PDB files 1DJX, 1PTG, and 1PTG, respectively; and prepared with the WebLab ViewerPro. The program SwissModel was used to construct the model structure for R69D shown in (C). Panel A shows two conservative histidine residues and other side-chains that form the calcium coordination sphere, and the bound inositol triphosphate within the active site. Panel B shows Arg69, His32, His82, side-chains of residues (Asp33, Asp67, and Glu117) that form hydrogen-bonding interactions with Arg69, and the bound inositol within the active site of the WT bacterial PI-PLC. Panel C represents the active site of the mutant R69D enzyme, constructed by SwissModel with energy minimization. Ins, inositol. InsP₃, D-myo-inositol 1,4,5-triphosphate.
Figure 2.1
Figure 2.2  (A) Activation of R69D btPLC by alkaline earth metal ions: calcium (○), magnesium (●), strontium (▼), and barium (V). All reactions included 0.5-1.0 µg R69D mutant in 40 mM HEPEPS, 1 mM EDTA, 2 mM PI, 8 mM DHPC, 0-1 mM free metal ion, pH 7.0 for 10 min at 37 °C. Solid lines represent non-linear fit of each data set to the Michaelis-Menten equation, \( V = V_{\text{max}} \frac{[M^{2+}]}{K_{d}^{\text{app}} + [M^{2+}]} \), and the parameters are summarized in Table 2. The activity of R69D in the absence of metal ions (0.29 U/mg) was deducted prior to plotting. (B) Activation of R69D btPLC by transition metal ions: cobalt II (○), manganese II (●), cadmium II (V), and zinc II (▼).
Figure 2.2
Figure 2.3 Arginine side chain (εNH) region of $^{15}$N-$^1$H HSQC spectra of uniformly $^{15}$N-labeled btPLC: (A) WT; (B) R69D; (C) R69D in the presence of 1 mM CaCl$_2$; (D) R69D in the presence of 5 mM CaCl$_2$. Horizontal arrows indicate the position of the signal from Arg71, vertical arrows indicate the position of the signal from Arg163, and the open circle with dashed line represents the missing signal from Arg69 in R69D.
Figure 2.3
Figure 2.4 $^{31}$P NMR assay of DPPsI hydrolysis catalyzed by the R69D mutant btPLC. (A) and (B) were $R_p$-DPPsI hydrolysates by R69D in the presence of $\text{Ca}^{2+}$ and in the presence of $\text{Ca}^{2+}$, respectively. The enzyme used was 65 µg in (A) and 6.5 µg in (B). (C) and (D) were $S_p$-DPPsI hydrolysates by R69D in the presence of $\text{Ca}^{2+}$ and in the presence of $\text{Ca}^{2+}$, respectively. The enzyme used was 2.9 mg in (C) and 0.98 mg in (D). Spectra were taken at 35 min intervals. The fifth spectrum in (C) was taken after 14 hr and 25 min incubation. The fifth spectrum in (D) was taken after 18 hr incubation with additional 1.96 mg R69D.
Figure 2.4
Figure 2.5 Activation of D33N/R69D btPLC by magnesium (●) and calcium (○). All reactions included 12-26 µg enzyme in 40 mM HEPEPS, 1 mM EDTA, 2 mM PI, 8 mM DHPC, 0-1 mM free metal ions, pH 7.0 for 10 min at 37 °C. Solid lines represent non-linear fit of each data set to the Hill equation, $V = V_{\text{max}} [M^{2+}]^n / ([K_d^{\text{app}}]^n + [M^{2+}]^n)$, and the parameters are summarized as follows: Mg$^{2+}$: $V_{\text{max}} = 1.35 \pm 0.05$ U/mg, $K_d^{\text{app}} = 0.73 \pm 0.04$ mM, n=1.5; Ca$^{2+}$: $V_{\text{max}} = 0.37 \pm 0.01$ U/mg, $K_d^{\text{app}} = 1.9 \pm 0.1$ mM, n=1.
CHAPTER 3

CLONING, EXPRESSION, PURIFICATION, AND A GENERAL CHARACTERIZATION OF PHOSPHOTIDYLINOSITOL PHOSPHOLIPASE C FROM \textit{STREPTOMYCES ANITBIOTICUS}


3.1 INTRODUCTION

Recently, a novel Ca\textsuperscript{2+}-dependent prokaryotic PI-PLC was identified from \textit{Streptomyces antibioticus} (saPLC1) \textsuperscript{3}. Similar to other prokaryotic PI-PLCs, saPLC1 is small in size (35 kDa) and uses PI but not multiply phosphorylated PI as the substrate \textsuperscript{3}. On the other hand, it also resembles eukaryotic PI-PLCs, because it is Ca\textsuperscript{2+} dependent, shows sequence homology to eukaryotic but not Ca\textsuperscript{2+}-independent prokaryotic PI-PLCs, and produces IP as the principle product from PI hydrolysis \textsuperscript{3}.

No sequence homology between saPLC1 and other bacterial PI-PLCs could be revealed by sequence alignment studies. In contrast, alignment of the sequence of saPLC1 with that of rat PI-PLC\textsuperscript{δ1} (\textbf{Figure 3.1}), for which there is a crystal structure (PDB entry code 1DIJ), suggests that saPLC1 contains most of the key catalytic residues
responsible for PI hydrolysis. His16 and His55 aligned with His311 and His356, which are the proposed general acid/base. Asn17, Glu39, and Asp41 aligned with Asn312, Glu341, and Asp343, which are responsible for Ca\(^{2+}\) binding as well as potential nucleophiles. In addition, the alignment reveals that saPLC1 lacks a conservative basic amino acid motif ((K/R) xxxKxK (K/R)) that is responsible for the binding of 4- and 5-phosphoryl groups of PIP\(_2\), which is highly conserved in eukaryotic PI-PLCs. This explains why saPLC1 hydrolyze PI but not PIP or PIP\(_2\).

As discussed in the previous chapter, although the functional switch of Arg69 to Asp69-Ca\(^{2+}\) has been successfully achieved, this switch was not able to convert a metal-independent PI-PLC into a mammalian like enzyme in the sense of the product profiles. It appears to us that some other factors might play a major role in evolving the “short circuit” one-step catalytic mechanism (metal-independent PI-PLCs) into the two-step sequential catalytic mechanism (natural occurring metal-dependent PI-PLCs). Since saPLC1 owns the same substrate specificity as btPLC, we believe that a systematic and quantitative comparison between these two enzymes will provide further insights into the catalytic differences between metal-independent and metal-dependent PI-PLC. Furthermore, the study on saPLC1 is likely to shine light on the problems arose in Chapter 2.

The cloning, expression, purification, and a general characterization of saPLC1 are described in this chapter. Note that the crystallization of native and Se-Met labeled saPLC1 was obtained by Dr. Thomas L. Selby at University of Central Florida.
3.2 MATERIALS AND METHODS

**Materials.** Phosphatidylinositol (PI) from bovine liver was purchased from Avanti Polar Lipids. L-α-[myo-inositol-2-³H(N)]-Phosphatidylinositol ([³H]-PI) was purchased from Dupont NEN. Oligonucleotides were purchased from Integrated DNA Technologies. The *E. coli* strains (XL-1 Blue) containing pE602 vectors comprising saPLC1 gene, was generous gifts from Dr. Yugo Iwasaki (Nagoya University, Japan.). The *E. coli* strains XL1 Blue and BL21(DE3)pLysS used for gene manipulation and protein overexpression were purchased from Stratagene. All DNA-modifying enzymes were from New England Biolabs (NEB). 99.9% atom D₂O and "100%" D₂O were purchased from Cambridge Isotope Laboratories. All other reagents were purchased from Sigma Chemical, Aldrich, Fisher, or Research Organics unless otherwise specified.

**Cloning of saPLC1.** As shown in *Figure 3.2*, the plasmid pE602, containing a *Streptomyces antibioticus* genomic fragment, was amplified with the primers, 5’–GGA ATT CCA TAT GGA GCC GGC CGC CAC GAC GTA C–3’ (*NdeI* site underlined) and 5’–CCC AAG CTT TCA TGC CCC GCG CGG CAC CAC–3’ (*HindIII* site underlined). The primers contain additional nucleotides at their 5’ends to ensure optimal digestion. The polymerase chain reaction (PCR) reaction mixture (50 µL) contained 120 ng pE602, 1 µM each primer, 200 µM dNTP’s (50 µM each), 1% DMSO, 1 × Native *pfu* buffer, and 2.5 U Native *pfu* DNA Polymerase. 25 thermal cycles were run as follows: 95°C for 45 seconds, 55°C for 45 seconds, 72°C for 2 minutes. The resulting ~1000 bp PCR product was purified with a PCR purification kit (QIAgen) and then digested simultaneously with
20 and 10 U respectively of Ndel and HindIII for 3 hours at 37°C in NEB buffer 2. This digested product was purified from a 1% agarose gel using QIAquick Gel Extraction Kit (QIAGen). Similarly, the pET28b was digested, dephosphorylated (by CIP at 37°C for 1 hour), and purified. Subsequently, this DNA fragment was ligated into the vector in the following reaction mixture (20 µL): 100 ng digested vector, 200 ng digested insert, 1000 U T4 DNA ligase, 1 × T4 DNA ligase buffer, 10 mM DTT. This reaction was incubated at 16°C for 23 hours and quenched by incubating at 70°C for 10 minutes. This ligation product (3 µL) was used to transform XL-Blue chemical competent cells. 3 colonies were picked, screened by digestion, and two were sequenced. The final plasmid, sequenced with an ABI PRISM sequencing kit (Perkin Elmer), was named pET28b-saPLC1. The (His)₆-tag at the N-terminal end of the recombinant saPLC1 is listed in Figure 3.3. The cDNA of the mature saPLC1 with the code amino acid sequence are shown in Figure 3.4.

**Expression and Purification of Recombinant saPLC1.** *E. coli* BL21(DE3)pLysS cells harboring pET28b-saPLC1 plasmid were grown at 37 °C in 4 liter of LB media containing 50 mg/L kanamycin. When the OD₆₀₀ of the culture reached 0.8, IPTG was added to a final concentration of 1 mM. Expression continued for additional 6-7 h at room temperature. Cells were collected by centrifugation and stored at −20 °C, which weakened the cell membranes in preparation for extraction. The frozen cell pellet (~12 g from 4 L culture) was thawed and resuspended in 50 mL of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The suspension was subject to
four cycles of 45-sec pulse sonication on ice, and the supernatant was separated from cell
debris by centrifugation (18,000 rpm for 1 h). The supernatant was incubated with 15
mL packed volume of Ni-NTA agarose (Qiagen), which was pre-equilibrated with lysis
buffer, for 3 hours at 4 °C to ensure complete mixing. The column was washed with 10-
column of lysis buffer, followed by washing with 5-column of wash buffer (50 mM
NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0), then eluted with 20-250 mM
imidazole gradient. Fractions containing saPI-PLC1 were collected and further purified
by S-100 gel filtration column (Pharmacia) in 20 mM Tris-HCl, 0.5 mM EDTA, pH 8.5.
The fractions contained the pure saPLC1, judged by SDS-PAGE followed by silver
staining, were combined and concentrated to ~10 mg/mL. The concentrated protein
solution was first dialyzed at 4 °C for 8 h in 4 L of 10 mM HEPES, pH 7.0, then 8 h
against 4 L of 1 mM HEPES, pH 7.0 twice. The samples then were lyophilized and
stored at –20 °C until further uses.

To produce and purify selenomethionine-derivatized saPLC1, pET28b-saPLC1 was
transformed into methionine auxotroph B834(DE3)pLysS cells (Novagen). Transformed
bacteria were grown in defined LeMaster media⁵⁷ containing 50 mg/L kanamycin
supplemented with 50 mg/L selenomethionine. When the OD₆₀₀ of the culture reached
0.6, IPTG was added to a final concentration of 1 mM. The purification procedure is the
same as native saPLC1 except that Tris(2-carboxyethyl) phosphine, hydrochloride was
used in the final dialysis buffer to prevent oxidation of selenomethionine.

