Investigation of the Mechanism of Lipid Interfacial Activation of Bacterial and Mammalian Phosphatidylinositol-specific phospholipase C

Author: Su Guo

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Investigation of the Mechanism of Lipid Interfacial Activation of Bacterial and Mammalian Phosphatidylinositol-specific phospholipase C

a dissertation

by

SU GUO

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Investigation of the Mechanism of Lipid Interfacial Activation of Bacterial and Mammalian Phosphatidylinositol-specific phospholipase C

Su Guo

Thesis Advisor: Professor Mary F. Roberts

Abstract

Phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves the substrate phosphatidylinositol through two steps: the first step occurs in the interface between lipid and solution, while the second step only takes place in water soluble environment. For interfacial catalysis, the enzyme should bind to the lipid surface first before engaging its substrate, therefore interfacial kinetics include both interfacial binding and an interfacial catalytic step.

The *Bacillus thuringiensis* PI-PLC is activated by binding to zwitterionic surfaces; phosphatidylcholine (PC) and two tryptophan residues (Trp47 in the two-turn helix B and Trp242 in a disordered loop) at the rim of the barrel structure, in particular, are critical for this interaction. The helix B region in PI-PLC orients the side chains of Ile43 and Trp47 so that they form a hydrophobic protrusion from the protein surface that likely facilitates initial membrane binding. An earlier crystal structure of the dimeric W47A/W242A mutant, which is unable to bind to PC, showed that the helix B region was reorganized into an extended loop. Whether this conformational change occurred in the wild type PI-PLC was tested with a series of
mutations targeting helix B residues and surrounding regions. Results strongly suggest that, while hydrophobic groups and presumably an intact helix B are critical for the initial binding of PI-PLC to membranes, disruption of helix B to allow enzyme dimerization is likely to play a role in the activated PI-PLC conformation.

Besides the helix B residues, a number of hydrophobic residues along the rim of the αβ-barrel and close to both helix B and the active site were also altered to assess their contribution to membrane binding and kinetic activation. Results showed that Tyr86 and Tyr88, but not Tyr118, contribute to the protein binding to PC vesicles. These residues are capable of cation-π interactions with the choline headgroup of the phospholipid PC.

Although mammalian PLCδ1 is a complex multidomain protein, the catalytic domain resembles the bacterial PI-PLC enzymes. Little work has been done to characterize the extent to which this domain contributes to membrane binding. A mutated protein that removes the very anionic X/Y linker region that covers the active site was constructed. The interfacial binding and the corresponding enzyme activity of this mutant against WT were measured in both micelles and large unilamellar vesicles. The results showed at µM protein concentration there was no large difference between the PLCδ1 and the deletion mutant in terms of vesicle binding. However, the deletion mutant showed much higher membrane binding affinity at nM concentrations. These results shed some light on the activation or inhibition role of the catalytic domain and pointed to a possible direction of future studies, for example examining specific mutant enzymes in the interfacial loop region.
this dissertation is dedicated to

my husband: Yu Zhao
Acknowledgements

Coming to the United States for graduate school was without doubt the biggest challenge I have faced in my life so far. I feel really fortunate that I was admitted to Boston College, where I have enjoyed research and life for the past seven years.

First of all I would like to thank Prof. Mary Roberts for all her education, support and help throughout these years. Mary welcomed me to her group as a foreign student who was having much difficulty understanding what people around were talking about. She is always there for me whenever and whatever help I might need. As my advisors, she taught me a lot of things about chemistry, biology and how to carry out research. Her influence on me will be a fortune for the rest of my life.

During the last two years of my research I have had the pleasure collaborating with Prof. Anne Gershenson at Brandeis University (later at University of Massachusetts at Amherst). Prof. Gershenson helped me a lot with FCS experiments. Her precise attitude in doing science impressed me very much.

Special thanks are expressed to Dr. Jianwen Feng and Ms. Xin Zhang, who have done beautiful work in the group and helped me initiate my research on bacteria PI-PLC. Their theses are where I find all the useful procedures and inspiration for my own work.