**Activity Assay of saPLC1 with [³H]-PI Substrate.** The activities of the enzymes
were measured according to the procedure reported earlier ¹⁵ with minor modifications.
[^3]H]-PI was mixed with unlabeled PI from bovine liver to obtain an overall PI concentration of 5 mM and a specific activity of ca. $1.25 \times 10^6$ cpm/mol, and detergent was added to a desired concentration. The reaction mixture contains 40 µL of this substrate solution, which was diluted with distilled water into different concentration (0-5 mM) and 40 µL of 100 mM HEPES, pH 7.0. An aliquot of 20 µL of enzyme solution was added to the reaction mixture and incubated at 37 °C for 10 min, the reaction then was stopped by adding 500 µL CHCl₃:CH₃OH:HCl (66:33:1). Phases were separated by a brief centrifugation and the radioactivity of 50 µL of the aqueous phase was measured by scintillation counting (Beckman). The concentrations of WT and mutants were adjusted so that substrate conversion does not exceed 10-30%. The enzyme solution was freshly prepared by dissolving the lyophilized enzyme in distilled water. The concentration of the enzyme was determined spectrophotometrically (extinction coefficient ($\varepsilon$) = 59 700 M⁻¹ cm⁻¹ at 280 nm). One unit (U) of enzyme activity is defined as the amount (mg) of enzyme that consumes one µmol of the substrate per minute at 37 °C. In the metal dependence study, the assay mixture contained 2 mM PI, 10 mM Triton X-100, 40 mM HEPES, 0-2 mM metal chloride solution, 1 mM EDTA, pH 7.0. WEBMAXC v2.10 was used to calculate the free metal ion concentrations in each assay system.
3.3 RESULTS AND DISCUSSION

3.3.1 Cloning, Expression and Purification of Unlabeled and Labeled saPLC1.

In order to express and purify the protein for kinetic and biophysical studies, we cloned the saPLC1 gene into the expression vector pET28b. The expression level of saPLC1 in *E. coli* under IPTG induction was high. Highly purified protein was obtained after two columns, a Ni-NTA affinity column and an S-100 gel filtration column. A silver nitrate stained SDS-PAGE showed saPLC1 to be greater than 99% pure and the typical yield was ~15 mg of pure enzyme from 1 L of cell culture. For storage, saPLC1 was extensively dialyzed against 1 mM HEPES, pH 7.0 (NaOH), lyophilized, and stored at -20 °C. Note that the DNA sequencing results for pE601 and pE602 vectors containing the saPLC1 gene prior to subcloning revealed a two-base-pair disagreement with the previously published result 3. Our sequencing results showed a CG → GC discrepancy at the DNA level, which leads to a proline to alanine mutation at position 243. WT and the “reversion mutant” (A243P) have been expressed, purified, and showed almost identical kinetic properties. Unless otherwise specified, the WT saPLC1 in our studies refers to the enzyme that has an alanine at position 243.

3.3.2 saPLC1 Crystal Structure Progress. The saPLC1 enzyme has been crystallized under the following conditions described (the legend of Figure 3.5). Diffraction experiments have been carried out on an in house R-Axis IV++ FRD located at The Scripps Research Institute, as well as The Stanford Synchrotron Radiation Lab (SSRL) beam line 9-11. Native crystals diffracted to 2.2 Å with 97% completeness (see
processing statistics below). Data was processed with DENZO and Scalepack (1).

Molecular replacement solutions, heavy atom soaks, and selenomethionine crystals to be used in MAD phasing are now being employed to complete the structure:

Space group = I222 a = 181.2, b = 181.2, c = 101.8 α = β = γ = 90

Resolution 2.35-2.26 I = 99.3 sigma = 28.8, R-factor (square) 0.277

3.3.3 Effect of Different Detergents on Enzyme Activity. The substrate, PI, is insoluble in aqueous phase; therefore, detergents are normally introduced to solubilize this substrate. The enzyme activity was first tested in Triton X-100 (non-ionic detergent), CHAPSO (Zwitterionic), and DHPC (lipid) at various concentrations (above CMC) to find an optimal assay condition. In the assay system, the exact concentration of PI was kept the same (2 mM), while the ratio of PI to detergents was varied. Within the concentration (2-80 mM) tested, Triton X-100, CHAPSO, and DHPC increase the saPLC1 activity initially, but decrease the enzyme activity at higher concentration (Figure 3.6). At the optimal Triton X-100 (10 mM), CHAPSO (10 mM), and DHPC (14 mM) concentration, the saPLC1 activity is 1500 U/mg, 1130 U/mg, and 1490 U/mg, respectively. Triton X-100 mixed micelles was chosen as the assay system in the study of saPLC1 because it (i) gives the same maximal activity as DHPC does, (ii) has low CMC value (0.25 mM), (iii) has minimal affinity for Ca$^{2+}$, and (iv) has rapid micelle exchange kinetics.

3.3.4 Effect of Different Metal Ions on saPLC1 Activity. Previously, saPLC1 has been shown to be activated by Ca$^{2+}$ but inhibited by heavy metal ions at a fixed
concentration of 1 mM. In this study, the metal concentration dependency was tested. This determines the ability of different metal ions to replace Ca²⁺. The activation by alkaline earth metal ions including Ca²⁺ was tested first. The results (Figure 3.7A) show that saPLC1 is activated not only by Ca²⁺, but also by Mg²⁺ and Sr²⁺ to various degrees. The apparent \( K_d \) for Ca²⁺, Mg²⁺, and Sr²⁺ are 12 ± 2 µM, 17 ± 13 mM, and 47 ± 7 µM, respectively. Ba²⁺ showed no activation effect on saPLC1 activity at concentrations up to 1 mM. It is known that Mg²⁺ requires a well-defined octahedral coordination geometry, whereas Ca²⁺ can form looser complexes with higher and variable coordination numbers, without directionality, and with variable bond lengths. The extremely high \( K_d \) for Mg²⁺ indicates that the coordination geometry of the metal binding site highly discriminates Mg²⁺ versus Ca²⁺.

In contrast to the alkaline earth metal ions, Co²⁺, Mn²⁺, and Cd²⁺ provide different activity-versus-metal ion concentration profiles (Figure 3.7B). These transition metal ions increase the activity of saPLC1 at low concentrations, reaching maxima at the concentrations of 50 µM, 100 µM, and 30 µM for Cd²⁺, Mn²⁺, and Co²⁺, respectively. The maximal activities are 310 U/mg for Cd²⁺, 540 U/mg for Mn²⁺, and 350 U/mg for Co²⁺ at their optimal concentrations. Other metal ions (Zn²⁺ and La³⁺) do not have detectable activation effects under our assay conditions.

To understand how the size of the metal binding site associates with the metal selectivity, metal ion (Ba²⁺ and La³⁺) inhibition studies were performed. Results showed that Ba²⁺ had no inhibitory effect at all; on the other hand, La³⁺ demonstrated a strong inhibitory effect on the Ca²⁺ activated saPLC1. The Lineweaver-Burk plot (Figure 3.8) indicates that La³⁺ is a strong competitive inhibitor with an apparent \( K_i \) about 5 µM. Ba²⁺
does not activate nor inhibit saPLC1. This could be that Ba^{2+} is too big to fit into the metal binding site. La^{3+} does not activate but competitively inhibits saPLC1. This suggests that La^{3+}, which has a similar ionic size and ligand preferences as Ca^{2+} but has a slow exchange rate with ligands^{59}, could bind to the metal binding site but could not support catalysis. Taken together, it seems that it is the size and ligand environment of the metal-binding site that determines the metal selectivity of saPLC1. The optimal metal concentrations (Ca^{2+} 1 mM and Cd^{2+} 50 µM) were chosen for the stereoselectivity studies in the next chapter.
**Figure 3.1** The amino acid sequence comparison between rat PI-PLC δ1 and saPLC1. The sequence alignment was performed by ClustalW. Gaps introduced to optimize the alignment are indicated by hyphens. The conserved active site residues are in bold. Asterisks indicate identical residues. Colons indicate conserved substitutions. Dots indicate semi-conserved substitutions.
<table>
<thead>
<tr>
<th></th>
<th>saPLC1</th>
<th>ratPI-PLCd1</th>
</tr>
</thead>
<tbody>
<tr>
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<td>EPAATYGSTSTGVRHAYEKEKRY-------FADALDSGAALLELDNSALGRSFRV</td>
<td>QP-LSHYLVSSS---HNTYLFEDQTFSTAYIRALCKGCRCLELDWDGP-QEPII</td>
</tr>
<tr>
<td>300</td>
<td>S HSNPLG--NNSHCGERANNASELRTKSQDQDFAG-----CLSMDRAWHDAS----</td>
<td>YGYTFTSKILFCVRAIRDLAYFQAPYPVILSLENHCSLEQQRVMARHLRAIGFILL</td>
</tr>
<tr>
<td>360</td>
<td>KIEMKDGFNAKGGRGPAEFDALIRQKLGDAVYGPQDILTGHATADAUEVRAAGWPSRADLA</td>
<td>DQPL-DGVTTSLP-SPEQLKGGKILL-GKDKLGPAGENGSEATDSDEVREAAEME</td>
</tr>
<tr>
<td>420</td>
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<td>-D---EAVRSQVQHKPKEDKLD----KLVPESDMIIYCKSVHGFSSPGTSGQAFYEMA</td>
</tr>
<tr>
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<td>ERYADPAIRPVVVFQDGAPTYNLNSIDTSYDTHRHYLLMTDAHNVPFPIDGTHPTEAE</td>
<td>SFES3RALR--LLQESGNGFVRHNVSLRIPAG----WRTDNSYSPVEMNGGQIV</td>
</tr>
<tr>
<td>540</td>
<td>ALARVRQLAHAHASFATADWYPLPSVLKTVVFRGA</td>
<td>ALN--FQTPGPEMDVYLGCQDNNGGCYVLKPA--</td>
</tr>
</tbody>
</table>

**Figure 3.1**
Figure 3.2  PCR of the saPLC1 gene and construction of the pET28b-saPLC1 vector
Figure 3.3 The (His)$_6$-tag (a) in the recombinant protein replaces the natural signal sequence (b) of *Streptomyces antibioticus* PI-PLC1.
Figure 3.4: The first amino acid sequence of the gene and the primary amino acid sequence of saPLC1. The first amino acid starts with the glutamate of the mature protein (Glu-27 of the pro-sequence).

```
gag ccg gcc gcc acg acg tac ggc acc aqc acg acg tcc gtg ggc gtc cac aac gcc tat gag
1 E P A A T T Y G T S T S V G V H N A Y E
61 aag gag aag tac cgc tac ttc gcg gac gcc ctc gac tgg gcc gcc gcc ctc gtc gaa ctc
121 gac ctc tgg tcc aat gcg ctc ggc ctc tcc gcg ctc ggc gtc cag cac aqc cag cgc ctc ggc
41 D W S N A L G R S W R V S H S N P L G
181 aac aac agc aac tgt gcg ggc gcc gcc aac gcc tcg gag ctc gcg acc aag agc gcg gac
241 cag ctc tgg ctc aat gcg tgc ctc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc
301 cgc ccc atc ctc aag atc gag atg aag gag gcg gtc ttc aac gcc aag ggc gcg gcc gcc gcc
361 ccc gcc gag ttc gcg ctc gcg ctc gcg ctc gac gcc gcc gcc gcc gcc gcc gcc gcc gcc
421 gac ctc tgg ctc aag atc gag atg aag gag gcg gtc ttc aac gcc aag ggc gcg gcc gcc gcc
481 cgc gcc gag ctc gcc gcc aag ttc ctc tcc gtc ctc cag gcg gcg gcc gcg gcg gcc gcg gcg gcg
541 R A D L A G K F L F E L I P G T V E E K
501 aac ccc ttc gac aag ctc aat gcg tgc gtc gag ctc gcc gcc gcc gcc gcc gcc gcc gcc gcc
561 gac ccc ctc gac gcg ctc gcg ctc gcg gtc ctc gcc gcc gcc gcc gcc gcc gcc gcc gcc
621 A Q K L A Q S T A F P A V H G A A P G
641 gac ccc ctc gcg ctc gcg ctc gcg ctc gtc ctc ctc gtc ctc gcc gcc gcg gcg gcg gcg gcg
681 D P R E R Y A D P A L R P W F V V F D G
721 gac gcg ccg acg tat ctc aac gcc agt atc gcg acc gcc tgg ttc aac gcc gcc gcc gcc gcc
741 D A P T Y L N S I D T S W Y D T R H Y
781 ctc ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg
821 L L I M T D A H N V P P V I D G T H P T
841 gag gcg gag gcg ctc gcg gcg ctc gcg ctc gcg gcg gcg gcg gcg gcg gcg gcg gcg gcg gcg
881 E A E A L A R V R Q L A A A H A S F A T
901 gcc gcg tgg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg ctc gcg
941 A D W Y P L P S V L K T V V P R G A -
```
Figure 3.5 Progresses in X-ray crystal structures of saPLC1. (A) Crystals of saPLC1 grown in bicine buffer (pH 9.1) with 1.5% (v/v) dioxane and 8% PEG-20,000. Protein concentration was 20 mg/ml in Tris-HCl buffer, pH 8.1. (B) Upper right corner of a representative diffraction diagram of crystallized saPLC1 collected on an R-Axis IV++ FRD (Molecular Structure Inc.) at The Scripps Research Institute. Plate distance = 200 mm, 2θ = 0, Exposure time was five minutes. (C) SeMet crystals of saPLC1 formed in Phosphate buffer with bicine buffer, pH 9.1, 1% dioxane and 5% PEG-20,000. These crystals diffracted to 2.4 Å on an in house FRD (R-Axis IV++). Crystals were then shipped to SSRL for data collection and MAD phasing.
Figure 3.6 The effects of different detergent: Ionic, zwitterionic, and nonionic.
**Figure 3.7** (A) Activation of saPLC1 by alkaline earth metal ions: calcium (○), magnesium (∇), and strontium (□). All reactions included 40 mM HEPEPS, 1 mM EDTA, 2 mM PI, 10 mM Triton X-100, 0-1 mM free metal ion, pH 7.0 for 10 min at 37 °C. Solid lines represent non-linear fit of each data set to the Michaelis-Menten equation, $V = V_{\text{max}} \frac{[M^{2+}]}{(K_a^{\text{app}} + [M^{2+}])}$. (B) Activation of saPLC1 by transition metal ions: manganese II (○), cobalt II (□), and cadmium II (∇).
Figure 3.7

A

B
Figure 3.8 Lineweaver-Burk plot for the La\(^{3+}\) inhibited Ca\(^{2+}\)-induced activity.
CHAPTER 4

THE UNUSUALLY HIGH NONBRIDGING THIO EFFECT AND INVERSE BRIDGING THIO EFFECT: IMPLICATIONS FOR THE CATALYTIC MECHANISM OF \textit{S. ANTIBIOTICUS} PLC1


4.1 INTRODUCTION

Sulfur substitution of a phosphoryl oxygen has been used extensively in mechanistic studies of enzymes and ribozymes that involve phosphoryl transfer reactions.\textsuperscript{14,16,21,60-64} The reliability of such applications for the attainment of mechanistic insight has however varied greatly owing to a lack of understanding of what thio effects ($k_O/k_S$) should be for enzymatic reactions. On one hand, thio effects and $R_p/S_p$ stereoselectivity have been used successfully to elucidate the detailed reaction mechanism of many enzymes. As a prominent example of the contrary, observation of a relatively small thio effect (ca. 3 or less) for the incorporation of dNTP$\alpha$S into DNA, and the somewhat increased thio effect for mismatches, have been used to conclude that the
chemical step is not rate-limiting. This was subsequently interpreted to support that an
induced-fit mechanism, where the conformational change is the rate limiting step, is
employed by DNA polymerases\textsuperscript{65-67}. Based on experimental and theoretical
considerations, our lab have recently suggested that the use of thio effects in this manner
has led to major misinterpretation of the catalytic mechanism of DNA polymerases\textsuperscript{68}.

Central to the proper interpretation of thio effects is knowledge of the range of their
possible magnitudes. In this regard, the magnitude of thio effects in chemical reactions
have been taken as a point of reference; these fall between 4 and 11 for phosphodiesters
containing sulfur at nonbridging positions\textsuperscript{64} and between $10^{-4}$ and $10^{-3}$ for thiophosphate
esters\textsuperscript{69}. However, it is not well recognized that the thio effect on an enzymatic reaction
is likely to fall in a much greater range, which has been illustrated by data reported in the
past two decades for various enzymes. A major goal of the studies in this chapter is to
examine the thio effects of saPLC1 and evaluate these values based on the rich
knowledge we have accumulated previously.

A thorough examination of thio effects, together with structural and mutagenic
information, for \textit{Bacillus thuringiensis} phosphatidylinositol-specific phospholipase C
(btPLC) previously enabled us to uncover a new mechanism of phosphodiester cleavage
illustrated in Figure 4.1.\textsuperscript{14,16,19-21} On the other hand, the magnitude of thio effects were
not compared to those in the corresponding base- acid-catalyzed chemical reaction since
alkyl inositol phosphates are almost completely resistant to chemical cyclization. In a
more recent work, the Brønsted-type LFER (linear free-energy relationship) study was
conducted using more reactive aryl (thio)phosphate ester\textsuperscript{70}. It was found that the rate
constants for cyclization of aryl inositol phosphates catalyzed by the imidazole buffer at

64
pH 7.0 were close to those of the corresponding $R_p$ and $S_p$-phosphorothioates. Hence, the thio effect ($k_O/k_S$) and $R_p/S_p$-stereoselectivity of chemical cyclization are close to unity. This result further supports that the observed differences between phosphate and phosphorothioate esters and between individual phosphorothioate $R_p$ and $S_p$-diastereomers in their enzyme-catalyzed cleavage were entirely due to specific enzyme interaction, and were not caused by the intrinsic properties of phosphates vs. phosphorothioates.

This chapter summarizes the thio effect studies on saPLC1. It is shown that the WT and mutant saPLC1 display unprecedented magnitudes of thio effects, reverse thio effects, and $R_p/S_p$ stereoselectivity. This information is important for proper application of thio effects as a mechanistic probe. Taking the magnitudes of the thio effects in both chemical reactions and enzyme catalyzed reaction as points of reference, we were able to propose a catalytic mechanism for saPLC1 that has a general application to other metal-dependent PI-PLCs.
4.2 MATERIALS AND METHODS

*Materials.* The synthesis of DOsPI ((2R)-1,2-dioctanoyloxypropanethio-3-(1-phospho-1D-myoinositol)) (2 in Figure 4.2) was reported previously \(^{71}\). The synthesis of DPPsI (1,2-dipalmitoyl-sn-glycero-3-(1-thiophospho-1D-myoinositol)) was reported previously\(^{44}\). For the reaction with \(R_p\)-DPPsI (3 in Figure 4.2), a \(R_p\) and \(S_p\)-DPPsI mixture containing less than 10% the \(S_p\) isomer was used. For the reaction with \(S_p\)-DPPsI (4 in Figure 4.2), the \(S_p\) isomer was obtained from the stereoisomer mixture by selectively removing the \(R_p\) isomer using *Bacillus thuringiensis* PI-PLC, followed by column chromatography on a silica gel column (Merck).

*Construction and Purification of mutant saPI-PLC1.* Mutation was introduced into saPLC1 by double-stranded site directed mutagenesis method (Stratagene). The mutagenic primers (Integrated DNA Technologies, Inc.) used were listed 5’ to 3’ with base substitutions underlined, only the forward primer sequence is shown for each mutant oligonucleotide, and the mutation produced is in parentheses: 5’- CGT CCG TGG GCG TCG CCA ACG CCT ATG AG -3’ (H16A), 5’- GGC GGG TCT CGG CCA GCA ACC CGC -3’ (H55A). The mutants were expressed and purified as described in Chapter 2.

*Steady-State Analyses Using \(^{3}H\)-PI Substrate.* \(^{3}H\)-PI was mixed with unlabeled PI from bovine liver to obtain an overall PI concentration of 5 mM and a specific activity of ca. \(1.25 \times 10^6\) cpm/mol, and Triton X-100 was also added to a final concentration of 25 mM. The reaction mixture contains 40 \(\mu\)L of this substrate solution, which was
diluted with distill water into different concentration (0-5 mM) and 40 μL of 100 mM HEPES, pH 7.0. An aliquot of 20 μL of enzyme solution was added to the reaction mixture and incubated at 37 °C for 10 min, the reaction then was stopped by adding 500 μL CHCl₃:CH₃OH:HCl (66:33:1). Phases were separated by a brief centrifugation and the radioactivity of 50 μL of the aqueous phase was measured by scintillation counting (Beckman). The concentrations of WT and mutants were adjusted so that substrate conversion does not exceed 10-30%. The enzyme solution was freshly prepared by dissolving the lyophilized enzyme in distilled water. The concentration of the enzyme was determined spectrophotometrically (extinction coefficient (ε) = 59 700 M⁻¹ cm⁻¹ at 280 nm). One unit (U) of enzyme activity is defined as the amount (mg) of enzyme that consumes 1 μmol of the substrate per minute at 37 °C. In the metal dependence study, the assay mixture contained 2 mM PI, 10 mM Triton X-100, 40 mM HEPES, 0-2 mM metal chloride solution, 1 mM EDTA, pH 7.0. WEBMAXC v2.10 was used to calculate the free metal ion concentrations in each assay system.

**Steady-State Analyses Using DOsPI. Activity Assays of saPLC1 with DOsPI**

*Substrate (Spectroscopic Assay).* In this assay, a phosphorothiolate analogue, DOsPI, was used to measure the reaction rate. The synthesis of DOsPI was reported elsewhere. This allows us to monitor the production of thio-DAG continuously by following the subsequent reaction of the free thiol with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)⁷². The enzyme solution was added to the substrate solution containing 0-1 mM DOsPI (DOsPI/Triton = 1:5), 40 mM HEPES (pH 7.0), 2 mM CaCl₂, 1 mM EDTA, and 1 mM DNTB, to start the reaction. After mixing for 30 seconds, the reaction mixture was
transferred to a cuvette that was pre-incubated (37 °C) in the spectrophotometer. The time course of the absorbance change at 412 nm was recorded. The linear portion of the curve was used to calculate the slope, which was then converted to the initial rate. \( \varepsilon = 12 \) 800 M\(^{-1}\) cm\(^{-1}\) for 5-thio-2-nitrobenzoic acid was used to calculate enzyme activity.

**Determination of the \( K_{i,\text{app}} \) for the \( R_P \) and \( S_P \) Stereoisomers of DPPsI.** To test whether the large rate difference between \( R_P \) and \( S_P \)-DPPsI arises from binding differences of the two stereoisomers to the active site of the enzyme, the inhibitory effects of these two stereoisomers on DOsPI hydrolysis were tested. The condition for the spectroscopic assay is as described above, except that a fixed Triton X-100 concentration (10 mM) was used. This assay was repeated in the presence of either 0.2 mM \( R_P \)-DPPsI or 0.2 mM \( S_P \)-DPPsI.

**Analyses of Stereochemistry by \(^{31}P \) NMR.** \(^{31}P \) NMR spectra were recorded at 202.44 MHz on a Bruker DRX-500 spectrometer at 27 °C. For all kinetic assays, a control spectrum (\( t = 0 \) min) was taken prior to the addition of enzyme. The amount of enzyme added to initiate the reaction depended on the substrates used and assay conditions. Unless otherwise specified, all assays were performed in a total volume of 500 µL containing 50 mM HEPES (pH 7.0), 10 mM PI, 50 mM Triton X-100, 20% D\(_2\)O, 2 mM CaCl\(_2\), and 1 mM EDTA. The optimal CdCl\(_2\) concentrations used for WT and H16A are 1.15 mM and 1.4 mM, respectively. Due to the limited quantity of the phosphorothioate analogs, \( \sim \) 3 mM DPPsI (DPPsI:Triton X-100 = 1:5) was used in the \(^{31}P \) NMR assay. Four or five µL of 500 mM Glucose 1-phosphate (Glu-1-P) was added
as an internal standard for quantitation. The rate of the reaction, expressed as the consumption of the substrate per minute, was calculated by comparing the integrated intensity of the products with that of the internal standard Glu-1-P.

**Structural Analyses Using 1D- and 2D-NMR.** All Spectra were obtained on a Bruker DMX-600 spectrometer at 27 °C. SaPLC1 samples were prepared as described in the previous section. Each lyophilized enzyme sample (~ 10 mg) was dissolved in 525 μL “100%” D₂O and incubated for 10 min to ensure exchange before the NMR data was collected.
4.3 RESULTS

4.3.1 Proton NMR Analyses of the Global Conformation. Both 1D $^1$H NMR and phase-sensitive NOESY spectra were obtained for the WT and mutant enzymes. The 1D $^1$H NMR spectra (Spectra not shown) of H16A and H55A displayed only minor perturbations. These enzymes were further analyzed by the NOESY spectra (shown in chapter 5 together with other mutants constructed in this dissertation work). The spectra show very similar NOE pattern in their aromatic-aromatic and aromatic-aliphatic regions. These results suggest that the global conformations of these mutants are largely preserved, and that the observed decreases in catalytic activity upon mutation are not likely to be due to the tertiary structural perturbation.