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Over the years I have collaborated with quite a few talented undergraduate students at
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>B. cereus</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td><em>Bacillus thuringiensis</em></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C2</td>
<td>protein kinase C conserved region 2</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>cIP</td>
<td>D-\textit{myo}-inositol 1,2-cyclic phosphate</td>
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<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>CSA</td>
<td>chemical shift anisotropy</td>
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<td>diC_{4}PI</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DOPA</td>
<td>1,2-dioleoyl-phosphatidic acid</td>
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<td>DOPG</td>
<td>1,2-dioleoyl-phosphatidyglycerol</td>
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<td>DOPS</td>
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<td>dithiothreitol</td>
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<td>E. coli</td>
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<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
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<td><em>L. monocytogenes</em></td>
<td><em>Listeria monocytogenes</em></td>
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<td>LUV</td>
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<td>1-oxyl-2,2,5,5-tetramethylpyrroline-3-methylmethanethiosulfonate</td>
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<td>PMe</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<td>------------</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-phosphatidylcholine</td>
</tr>
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<td>QFF</td>
<td>Q-sepharose fast flow</td>
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<td>TX-100</td>
<td>Triton X-100</td>
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<td>phospholipase C-related, catalytically inactive proteins</td>
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<td>( X_{PC} )</td>
<td>mole fraction of PC</td>
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Chapter 1

Introduction

II. Membrane and lipids

Biological membranes form the boundary of cells and separate the cells from the outside. Cell membranes have selective permeability. They contain transport systems that allow specific molecules to be internalized and unwanted compounds to be exported from the cell. The main components of these selective permeability barriers are lipids and proteins. The lipid bilayer forms the basic matrix of biological membranes and proteins are embedded in this sea of lipids.\(^1\) The dry weight ratio of protein to lipid ranges from 1:4 to 4:1. Carbohydrates that are covalently linked to lipids and proteins are also part of the membrane. Membranes play a variety of biological roles including 1) defining the boundaries and serving as permeability barriers; 2) sites of specific functions; 3) detection and transmission of chemical and electrical signals; 4) regulation of the transport of solutes; and 5) mediation of cell-to-cell communications.

The formation of a bilayer structure by the amphiphilic lipid molecules in aqueous media is an energetically preferred process based on the hydrophobic effect. There are mainly three types of membrane lipids: cholesterol (in mammals but often other sterols in different eukaryotes, e.g., plants), glycolipids, and phospholipids.

Cholesterol is a lipid based on a steroid nucleus and built from four fused hydrocarbon rings (Figure 1-1A). A hydroxyl group is linked to the steroid at one end, and a hydrocarbon tail is attached at the other end. Cholesterol is an important
component of mammalian cell membranes and plays essential role in regulating membrane permeability and fluidity. Cholesterol is also an important precursor for the synthesis of steroid hormones, bile acids, and fat-soluble vitamins including Vitamins A, D, E and K. In the mammalian cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction.

**Figure 1-1. Structure of cholesterol and glycolipids.**

A. cholesterol:

B. Glycolipid (sphingosine moiety is highlighted in red):

Glycolipids are sugar-containing lipids that are usually derived from sphingosine with one or more sugars linked to the hydroxyl group of the sphingosine backbone (Figure 1-1B). Sugars can also be attached to a diacylglycerol backbone. Glycolipids always lie on the extracellular side of the plasma membrane and serve as markers for cellular recognition.
A. Phospholipids

1. Structure of phospholipids

Figure 1-2. General structure and classification of phospholipids.

Phospholipids, the major type of membrane lipids, are amphiphilic molecules that are composed of three components: a backbone that is in most cases glycerol, and two hydrophobic fatty acyl chains as well as a phosphate-containing polar or charged head group that are covalently linked to the glycerol backbone (Figure 1-2). Phospholipids are classified according to the identity of their polar head group; they can contain a wide variety of fatty acids serving as the hydrophobic tails. The
common head groups are the amino acid serine, ethanolamine, choline, glycerol, methanol, and the inositol. The net charge of a phospholipid depends on the head group and varies from 0 to -2 under physiological conditions for the lipids shown.

Phospholipids based on backbones other than glycerol also occur. Sphingomyelin, which is found in animal cell membranes, for example, is generated from sphingosine, the common backbone for glycolipids (Figure 1-1B). In humans, sphingomyelin is believed to be the only cell membrane phospholipid not derived from glycerol. As shown in Figure 1-3, the fatty acid is linked to the sphingosine backbone by an amide bond and phosphorylcholine is link to the hydroxyl group of sphingosine by an ester bond. The functions of sphingomyelin remained unclear until recently, when this molecule and some of its precursors and hydrolysis products were found to have roles in signal transduction (particularly apoptosis)².

Figure 1-3. Structure of sphingomyelin.