4.3.2 Steady State Analyses of WT and Mutants Using $[^3]$H]PI. Alignment of the sequence of saPLC1 with the catalytic domain of rat PI-PLCδ1, for which there is a crystal structure (PDB entry code 1DIJ), indicated that His16 and His55 are homologous to His311 and His356, which were proposed to function in general acid/base catalysis. The replacements of His16 and His55 by alanine residues resulted in a great reduction in $V_{\text{max}}$ by a factor of $10^3$-$10^5$ relative to that of WT (Table 4.1), supporting the catalytic importance of those residues predicted by sequence alignment studies. Furthermore, the $K_m$ values of H16A and H55A are within the same order of magnitude as that of WT, which suggests that the substrate binding is not likely affected upon these single
mutations. The Michaelis-Menten plot of the WT saPLC1, where $V_{\text{max}}$ and $K_m$ is extrapolated, is shown in Figure 4.3.

\[4.3.3 \text{ Steady State Analyses of WT and Mutants Using D\textit{O}s\textit{P}I.}\] The steady-state kinetics analyses (with D\textit{O}s\textit{P}I) of the mutants were also performed using a more sensitive spectroscopic method (Material and Method). The Michaelis-Menten plot of the WT saPLC1, where $V_{\text{max}}$ and $K_m$ is extrapolated, is shown in Figure 4.4. The $V_{\text{max}}$ and $K_m$ values were compared to the values obtained from the radioactivity assay. The data in Table 4.1 indicate that, with the exception of H55A, the changes in $V_{\text{max}}$ upon mutation relative to that of WT paralleled to those obtained with $[^3\text{H}]-\text{PI}$. The $V_{\text{max}}$ values of WT toward PI and D\textit{O}s\textit{P}I are 1100 and 650 U/mg, respectively, giving a thio-effect ($k_O/k_S$) of 2. Strikingly, however, H55A displays an inverse thio effect ($k_O/k_S$) of 0.0019, where $k_O$ and $k_S$ is 0.27 and 107 U/mg, respectively. To the best of our knowledge, this is the largest inversed thio effect reported for any enzyme catalyzed phosphoryl transfer reaction.

This inverse thio effect shown by H55A was further demonstrated using $^{31}\text{P}$ NMR. The time courses for the conversion of PI to IP are shown in Figure 4.5, where the amount of H55A (20 µg) used is 400-fold higher than that of WT (0.5 µg). Even at this level of enzyme, the conversion of PI to IP is slower for H55A, as evidenced by the relative peak intensities between spectrum b (WT, 6 min) and spectrum f (H55A, 6 min). It takes 11 times longer for H55A (spectrum h, 66 min) to reach the similar extent of conversion as WT in spectrum b. This result indicates that the activity of H55A toward PI is much slower than that of WT by up to ca. 4400 times. In Figure 4.6, the time
course for the cleavage of DOsPI was compared between WT and H55A using the same amount (0.05 µg) of enzyme. The extent of DOsPI conversion by H55A in spectrum h (66 min) is about a quarter of that by WT in spectrum b (6 min). It seems that the activity of H55A is ~7 folds lower than that obtained through continuous assay. This discrepancy might be due to several variants in the spectroscopic assay: higher temperature, the presence of DTNB, and the absence of D₂O.

Based on its activity toward [³H]-PI, H55A is 400-fold more active than would be expected. This unique phenomenon is likely related to the lower pKₐ of the thiol leaving group as compared to hydroxyl leaving group of diacylglycerol. The implication of this property on the catalytic mechanism of saPLC1 is further addressed in the Discussion.

### 4.3.4 Ca²⁺-saPLC1 Displays An Extraordinary Stereospecificity.

In the first set of experiments, Rₚ and Sₚ-DPPsI were subjected to the reaction catalyzed by Ca²⁺-activated saPLC1. 0.27 µg enzyme was added to the reaction mixtures to initiate the reaction. As shown in Figure 4.7, Rₚ-DPPsI (57.4 ppm) reacted slower than PI (control experiment); in addition, it produced both IcPs (73.4 ppm) and IPs (45.3 ppm) simultaneously. Using the trans-IcPs produced from Rₚ-DPPsI by btPLC as an internal control, the peak at 73.4 ppm was verified as trans-IcPs. In contrast, Sₚ-DPPsI (57.7 ppm) was only partially hydrolyzed after incubation with 2.7 mg of WT enzymes for 22 days. The rates of the reactions estimated from the ³¹P NMR spectra are listed in Table 4.2. It was found that the WT enzyme demonstrates a normal Rₚ-thio effect (kₒ/kₐₒ = 47) but an extraordinarily high Sₚ-thio effect (kₒ/kₐₚ = 1.6 × 10⁸). Subsequently, it shows an extremely high stereoselectivity (kₐₒ/kₐₚ = 6.2 × 10⁶) in the presence of Ca²⁺.
4.3.5 Cd^{2+}-saPLC1 Shows A Relaxed Stereospecificity. In the previous chapter, we have shown that saPLC1 was activated by various metal ions, among which more sulfurphilic metal ions (Mn^{2+}, Co^{2+}, and Cd^{2+}) show activation effects as well. Mn^{2+} and Co^{2+} are paramagnetic therefore preclude the observation of phosphorus by NMR. For this reason, only Cd^{2+} substitution was used in our study. With Cd^{2+}, no obvious change was observed for PI and Rp-DPPsI hydrolysis, whereas the activity toward Sp-DPPsI was partially restored (Figure 4.7). The $R_P$- and $S_P$-thio effect in the presence of Cd^{2+} are 50 and $4.2 \times 10^5$, respectively. Therefore, substitution of Ca^{2+} by Cd^{2+} relaxed the stereoselectivity of saPLC1 by 1000-fold.

4.3.6 H16A Displays Unusual Nonbridging Effect. To probe the possible transition state interaction between the phosphate moiety and His16, the nonbridging thio effect of the H16A mutant was also tested. Surprisingly, H16A-Ca^{2+} shows similar activities toward PI and Rp-DPPsI, subsequently, an $R_P$-thio effect ($k_O/k_{Rp}$) that is close to unity (Table 4.2). In contrast, H16A-Cd^{2+} strongly prefers PI versus Rp-DPPsI, consequently, an $R_P$-thio effect ($k_O/k_{Rp}$) of $7.9 \times 10^3$. The phosphodiesterase activities of H16A-Ca^{2+} and H16A-Cd^{2+} toward Sp-DPPsI were also examined, yet too low to be detected. However, after extend incubation time with excess amount of enzymes, Sp-DPPsI (57.7 ppm) was slowly converted to a product that gives an upfield signal (57.1 ppm) (Figure 4.8 and Figure 4.9). This compound at 57.1 ppm should not be Rp-DPPsI, which usually shows a signal at $\sim$ 57.3 ppm. Analysis of the reaction product using Mass
Spectrometry indicates that this product (57.1 ppm) is deacylated $S_p$-DPPsI (Figure 4.10).

4.3.7 $R_p$- and $S_p$- Stereoisomers of DPPsI are both Competitive Inhibitors of DOsPI. Could the large rate difference between $R_p$ and $S_p$-DPPsI arise from differential binding of the two diastereomers to the enzyme active site? To answer this question, the two diastereomers were used as inhibitors in the continuous assay with DOsPI as the substrate. Since DPPsI does not contain a free thiol, it cannot couple with DTNB. The enzyme will therefore be “inhibited” because it will bind to DPPsI and shows lowered activity towards DOsPI. The Lineweaver-Burk plots for the WT enzyme in the absence and in the presence of 2.5 mM DPPsI (either $R_p$ or $S_p$) is shown in Figure 4.11. The $K_i$ for each diastereomer is calculated by using the equation:

$$K_m' = K_m (1 + [I_0]/K_i)$$

Where $K_m'$ is the apparent $K_m$ in the presence of an initial concentration of a competitive inhibitor. The $K_m$ value for DOsPI is 0.148 mM. The $K_i$ value for $R_p$ and $S_p$-DPPsI determined from this plot are 0.161 mM and 0.190 mM, respectively. The determined $K_i$ values are also similar to the $K_m$ value (0.117 mM) of the natural substrate at the same assay condition. The enzyme therefore has similar affinities for the thio-analogs as it does for the natural substrate. The results indicate that the concentrations of $R_p$ and $S_p$-DPPsI used in stereoselectivity studies are saturating, and the large different in rate between $R_p$ and $S_p$-DPPsI should not be explained by simple steric arguments.
4.3.8 Chemical Cyclization of DOsPI. Chemically more liable \((2.4 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1})\) compounds, \(p\)-nitrophenyl 1-inostiol phosphate (NPIP) and its phosphorothiolate analogs, have been used as a model system to understand the reactivity of inositol phosphodiester toward P-O bond cleavages. It was found that the non-enzymatic chemical reaction preceded the same in-line mechanism, which results in inversion of configuration at phosphorus, analogous to the enzymatic reaction. Although the chemical stability of DOsPI has been estimated based on the bond energy between P-O and P-S, the catalytic rate for the chemical cyclization of DOsPI is not available. In this work, the imidazole catalyzed DOsPI cleavage was examined. This enables us to have a point of reference for comparing the bridging thio effect in enzymatic reactions. Similar to NPIP, DOsPI was cleaved at 80 °C \((k = 6.1 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1})\) to afford two products giving rise to \(^{31}\text{P}\) NMR signals at 18.3 ppm (1,6-IcP) and 17.5 ppm (1,2-IcP) at a 1:5 ratio (Figure 4.12). In comparison, the non-enzymatic hydrolysis of PI in the same condition is 1000-fold slower \((5.17 \times 10^{-9} \text{ M}^{-1} \text{ s}^{-1}, \text{extrapolated from Brønsted plot reported recently})\). In another word, the chemical reactivity of phosphorothiolate analog of PI (DOsPI) is estimated to be ca. \(10^3\) more reactive than PI. This result agrees with the previous studies, where the chemical reactivity of thiolphosphate esters was shown to be ca. \(10^3-10^4\) more reactive than that of corresponding oxyesters. On the basis of theoretical and experimental calculations, the bridging thio effect \((k_O/k_S)\) for the chemical cyclization of PI is 0.001.
4.4 DISCUSSIONS

4.4.1 Implication of the Exceedingly High Stereoselectivity. Stereoselectivity of an enzyme toward $R_P$ and $S_P$ isomers of the substrate phosphorothioate analogs, coupled with varied metal ions, has long been established as a useful tool in probing detailed interactions between metal ions and the phosphate moiety at the active site of enzymes 60-62. A number of enzymes showed a “reversal of stereoselectivity” upon substitution of Mg$^{2+}$ by Cd$^{2+}$, presumably due to the fact that Mg$^{2+}$ prefers oxygen ligands and Cd$^{2+}$ prefers sulfur ligands75; this has been used as evidence for the involvement of that phosphoryl group in metal ligation during catalysis. However, such a metal-dependent stereospecificity reversal was not always observed, instead, a relaxed stereoselectivity was more commonly seen60,62,76. This could be caused by the high geometric constraint near the phosphate moiety, such that Cd$^{2+}$ is forced to bind to the oxygen even though it prefers sulfur.

Previously the stereoselectivity of several mammalian PI-PLC isozymes have only been qualitatively tested. It was shown that they preferred $R_P$-DPPsI in the presence of Ca$^{2+}$ and their product profiles showed no changes upon the phosphorothioate analog substitution 43,44. These experimental results together with the structural information 5,6 have been used to predict the important catalytic roles of the Ca$^{2+}$ cofactor. It was proposed that Ca$^{2+}$ is 1) to lower the pK$_a$ of the 2-OH group of the inositol moiety facilitating its deprotonation and subsequent nucleophilic attack on the 1-phosphate, 2) to stabilize the negatively charged transition state via interacting pro-$Sp$ oxygen, 3) responsible for retaining cyclic product intermediate due to the bidentate (Ca$^{2+}$ to both
pro-Sp and 2-OH of inositol) interactions. On the other hand, neither the quantitative stereoselectivity nor the metal substitution approach was further pursued due to the enzymes’ stringent requirement for Ca$^{2+}$. Fortunately, the promiscuity of saPLC1 in metal ion selectivity allows us to further characterize the metal binding site and examine the possible mechanistic roles of the metal cofactor. Further analyses of the data in Table 4.2 give the following interesting points:

(i) Trans-IcPs was produced from Rp-DPPsI by saPLC1; therefore the stereochemical course of the first step of the reaction (the conversion of PI to IcP) is inversion of configuration at phosphorus. Thus saPLC1 most likely uses the same in-line attack double displace mechanism proposed for other PI-PLCs $^{12,43}$, as demonstrated in Chapter 1 (Figure 1.4).

(ii) Substitution of Ca$^{2+}$ by Cd$^{2+}$ in WT saPLC1 enhances the activity toward Sp-DPPsI by a factor of 400, but does not affect the activity toward PI or Rp-DPPsI, thus the Sp-thio effect and the $R_p/S_p$ stereoselectivity are decreased by the same magnitude. Such a large change in stereoselectivity toward DPPsI is strong evidence that the stabilization of the pentacoordinate transition state is through direct metal ion coordination to the pro-Sp oxygen. This is illustrated in Figure 4.13.

(iii) Since the nonbridging thio effect of a chemical reaction is close to unity, the relatively small $R_p$-thio effect ($k_{O}/k_{R_p} = 50$) shown by the WT enzyme might be caused by some unfavorable interactions between the pro-$R_p$ oxygen and active site residues. In contrast, withdrawing a transition state stabilization interaction usually causes a much larger effect on the nonbridging thio effect. A drastic decrease in activity upon H16A mutation suggests that His16 might be involved in the interaction with the pro-$S_p$ oxygen.
of the phosphate moiety. However, additional experiments are required to understand the nature of these interactions.

(iv) The $R_p$-thio effect ($k_O/k_Rp = 1.1$) of H16A is close to unity in the presence of Ca$^{2+}$, whereas this $R_p$-thio effect increases to $7.9 \times 10^3$ upon Cd$^{2+}$ activation. This latter value is significantly higher than that of WT ($k_O/k_Rp = 50$) in the presence of Ca$^{2+}$ or Cd$^{2+}$. We postulate that the small $R_p$-thio effect demonstrated by Ca$^{2+}$-H16A originates from an increased flexibility at the active site due to the alanine mutation, which allows the enzyme to accommodate a sulfur atom at the pro-R position. A rotation of the sulfur atom toward the thio-philic Cd$^{2+}$ ion, however, leads the leaving group to an equatorial position. This conformation is not favorable for the in-line attack double displacement mechanism, since both nucleophile and leaving group need to be in the apical position for the reaction to proceed. Furthermore, this rotation might also diminish the proper function of the general acid. Taken together, Cd$^{2+}$-H16A has an exceedingly low activity toward $R_p$-DPPsI. This is further demonstrated in Figure 4.14.

### 4.4.2 Implication of the Large Inverse Thio Effect by H55A

It was predicted from the crystal structure of mammalian PI-PLCδ1 complexed with inositol 1,4,5-triphosphate (InsP$_3$) that His365 acts as the general acid, protonating the C$_3$ hydroxyl group of diacylglycerol in the phosphotransfer reaction. It was shown that mutation at His365 could cause ~ $10^4$-fold decreases in activity. No functional analyses were further pursued. However, the catalytic differences between His365 (the counterpart in saPLC1 is His55) in PLC-δ1 and His82 in btPLC might contribute to the mechanistic differences between metal-dependent PI-PLCs and metal-independent PI-PLCs.
In this study, the catalytic function of His55 was analyzed using thio effect. Mutation of His55 to alanine results a 4000-fold decrease in activity toward the natural PI, but only 6.3-fold decrease with the phosphorothiolate analogues, DOsPI. Thus H55A demonstrates an extremely high inverse thio effect \( (k_O/k_S = 0.0017) \). This inverse thio effect is most likely related to the lower pK\(_a\) of the thiol (ca. 9.51) \(^{77}\) compared to the hydroxyl leaving group of DAG (ca. 14.2) \(^{78}\). By contrast, the WT and other mutant saPLC1 show a small thio effect \( (k_O/k_S = 0.5 \sim 2) \). It seems that the observed rates of the phosphorothiolate cleavage are the results of two compensating effects: (i) the presence of sulfur on the leaving group reduces stabilization of the transition state by the general acid due to the loss of hydrogen bonding to the leaving group; but (ii) the lower pK\(_a\) of the thiol group allows more negative charge developing on the sulfur atom and, hence, stabilizes the transition state. These two effects seem to balance out in the WT and most mutant saPLC1. Upon alanine substitution at His55 (withdrawal of the first effect), the second effect seems to take control of the thio effect. Therefore, H55A has a 1000-fold increased activity toward DOsPI compared to PI. It is interesting to note that the thio effect demonstrate by H55A \( (k_O/k_S = 0.0017) \) is comparable to that of the nonenzymatic chemical reaction \( (k_O/k_S = 0.001) \).

Similar thio effects have been observed for btPLC, where the corresponding values are 12 \sim 24 \) for the WT and most mutants but 0.1 for D33A and H82A. \(^{14,19}\) The results from cross examining the bridging and nonbridging thio effect had led to the recognition of a novel catalytic triad (Arg69-Asp33-His82), which plays a dual catalytic task: interacting the \textit{pro}-S\(_p\) oxygen and protonation of the DAG leaving group \(^{21}\). Therefore, the interdependence of the three catalytic residues is also responsible for the observed
rates of the phosphorothiolate cleavage (third effect) – weaker interactions to the bridging oxygen could also translate into weaker nonbridging interaction resulting in lower activity for phosphorothiolate analog than for phosphate substrate. In btPLC, $k_O/k_S$ should be the additive effect of the three effects described above. The inverse thio effects ($k_O/k_S = 0.1$) shown by D33A and H82A are controlled by the second effect.

Based on the small thio effect, which is close to unity, by the WT and mutant enzymes and an extremely high inverse thio effect, which is similar to that of a nonenzymatic chemical reaction, shown by H55A, we hypothesize that His55 functions independently from other part of the catalytic machinery. This property of His55 might closely relate to its reversal role (general base) in the second step of the reaction.

4.5 CONCLUSION

Taken together, the observed thio effects for saPLC1 support the transition state structure shown in Figure 4.15. Most importantly, we have observed the strongest bridging and nonbridging thio effects ever recorded, and our results demonstrate that the magnitude of enzymatic thio effects can vary greatly (from 0.002 to 20 for the bridging thio effect and from 1 to $10^8$ for the nonbridging thio effect). Likewise, we have shown that the $R_p/S_p$ stereoselectivity of an enzyme may span a large range of values (1 to $10^6$). A change in the microenvironment of the active site (such as Arg to Lys, or Ca$^{2+}$ to Cd$^{2+}$), or in the fine structure of the substrate, is sufficient to perturb these values by several orders of magnitude. Such information is important for proper application of thio effects as a mechanistic probe.
Furthermore, on the basis of the exceeding high non-bridging thio effect and inverse bridging thio effect, we postulate that this metal dependent PI-PLC accelerates the chemical reaction in a slightly different way from that of the metal independent PI-PLC. It appears that this metal dependent saPLC1 lowers the $\Delta G^\ddagger$ via inputting much transition state stabilization energy through pro-$S$ oxygen and the general acid (His55) is fairly independent of other part of the catalytic machinery. This might be advantageous for the enzyme in efficient catalyzing the sequential two-step reaction.
### Table 4.1 Summary of Bridging Thio Effects for WT and mutant saPLC1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}^c$</th>
<th>$K_{\text{m, app}}^e$</th>
<th>$V_{\text{max}}^d$</th>
<th>$K_{\text{m, app}}^e$</th>
<th>$k_O/k_S^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1122 ± 22</td>
<td>58 ± 7</td>
<td>678 ± 28</td>
<td>24 ± 5</td>
<td>1.7</td>
</tr>
<tr>
<td>H16A</td>
<td>0.024 ± 0.001</td>
<td>16 ± 3</td>
<td>0.044 ± 0.002</td>
<td>6 ± 2</td>
<td>0.54</td>
</tr>
<tr>
<td>H55A</td>
<td>0.273 ± 0.005</td>
<td>23 ± 3</td>
<td>107 ± 8</td>
<td>36 ± 10</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

$a$ Measured at 37 °C, 0-2.0 mM PI(or DOsPI) and PI(or DOsPI)/Triton X-100 = 5 in 40 mM HEPES, 2 mM CaCl₂, 1 mM EDTA, pH 7.0. Activities are expressed in µmoles mg⁻¹ min⁻¹. $b$ Natural phosphatidylinositol, where the chain length of DAG may vary from that of DPPI. The chain length differences between PI and DOsPI might affect the kinetic parameters slightly. However, the mechanistic interpretation was drawn mainly from the comparison between thio effects. Thus the possible chain length effect was not further pursued. $c$ Maximal activity toward PI determined by the radioactivity assay$^{15}$. $d$ Maximal activity toward DOsPI obtained by the spectroscopic assay$^{15}$. $e$ In mM. $f$ Bridging thio effect. The values for N17A, E39A, E39Q, and D41A are 0.94, 0.53, 1.3, and 1.3, respectively.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>WT</th>
<th>WT</th>
<th>H16A</th>
<th>H16A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metal Ions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>646</td>
<td>666</td>
<td>0.0041</td>
<td>0.038</td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>13.7</td>
<td>13.3</td>
<td>0.0038</td>
<td>4.8 × 10(^{-6})</td>
</tr>
<tr>
<td>k(_O)(^{b})</td>
<td>47</td>
<td>50</td>
<td>1.1</td>
<td>7.9 × 10(^3)</td>
</tr>
<tr>
<td>k(_S)(^d)</td>
<td>4.0 × 10(^{-6})</td>
<td>1.6 × 10(^{-3})</td>
<td>ND (^f)</td>
<td>ND</td>
</tr>
<tr>
<td>k(_R)(^e)</td>
<td>6.2 × 10(^6)</td>
<td>4.3 × 10(^3)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Measured by \(^{31}\)P-NMR \(^48\) at 27 °C, in 40 mM HEPES, 1 mM EDTA, optimal metal ion concentrations, saturating substrate concentrations, pH 7.0. Activities are expressed in µmoles mg\(^{-1}\) min\(^{-1}\). \(^b\) The specific activities should be close to maximal activities, since the substrate concentrations used in the assays are well above the \(K_{m,app}\) value for PI and \(K_{i,app}\) values (0.161 mM and 0.190 mM for \(R\)\(_P\) and \(S\)\(_P\)-DPPsI, respectively) for DPPsI. \(^c\) \(R\)\(_P\)-thio effect. \(^d\) \(S\)\(_P\)-thio effect. \(^e\) \(R\)\(_P\)/\(S\)\(_P\) stereoselectivity. \(^f\) ND, non-detectable.

**Table 4.2** Summary of Nonbridging Thio Effects for WT saPLC1 and Mutant H16A in the Presence of Different Metal Ions \(^a\)
Figure 4.1 The proposed transition states of PI-PLC from *Bacillus thuringiensis*. DAG = diacylglycerol.
Figure 4.2 Structures of the substrate and substrate analogues used in this work. DPPI, 1,2-dipalmitoyl-sn-glycero-3-(1-phospho-1\textit{D}-myo-inositol). DOsPI, (2\textit{R})-1,2-dioctanoyloxypropanethio-3-(1-phospho-1\textit{D}-myo-inositol). DPPsI, 1,2-dipalmitoyl-sn-glycero-3-(1-thiophospho-1\textit{D}-myo-inositol).
Figure 4.3  Michaelis-Menten plot for WT saPLC1 using substrate PI.
**Figure 4.4** Michaelis-Menton plot for WT saPLC1 using substrate DOsPI.
Figure 4.5 The time course of the conversion of PI to IP monitored by $^{31}$P NMR, where the amount of H55A (20 μg) is 400-fold higher than that of WT (0.05 μg).
Figure 4.6 The time course for DOsPI cleavage monitored by $^{31}$P NMR, where same amount of WT (0.05 µg) and H55A (0.05 µg) are used.
Figure 4.7 $^{31}$P NMR assay of DPPsI hydrolysis catalyzed by WT saPLC1. (A) and (B) were $R_p$-DPPsI hydrolyses by WT saPLC1 in the presence of $Ca^{2+}$ and $Cd^{2+}$, respectively. (C) and (D) were $S_p$-DPPsI hydrolyses by WT saPLC1 in the presence of $Ca^{2+}$ and $Cd^{2+}$, respectively. Spectra were taken at 50 min intervals, except that the sixth spectra in (C) and (D) were taken after 22-day incubation.
**Figure 4.8** $^{31}$P NMR assay of DPPsI hydrolysis catalyzed by saPLC1 mutant (H16A) in the presence of Ca$^{2+}$. 
Figure 4.9 $^{31}$P NMR assay of DPPsI hydrolysis catalyzed by saPLC1 mutant (H16A) in the presence of $\text{Ca}^{2+}$.
Figure 4.10 Determination of the product from $S_b$-DPPsI catalyzed by saPLC1 (H16A) by mass spectroscopic method.
Figure 4.11 Lineweaver-Burk plots for WT saPLC1 using \( R_P \) and \( S_P \)-DPPsI as inhibitors.

The \( K_{i,\text{app}} \) of each stereoisomer is calculated by using the equation: \( K_{m,\text{app}}' = K_{m,\text{app}} (1 + [I_0]/K_{i,\text{app}}) \), where \( K_{m,\text{app}}' \) is the \( K_{m,\text{app}} \) in the presence of an initial concentration of the competitive inhibitor \([I_0] \). The \( K_{m,\text{app}} \) of DOsPI is 0.148 mM. The \( K_{i,\text{app}} \) values of \( R_P \) and \( S_P \)-DPPsI determined from this plot are 0.161 mM and 0.190 mM, respectively. Note that the \( K_{m,\text{app}} \) of PI is 0.117 mM at this assay condition.
Figure 4.12 Imidazole buffer catalyzed cyclization of DOsPI. Conditions: 0.5 M imidazole, pH 7.0, 80 °C.
Figure 4.13 The effects of substrate and metal substitution on the catalytic activity of the WT saPLC1.
Figure 4.13 The effects of substrate and metal substitution on the catalytic activity of the H16A mutant saPLC1.
Figure 4.15 The proposed transition state of saPLC1. DAG = diacylglycerol.
CHAPTER 5

THE PROPOSED GENERAL ACID/GENERAL BASE CATALYTIC MECHANISM OF S. ANTIBIOTICUS PLC1 AND THE FUTURE PERSPECTIVE

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5.1 INTRODUCTION

On the basis of current mechanistic studies on mammalian PI-PLC enzymes, it was proposed that PI-PLC-catalyzed cleavage of phosphatidylinositol 4,5-bisphosphate results first in formation of the cyclic intermediate, inositol 1,2-cyclic-4,5-trisphosphate, which is subsequently hydrolyzed to afford inositol 1,4,5-triphosphate secondary messenger\textsuperscript{12,18,58}. In contrast to inositol 1,4,5-triphosphate, the potency of cyclic inositol phosphates to mobilize calcium is at least an order of magnitude less \textsuperscript{79}. On the other hand, the cyclic product intermediates are poor substrates for the phosphatase and kinase that metabolize IP\textsubscript{3}, therefore can eventually accumulate to reach a higher concentration after prolonged stimulation \textsuperscript{80-82}. The available evidence indicates that cells cannot avoid making cyclic inositol phosphates; in certain cells, cyclic phosphates are even the major product of phosphoinositide cleavage \textsuperscript{26-28}. Although the exact physiological roles of
these cyclic inositol phosphates are not clear, these molecules seem to have greater functional significance than current state of knowledge might have indicated.

In order to elucidate the possible mechanism – how the “leaking” production of cyclic inositol phosphates is controlled and regulated, much effort has been devoted to tackling this problem. It was proposed that whether cyclic inositol phosphates could be efficiently hydrolyzed is influenced by how well these intermediates are hampered from dilution into the bulk solvent. Since the metal-independent bacterial PI-PLC could only efficiently catalyze the conversion of PI to IcP, it seems that the difference between the arginine residue and the calcium cofactor might induce the mechanistic variation. In the crystal structures, calcium showed a promiscuity in coordination geometries, where both regular octahedral (1,4,5-IP3 complex) and square-pyramidal (cICH2P complex) were observed. This promiscuity enables Ca2+ to compromise the geometric changes in the active site during the course of the reaction, thus form a strong interaction with the product intermediate. The low off-rate of the product intermediate was proposed to be related to this promiscuity of calcium. In contrast, an arginine side-chain, which exerts hydrogen bonding interactions, might be lacking this promiscuity.

The results from Chapter 2 indicate that an arginine to calcium switch is insufficient to convert btPLC to a mammalian like and suggest that some other differences in the active site might be responsible for the catalytic behavior of mammalian PI-PLCs. This hypothesis is further supported, though fairly weakly, by the results from the thio effect studies in Chapter 4 – both Ca2+ and His16 are involved in the interaction with the phosphate moiety. This additional interaction from His16 could (i) facilitate transition state stabilization, which in turn allow His55 to play double roles
(GA/GB) in two subsequent reactions, and (ii) lock IcP in the active site before it undergoes further hydrolysis.

One major goal of this work is to establish a kinetic model to further understand the catalytic and regulatory mechanism of the more complicated mammalian PI-PLCs. The results from thio effects and mutagenesis studies in Chapter 4 outlined the catalytic mechanism of saPLC1. In the absence structural information, the site-directed mutagenesis based on sequencing alignments is one of the most straight forward approaches in confirming the catalytic similarity between two homologous enzymes. This approach is thoroughly used in this chapter.

Another issue, which baffled us, is whether an “induce-fit mechanism” is used by PI-PLCs to achieve the $10^{12}$-fold rate enhancement. Superimposing the X-ray crystal structures of both the free enzyme and enzyme-substrate analog complexes indicates that there are only subtle changes upon substrate analog binding. However, this cannot argue against the “induce-fit mechanism”, since the analogs used lack the hydrophobic leaving group. To comprehend the conformational change, if any, upon substrate binding, would help us to understand how the activity of the enzyme is regulated and how the release of IcP is controlled. We attempted to solve this puzzle using NMR techniques in this study. The results from the binding studies, along with the kinetic studies with water soluble phosphodiesters, suggest that a localized structural change might exist upon substrate binding.
5.2 MATERIAL AND METHODS

Materials. All other reagents were the same as in previous chapters unless otherwise specified. D-Ins(1:2cyc)P was prepared through enzymatic digestion (btPLC) of raw PI (50%). PI (2 g) was dispersed in 20 mL of 20 mM Tris-HCl (pH7.5) containing 4% Triton X-100, 50% D$_2$O. btPI-PLC (1 µg) was added and the mixture incubated at room temperature and the progress of the reaction was followed by $^{31}$P NMR. After the reaction was nearly complete, the reaction mixture was extracted by adding 20 mL each of methanol and chloroform followed by vigorous stirring. After brief centrifugation, the aqueous layer was collected and reextracted twice with chloroform. The mixture was then lyophilized and redissolved in desire amount of distilled water. Gro-PI was obtained by deacylation of raw PI (50%) with methylamine in a methanol solution at room temperature over the course of 24 hours. The fatty acid methylamide was removed by extraction (3 times) of the aqueous solution of the reaction mixture with a methanol/chloroform mixture (4:6), and the aqueous phase was concentrated to give the pure product.

Site-directed Mutagenesis, Expression, and Purification of Recombinant saPI-PLC1. The mutagenic primers (Integrated DNA Technologies, Inc.) used were listed 5’ to 3’ with base substitutions underlined, only the forward primer sequence is shown for each mutant oligonucleotide, and the mutation produced is in parentheses: 5’- CGT CCG TGG GCG TCG CCA ACG CCT ATG AG -3’(H16A); 5’- GTG GGC GTC CAC GC GCC TAT GAG AAG -3’ (N17A); 5’- GGG CGT CCA CCA AGC CTA TGA GAA GG
–3’ (N17Q); 5’- CCG CCC TGC TCG CCC TCG ACC TCT G -3’ (E39A); 5’- CCG CCC TGC TCG ACG TCG ACC TCT G –3’ (E39D); 5’- CCG CCC TGC TCG ACC TCT G –3’ (E39Q); 5’- CCT GCT CGA ACT CGC CCT CTG GTC C -3’ (D41A); 5’- GCT CGA ACT CGA ACT CTG GTC CAA TG –3’ (D41E); 5’- CCT GCT CGA ACT CAA CCT CTG GTC C –3’ (D41N); 5’- GGC GGG TCT CGG C CA GCA ACC CGC -3’ (H55A); 5’- CGA CGG CGA CGC GCC GAC GTA TCT C -3’(A243P). All mutations were verified by sequencing and each mutant was completely sequenced to confirm that no unwanted mutation had been introduced.

**Steady-State Analyses Using [3H]-PI Substrate.** [3H]-PI was mixed with unlabeled PI from bovine liver to obtain an overall PI concentration of 5 mM and a specific activity of ca. 1.25 × 10^6 cpm/mol, and Triton X-100 was also added to a final concentration of 25 mM. The reaction mixture contains 40 µL of this substrate solution, which was diluted with distill water into different concentration (0-5 mM) and 40 µL of 100 mM HEPES, pH 7.0. An aliquot of 20 µL of enzyme solution was added to the reaction mixture and incubated at 37 °C for 10 min, the reaction then was stopped by adding 500 µL CHCl3:CH3OH:HCl (66:33:1). Phases were separated by a brief centrifugation and the radioactivity of 50 µL of the aqueous phase was measured by scintillation counting (Beckman). The concentrations of WT and mutants were adjusted so that substrate conversion does not exceed 10-30%. The enzyme solution was freshly prepared by dissolving the lyophilized enzyme in distilled water. The concentration of the enzyme was determined spectrophotometrically (extinction coefficient (ε) = 59 700 M^-1 cm^-1 at
280 nm). One unit (U) of enzyme activity is defined as the amount (mg) of enzyme that consumes 1 µmol of the substrate per minute at 37 °C. In the metal dependence study, the assay mixture contained 2 mM PI, 10 mM Triton X-100, 40 mM HEPES, 0-2 mM metal chloride solution, 1 mM EDTA, pH 7.0. WEBMAXC v2.10 was used to calculate the free metal ion concentrations in each assay system. In pH-activity dependence experiments, the combination of 50 mM each Tris (pK_a 8.1), MOPS (pK_a 7.2), and MES (pK_a 6.27) was used in pH buffering.

**Steady-State Analyses Using DOsPI.** Activity Assays of saPLC1 with DOsPI Substrate (Spectroscopic Assay). In this assay, a phosphorothiolate analogue, DOsPI, was used to measure the reaction rate. The synthesis of DOsPI was reported elsewhere. This allows us to monitor the production of thio-DAG continuously by following the subsequent reaction of the free thiol with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The enzyme solution was added to the substrate solution containing 0-1 mM DOsPI (DOsPI/Triton = 1:5), 40 mM HEPES (pH 7.0), 2 mM CaCl_2, 1 mM EDTA, and 1 mM DNTB, to start the reaction. After mixing for 30 seconds, the reaction mixture was transferred to a cuvette that was pre-incubated (37 °C) in the spectrophotometer. The time course of the absorbance change at 412 nm was recorded. The linear portion of the curve was used to calculate the slope, which was then converted to the initial rate. ε = 12 800 M^{-1} cm^{-1} for 5-thio-2-nitrobenzoic acid was used to calculate enzyme activity.

**31P NMR Spectroscopy.** 31P NMR spectra were recorded at 202.44 MHz on a Bruker DRX-500 spectrometer at 27 °C. For all kinetic assays, a control spectrum (t = 0
min) was taken prior to the addition of enzyme. The amount of enzyme added to initiate the reaction depended on the substrates used and assay conditions. IcP hydrolyses were performed in a total volume of 500 µL containing 50 mM HEPES (pH 7.0), 5-200 mM IcP, 50 mM Triton X-100, 20% D2O, 2 mM CaCl2, and 1 mM EDTA. Gro-PI hydrolyses were performed in a total volume of 1000 µL containing 50 mM HEPES (pH 7.0), 5-200 mM IcP, 50 mM Triton X-100, 20% D2O, 2 mM CaCl2, and 1 mM EDTA. 5 µL of 500 mM Glucose 1-phosphate (Glu-1-P) was added as an internal standard for quantitation. The rate of the reaction, expressed as the consumption of the substrate per minute, was calculated by comparing the integrated intensity of the products with that of the internal standard Glu-1-P. Reactions usually were monitored for 3-6 hours.

**NMR Titration.** Uniformly 15N-labeled WT and R69D were expressed in M9 minimal media using 15NH4Cl as a single nitrogen source and purified as described above. Two-dimensional 15N-1H HSQC spectra of the uniformly 15N-labeled protein at Ca2+ concentration ranging from 0 to 5 mM were obtained on a Bruker DRX-800 spectrometer at 37 °C. Each sample contained 0.4 mM enzyme and 50 mM HEPES buffer in 90% H2O/10% D2O, pH 7.5.

**Structural Analyses Using 1D- and 2D-NMR.** All Spectra were obtained on a Bruker DMX-600 spectrometer at 27 °C. SaPLC1 samples were prepared as described in the previous section. Each lyophilized enzyme sample (~ 10 mg) was dissolved in 525 µL “100%” D2O and incubated for 10 min to ensure exchange before the NMR data was collected.
5.3 RESULTS AND DISCUSSIONS

5.3.1 Proton NMR Analyses of the Global Conformation. Both 1D $^1$H NMR and phase-sensitive NOESY spectra were obtained for the WT and mutant enzymes. The 1D $^1$H NMR spectra (Spectra not shown) of H16A, N17A, E39A, E39Q, D41A, and H55A displayed only minor perturbations. These enzymes were further analyzed by the NOESY spectra shown in Figure 5.1. The spectra show very similar NOE pattern in their aromatic-aromatic and aromatic-aliphatic regions. These results suggest that the global conformations of these mutants are largely preserved, and that the observed decreases in catalytic activity upon mutation are not likely to be due to the tertiary structural perturbation.

5.3.2 Steady-state Analyses of WT and Mutants Using $[^3]$H-PI and DOsPI.

Alignment of the sequence of saPLC1 with the catalytic domain of rat PI-PLCδ1, for which there is a crystal structure (PDB entry code 1DIJ), suggests that saPLC1 contains most of the key catalytic residues responsible for PI hydrolysis. His16 and His55 aligned with His311 and His356, which were proposed to function in general acid/base catalysis. Asn17, Glu39, and Asp41 align with Asn312, Glu341, and Asp343, which were suggested to be responsible for Ca$^{2+}$ binding as well as potential nucleophiles. In order to examine the catalytic importance of the conservative residues, all these residues were individually replaced by alanine. The maximal activity ($V_{\text{max}}$) and the apparent $K_m$ of the WT and mutant saPLC1 toward PI were obtained (Table 5.1). All mutants display decreases in $V_{\text{max}}$ by a factor of $10^3$-$10^5$ relative to that of WT, supporting the catalytic
importance of these residues predicted by sequence alignment studies. The $K_m$ values of these mutants are within the same order of magnitude as that of WT, which suggests that the substrate binding is not likely affected upon these single mutations. Further supports for specific functional roles of these residues are provided by additional experiments described in the next section.

5.3.3 Possible Functional Roles of Three Critical Acidic Residues. The residues Asn17, Glu39, and Asp41 of saPLC1 align with Asn312, Glu341, and Asp343 of PLC-δ1, which were suggested to be responsible for Ca$^{2+}$ binding as well as potential nucleophiles$^{10}$. To better understand the catalytic metal binding site and, most importantly to investigate the possibility of Glu39 (the counterpart of Glu341 in PI-PLCδ1) being a potential general base in phosphotransferase reaction, we applied additional amino acid replacements at the following residues: Asn17 was mutated to glutamine, Glu39 to aspartate and glutamate, and Asp41 to glutamate and asparagine. If these residues are involved in metal binding, upon these mutations, one or several properties of the metal binding site would be altered, e.g., size, coordination number and geometry, and ligand preference. Substitution of aspartate with asparagine or extension of asparagine to glutamine (or aspartate to glutamate) by one methyl unit could be as deleterious as deleting the carboxylic group. In order to test this hypothesis, both steady-state kinetic analyses and Ca$^{2+}$ concentration dependence studies were performed. As listed in Table 5.1, mutating these acidic residues generally leads to increased $K_{d,app}$ toward Ca$^{2+}$. The effects are greater for amide substitutions (of carboxylate side chain). This could be due to the fact that amide is strongly unfavorable to Ca$^{2+}$. At saturating
Ca$^{2+}$ concentrations, the $V_{\text{max}}$ values of these conservative mutants, except E39Q, were similar to those of alanine mutants.

The impacts (catalytic rate and metal ion preference) of amino acid substitution at Asn17, Glu39, and Asp41 provide experimental evidence that these acidic residues are important in metal coordination. It seems that the precise coordination distances and geometry are both critical in modulating the metal cofactor to the proper position (so that it can coordinate both the inositol 2-OH and pro-$S$ oxygen of the phosphate moiety) for efficient catalysis. It is interesting to note that the E39Q mutation causes a $10^6$-fold decrease in $V_{\text{max}}$ compared to WT, whereas the E39D substitution reduces ($10^3$-fold) $V_{\text{max}}$ to a much lesser extent. On the basis of the structural information $^5,6$, Glu341, the counterpart of Glu39 in mammalian PI-PLC-$\delta_1$, was suggested to be the general base in the phosphotransferase reaction. On the basis that Gln39 could coordinate Ca$^{2+}$ but could not function as a nucleophile, whereas Asp39 could function as a nucleophile though less efficiently; our results favor the hypothesis that Glu39 acts both as a metal ion ligand and a general base in the phosphotransferase reaction (Figure 5.2). A single mutation might lead to a local conformational change or perturbed the hydrogen bond network within the active site; therefore additional structural and functional studies are necessary to identify the exact functional roles of Glu39.

5.3.4 Activities toward Water-Soluble Phosphodiesters. To estimate the effect of the leaving group (the presence of hydrophobic leaving group) on substrate reactivity, we tested the activity of saPLC1 toward deacylated substrate analog: sn-glycero-3-(1-phospho-1D-myoinositol) (GPI). This change in hydrophobicity is achieved without a
significant change in pK\textsubscript{a} of the leaving group (diacylglycerol vs. glycerol). Like PI hydrolysis by saPLC1, which yields acyclic phosphate product, the hydrolysis of GPI by saPLC1 only produces IP. As shown in Figure 5.3, the WT saPLC1 has low \( V_{\text{max}} \) and high \( K_{\text{m}} \) toward GPI (\( V_{\text{max}} = 19.7 \pm 1.3 \) U/mg, \( K_{\text{m}} = 26.3 \pm 4.4 \) mM). Therefore, the removal of fatty acids from the diacylglycerol moiety results in a 10\(^2\)–fold decrease in \( V_{\text{max}} \) and a 10\(^2\)–fold increase in \( K_{\text{m}} \), consequently, a 10\(^4\)–fold reduction in catalytic efficiency (\( k_{\text{cat}}/K_{\text{m}} \)). A similar result has been reported previously for mammalian PI-PLC-\(\delta\)1 and btPLC\(^{21,58,84}\). The kinetic parameters of these three PI-PLC enzymes are summarized in Table 5.2.

If a sequential mechanism of PI hydrolysis for saPLC1 is operational, IcP must be a substrate for this enzyme. The ability of saPLC1 to hydrolyze IcP was thus tested. The dependence of the enzyme activity on IcP concentration is shown in Figure 5.4. The \( K_{\text{m}} \) value for IcP is comparable to that for GPI. Surprisingly, however, \( V_{\text{max}} \) toward IcP is 10\(^3\)–fold lower than that for GPI (Table 5.2), which leads to a 10\(^3\)–fold decrease in catalytic efficiency (\( k_{\text{cat}}/K_{\text{m}} \)). The kinetic parameters summarized in Table 5.2 raise several important points.

(i) All these enzymes show 10\(^{2-3}\)–fold decreases in \( V_{\text{max}} \) and 10\(^{2-3}\)–fold increases in \( K_{\text{m}} \) toward deacylated substrates analogs, resulting drastic reduced catalytic efficiencies (\( k_{\text{cat}}/K_{\text{m}} \)). It is evident that the presence of hydrophobic leaving group is important for retaining the high catalytic efficiency.

(ii) Both mammalian PI-PLC\(\delta\)1 and btPLC demonstrate higher activity toward IcP than toward GPI, possibly because the strain energy of IcP (due to the formation of five-member ring) can be released during the hydrolysis of IcP to generate I-1-P\(^{23}\). In stark
contrast, saPLC1 shows $10^3$–fold higher $k_{\text{cat}}$ toward GPI than toward IcP, while bearing similar $K_m$ toward both substrates. Two possible explanations can be proposed for saPLC1:

\[
\begin{align*}
\text{IcP} + E & \underset{k_{-1}}{\underset{k_1}{\rightleftharpoons}} \text{IcP}E \\
& \overset{k_2}{\rightarrow} \text{IP E}
\end{align*}
\]

\[
\begin{align*}
\text{GPI} + E & \underset{k_{-1}'}{\underset{k_1'}{\rightleftharpoons}} \text{GPI}E \\
& \overset{k_2'}{\rightarrow} \text{IcP E} \overset{k_3'}{\rightarrow} \text{IP E}
\end{align*}
\]

(a) If $K_m$ values are good representation of $K_d$ values ($k_{-1} >> k_2$) in both cases, saPLC1 should be extremely incompetent in hydrolyzing IcP. It is possible that the initial protonation states of the general acid and general base are to accommodate the phosphotransferase reaction, thus disfavor of the second step of the reaction. Consequently, IcP cannot readily be hydrolyzed by saPLC1. Alternatively, (b) if $k_{-1} \approx k_2$ or $k_{-1} << k_2$, the high $K_m$ value ($K_m = (k_2 + k_{-1}) / k_1$) toward IcP indicates a fast hydrolysis step versus an extremely slow binding step. This binding step, instead of the chemistry, is the rate-limiting step for IcP hydrolysis.

(iii) BtPLC can catalyze both the transphosphorylation of PI to form IcP and IcP hydrolysis to form IP. But btPLC evolved primarily to catalyze transphosphorylation rather than hydrolysis. In order to regenerate the enzyme after the “short circuit” one step reaction, btPLC needs to have an iso mechanism \(^{85}\) in which the protonation states of the unliganded enzyme are inter-converted by a pathway that does not involve substrates.
molecules. Theoretically, this iso mechanism (the reverse reaction of this pathway) facilitates IcP hydrolysis by btPLC.

5.3.5 Parameters that Affect Product profiles. Several factors that affect the product profile of mammalian PI-PLCs were tested on saPLC1. Temperature, pH, and Ca\(^{2+}\) concentration did not affect the product profile of saPLC1.

\[ \begin{align*}
    \text{E PI} & \xrightarrow{k_1} \text{E IcP} \xrightarrow{k_2} \text{E IP} \\
    & \quad \downarrow k_{on} \quad \downarrow k_{off} \\
    & \quad \downarrow \text{IcP}
\end{align*} \]

As explained previously, whether IcP can be released from the active site depends on the relative microscopic rate constants – \(k_2\) versus \(k_{off}\). Can mutations, which drastically decrease \(k_1\) and \(k_2\), trigger the release of IcP? The answer is no. \(^{31}\)P-NMR analyses showed that all mutants (N17A, E39A, E39Q, D41A, H55A), except H16A, maintain the same product profile as the WT enzyme. PI hydrolysis by E39Q and H55A are demonstrated in Figure 5.5. Assuming these mutations do not perturb IcP binding \((k_{off})\), the reduced \(k_2\) value should still be substantially higher than \(k_{off}\). The observed IcP production by H16A should come from two effects: decreased \(k_2\) and increased \(k_{off}\). On the basis of the stereochemical studies in Chapter 4, His16 was suggested to interact with the pro-Sp oxygen. We speculate that this interaction is also crucial for IcP binding, which leads to an extremely low \(k_{off}\).
5.3.6 $^{15}\text{N}-^1\text{H}$ HSQC NMR as A Monitor of Global Protein Structure. NMR spectroscopy is widely used to investigate protein-ligand interactions\textsuperscript{86,87} due to its ability to monitor conformational changes of both proteins and small ligands in solution. In addition, heteronuclear NMR enable us to specifically monitor the conformation changes of the molecule of the interest.

The ligand used in our investigation was 2-amino-2-deoxy-dhPI, which is stable for days even in the presence of excess enzymes. Inhibition studies (Figure 5.6) showed that this substrate analog is a competitive inhibitor of the substrate. 2-amino-2-deoxy-dhPI has a small $K_{\text{I,app}}$ value (0.232 mM), which is comparable to the $K_{\text{m,app}}$ value of DOsPI at the same assay condition. In this analog, the aryl groups are attached to the glycerol moiety via ether bonds rather than ester bonds. This leads to a high CMC value, which ensures the monomeric form of the analog in the titration range of the experiments (one approach to prevent the line-boarding effect).

In the $^{15}\text{N}-^1\text{H}$ HSQC spectrum of the uniformly $^{15}\text{N}$ labeled saPLC1, there are 6 and 201 signals in the arginine side chain ($^6\text{NH}$) region and the amide backbone region, respectively (Figure 5.7). Without total NMR assignment of the backbone amide signals, the $^1\text{H}-^{15}\text{N}$ HSQC spectrum is not very informative about the changes occurring in the protein. On the other hand, the change in the spectrum could provide useful information for the binding event. In the absence of Ca$^{2+}$, no obvious spectroscopic changes could be observed in the $^{15}\text{N}-^1\text{H}$ HSQC spectrum upon addition of 0.2 mM 2-amino-2-deoxy-dhPI (Figure 5.6). Upon adding calcium ions to this system, about 30 signals in the amide backbone region shift or move to different positions (Figure 5.8).
These results suggest that 2-amino-2-deoxy-dhPI binds to the enzyme in a Ca	extsuperscript{2+} -
dependent manner.

As a control, we also examined the changes in the $^{15}$N-$^1$H HSQC spectrum of
saPLC1 upon Ca	extsuperscript{2+} titration. As shown in Figure 5.9, about 50 signals in the amide
backbone region are subject to drastic changes upon Ca	extsuperscript{2+} addition. This phenomenon is
common for enzymes that use metal ions as cofactors. Interestingly, overlaying three
spectra (free enzyme, enzyme-Ca	extsuperscript{2+}, enzyme-Ca	extsuperscript{2+}-inhibitor) shows that most of the
signals, which were subject to changes upon inhibitor binding, experience alternations
upon calcium binding. This ultimately suggests that Ca	extsuperscript{2+} and 2-amino-2-deoxy-dhPI
binds to saPLC1 in a closely related region, possibly the active site.

Since our final goal is to solve the complex structure of saPLC1 by X-ray or NMR,
this set of preliminary binding studies provides promising results for the future work.

5.4 CONCLUSION AND FUTURE PERSPECTIVES

The mutagenesis studies in this chapter further support that saPLC1 utilizes a
similar catalytic mechanism as that of mammalian PI-PLCs. On the basis of the different
catalytic behaviors toward PI, GPI, and IcP, saPLC1 might utilize a “switch” that tightly
controls the binding of the product intermediate. We hypothesize that saPLC1 possesses
a transient conformation/protonation state between the first and second step of the
reaction. This conformation, which is responsible for the tight binding toward IcP in
saPLC1, might not exist in btPLC. How exactly this transient state is perturbed or
regulated, which in turn leads to altered microscopic rate constants ($k_2$ and $k_{off}$), is a very
intriguing question. More detailed mechanistic and structural studies on this small
saPLC1 will be extremely beneficial for comprehending how IcP release is regulated in different mammalian PI-PLC isozymes.
Table 5.1 Summary of Kinetic Data for the WT and mutant saPLC1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}^b$</th>
<th>$K_{\text{m, app}}^b$</th>
<th>$K_d (\text{Ca}^{2+})^c$</th>
<th>$V_{\text{max}}^b$</th>
<th>$K_{\text{m, app}}^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1122 ± 22</td>
<td>58 ± 7</td>
<td>4.2 ± 0.9</td>
<td>678 ± 28</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>H16A</td>
<td>0.024 ± 0.001</td>
<td>16 ± 3</td>
<td>65 ± 20</td>
<td>0.044 ± 0.002</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>N17A</td>
<td>1.03 ± 0.02</td>
<td>39 ± 6</td>
<td>11 ± 3</td>
<td>1.09 ± 0.02</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>N17Q</td>
<td>2.45</td>
<td>ND$^d$</td>
<td>435 ± 54</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E39A</td>
<td>0.112 ± 0.006</td>
<td>125 ± 32</td>
<td>25 ± 8</td>
<td>0.21 ± 0.01</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>E39D</td>
<td>2.08 ± 0.04</td>
<td>12 ± 2</td>
<td>4.4 ± 0.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E39Q</td>
<td>0.0077 ± 0.0004</td>
<td>76 ± 19</td>
<td>196 ± 62</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D41A</td>
<td>0.053 ± 0.004</td>
<td>205 ± 72</td>
<td>6.0 ± 0.9</td>
<td>0.041 ± 0.001</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>D41E</td>
<td>0.049 ± 0.001</td>
<td>17 ± 3</td>
<td>26 ± 6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D41N</td>
<td>0.026</td>
<td>ND</td>
<td>96 ± 19</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H55A</td>
<td>0.273 ± 0.005</td>
<td>23 ± 3</td>
<td>26 ± 9</td>
<td>107 ± 8</td>
<td>36 ± 10</td>
</tr>
</tbody>
</table>

$^a$Measured at 37 °C, 0-2.0 mM PI(or DOsPI) and PI(or DOsPI)/Triton X-100 = 5 in 40 mM HEPES, 2 mM CaCl$_2$, 1 mM EDTA, pH 7.0. Activities toward PI and DOsPI are determined by the radioactivity assay and spectroscopic assay, respectively. $^b$V$_{\text{max}}$ and $K_{\text{m}}$ values are estimated from the equation $1/V = K_{\text{m, app}} / V_{\text{max}} [S] + 1 / V_{\text{max}}$, where [S] is the initial substrate concentration. $V_{\text{max}}$ values are expressed in µmoles mg$^{-1}$ min$^{-1}$. $K_{\text{m}}$ values are expressed in µM. $^c$In µM. $^d$ND = not determined.

Table 5.1 Summary of Kinetic Data for the WT and mutant saPLC1$^a$
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$V_{\text{max}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(U mg$^{-1}$)</td>
<td>(mM)</td>
<td>(M$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>saPLC1$^a$</td>
<td>Gro-PI</td>
<td>19.7 ± 1.3</td>
<td>26.3 ± 4.4</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td>W/Triton</td>
<td>0.0178 ± 0.0008</td>
<td>37.1 ± 4.7</td>
<td>0.294</td>
</tr>
<tr>
<td></td>
<td>IcP W/Triton</td>
<td>0.0149 ± 0.0006</td>
<td>46.3 ± 5.3</td>
<td>0.322</td>
</tr>
<tr>
<td></td>
<td>IcP W/O Triton</td>
<td>4.8 ± 0.6</td>
<td>4.4 ± 1.1</td>
<td>1300</td>
</tr>
<tr>
<td>Mammalian</td>
<td>GPIP</td>
<td>0.79 ± 0.08</td>
<td>1.8 ± 0.4</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>GPI</td>
<td>0.001</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>IcP</td>
<td>0.40 ± 0.03</td>
<td>25.2 ± 2.9</td>
<td>19</td>
</tr>
<tr>
<td>btPLC</td>
<td>GPI</td>
<td>1.2</td>
<td>88</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>IcP</td>
<td>20</td>
<td>90</td>
<td>130</td>
</tr>
</tbody>
</table>

$a$ The reaction rate were determined by $^{31}P$ NMR. Assay conditions include 50 mM HEPES, pH 7.0, 20% D$_2$O, 2 mM CaCl$_2$, 1 mM EDTA, with or without 50mM Triton X-100, 27 °C. 3 µg and 540 µg of saPLC1 were used for substrates Gro-PI and IcP, respectively. $V_{\text{max}}$ and $K_m$ values are estimated from the equation $1/V = K_m^{\text{app}} / V_{\text{max}} [S] + 1 / V_{\text{max}}$, where [S] is the initial substrate concentration. $^b$ ref. $^{58}$ $^c$ ref. $^{21}$ $^d$ ref. $^{84}$

$^e$ Calculated based on the molecular weight of saPLC1, rat Δ (1–132) PI-PLCδ1, and btPLC are 36.7 kDa, 70.5 kDa, and 34.5 kDa, respectively.

**Table 5.2** Kinetic Parameters for Hydrolysis of Water-Soluble Phosphodiesters by saPLC1, mammalian PI-PLCδ1, and btPLC.
Figure 5.1  Two-dimensional NOESY spectra of the WT and the mutant saPLC1 in D$_2$O at 600 MHz: (A) WT, (B) H16A, (C) N17A, (D) E39A, (E) E39Q, (F) D41A, and (G) H55A. The samples contained 0.5 mM enzyme and 1 mM HEPES (pH 7.0).
Figure 5.2 The proposed transition state of saPLC1. DAG = diacylglycerol.
Figure 5.3 Specific activity versus GPI concentration for WT saPLC1. Assay condition include 50 mM HEPES, pH 7.0, 20% D$_2$O, 2 mM CaCl$_2$, 1 mM EDTA, with 50mM Triton X-100, 27 °C and 3 µg of saPLC1. The curve was drawn by fitting the data with the Michaelis-Menton equation $1/V = K_m^{app} / V_{max} [S] + 1/ V_{max}$, and the parameters are summarized in Table 2.
Figure 5.4  Specific activity versus lcP concentration for WT saPLC1 in the presence (○) and in the absence (●) of 50mM Triton X-100. Assay conditions include 50 mM HEPES, pH 7.0, 20% D₂O, 2 mM CaCl₂, 1 mM EDTA, 27 °C, and 540 µg of saPLC1. The curves were drawn by fitting the data with the Michaelis-Menten equation \( \frac{1}{V} = \frac{K_{m}^{app}}{V_{\text{max}}} + \frac{1}{V_{\text{max}}} [S] + \frac{1}{V_{\text{max}}} \), and the parameters are summarized in Table 2.
Figure 5.5 $^{31}$P NMR assay of PI hydrolysis catalyzed by 10 mg E39Q (A) and 2.7 mg H16A (B). Spectrum (a) was taken before addition of enzyme. Spectrum A(b) was taken 27 min after addition of enzyme. Spectra A(c-e) were taken every 100 min. Spectrum B(b) was taken 6 min after addition of enzyme. Spectra B(c-e) were taken every 30 min.
Figure 5.6 Lineweaver-Burk plots for WT saPLC1 using 2-amino-2-deoxy-dhPI as inhibitors. The $K_{i,app}$ of this nonhydrolysable inhibitor is calculated by using the equation: $K_{m,app} = K_{m,app} (1 + [I_0]/K_{i,app})$, where $K_{m,app}$’ is the $K_{m,app}$ in the presence of an initial concentration of the competitive inhibitor $[I_0]$. The $K_{i,app}$ values of 2-amino-2-deoxy-PI determined from this plot are 0.232 mM. The $K_{m,app}$ of DOsPI at this assay condition is 0.03 mM.
Figure 5.7 Backbone amide region and arginine side chain ($^6$NH) region of $^{15}$N-$^1$H HSQC spectra of uniformly $^{15}$N labeled saPLC1 acquired at 800 MHz at 310 K. $^{15}$N-$^1$H HSQC spectra of saPLC1 in the absence (blue) and in the presence (red) of 0.2 mM 2-amino-2-deoxy-dhPI. Samples are in 90% D$_2$O/10%H$_2$O, 50 mM HEPES, 1 mM EDTA, pH 7.0. The protein concentration is ca. 0.2 mM.
Figure 5.8 Backbone amide region and arginine side chain (εNH) region of $^{15}$N-$^1$H HSQC spectra of uniformly $^{15}$N labeled saPLC1 acquired at 800 MHz at 310 K. $^{15}$N-$^1$H HSQC spectra of saPLC1 in the absence (blue) and in the presence (red) of 0.2 mM 2-amino-2-deoxy-dhPI. Samples are in 90% D$_2$O/10%H$_2$O, 50 mM HEPES, 1 mM EDTA, 1.5 mM CaCl$_2$, pH 7.0. The protein concentration is ca. 0.2 mM.
Figure 5.9 Backbone amide region and arginine side chain (εNH) region of $^{15}$N-$^1$H HSQC spectra of uniformly $^{15}$N labeled saPLC1 acquired at 800 MHz at 310 K. $^{15}$N-$^1$H HSQC spectra of saPLC1 in the absence (blue) and in the presence (red) of 0.5 mM free Ca$^{2+}$. Samples are in 90% D$_2$O/10%H$_2$O, 50 mM HEPES, 1 mM EDTA, pH 7.0. The protein concentration is ca. 0.2 mM.
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