2. Aggregation of phospholipids

In the amphiphilic structure of membrane lipids, the two hydrophobic fatty acyl chains are approximately parallel to each other, while the hydrophilic head group points in the opposite direction. A consequence of the amphiphilic nature of the molecules is the formation of membrane structures by lipids. The polar head groups like to contact water, while the hydrophobic tails like to interact with one another, in
preference to water. The structure and size of the aggregates produced depend on size, and shape of the lipids. At the air-water interface of aqueous media, phospholipids always form monolayers with the heads pointing toward the water sub-phase and the chains toward the air. In aqueous solution, phospholipid molecules are present as monomers as well as aggregates with different forms present depending on the length and number of the fatty acid chains (Figure 1-4): a micelle is a globular structure in which the lipids’ head groups are surrounded by water and hydrocarbon chains kept inside, a vesicle is a small membrane-enclosed sack that can be visualized as a bubble of liquid within another liquid.

**Figure 1-4. Physical states of phospholipids in aqueous solution.**

In general, short-chain phospholipids with chain length no longer than eight carbons tend to form micelles where the shape of micelles varies with the chain length,
the concentration of lipid, and the surface area of the head group. Long-chain phospholipids form bilayer vesicles. When phospholipids are dispersed in water, they from multilamellar vesicles (MLV) which have more than one phospholipid bilayer (much like the form of an onion with multiple layers). If a vesicle has a single bilayer it is called a unilamellar vesicle. Multilamellar vesicles (MLVs) formed spontaneously, while unilamellar vesicles require input of energy to convert them to single bilayer particles. Small unilamellar vesicles (SUVs), with diameters in the range of 25–50 nm, can be prepared by sonication from MLVs. Large unilamellar vesicles (LUVs), typically from 100 nm to 1 µm in diameter, are usually prepared by extrusion through polycarbonate filters (although other methods of formation exist for specific lipids).

A wide variety of factors, including hydrocarbon unsaturation, head group size, temperature, ionization, and hydration, can modulate the structural preferences of the lipid system. This behavior can be related to a generalized ‘shape’ property of lipid molecules. The dimensionless shape parameter $S = \frac{v}{a_0 l_c}$ is defined as a basic packing property. Here $a_0$ is the optimal surface area per molecule at the lipid-water interface; $v$ represents the volume of the hydrophobic chains per molecule; $l_c$ is the maximum length of the fully extended acyl chains. The S parameters that are related to the corresponding molecular shapes are summarized in Table 1-1. This simple treatment does an excellent job of predicting particle morphology for lipids.
Table 1-1. Shape factor and the corresponding phase.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Lipid Shape</th>
<th>Shape Factor</th>
<th>Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micellar</td>
<td><img src="image" alt="Micellar" /></td>
<td>S&lt;1</td>
<td>Inverted Cone</td>
</tr>
<tr>
<td>Bilayer</td>
<td><img src="image" alt="Bilayer" /></td>
<td>S=1</td>
<td>Cylindrical</td>
</tr>
<tr>
<td>Hexagonal</td>
<td><img src="image" alt="Hexagonal" /></td>
<td>S&gt;1</td>
<td>Cone</td>
</tr>
</tbody>
</table>

3. **Function of phospholipids**

Phospholipids play multiple roles in cells.\(^6\) First, as the major class of membrane lipids, phospholipids form the plasma membrane bilayer and intracellular membranes. Secondly, they work as the supporting matrix and surface for many catalytic processes and directly affect membrane protein structure and function. For example, they help some peripheral proteins anchor to the membrane. Clusters of particular phospholipids have been implicated as platforms for attracting proteins involved in diverse pathways. Thirdly, they are involved in signal transduction. For example, phosphatidylserine plays a role in the eukaryotic cell as an indicator of
apoptosis or programmed cell death. Lastly, they provide precursors for macromolecular synthesis.

**III. Role of peripheral membrane proteins in cells**

Peripheral membrane proteins refer to those that are only temporarily adsorbed on the biological membrane. These proteins attach to the peripheral regions of the lipid bilayer or to the integral membrane proteins embedded in the bilayer. For example, many regulatory subunits of ion channels or transmembrane receptors may be defined as peripheral membrane proteins.

Eukaryotic cells contain a large number of distinct compartments or organelles separated from the bulk cytoplasm by bilayer membranes. During membrane trafficking, proteins form transport vesicles which then move to and fuse with the correct acceptor organelle. A large number of proteins are involved in these processes, most of which, however, are peripheral membrane proteins that are recruited directly from the cytosol rather than integral membrane proteins. An explanation is that the peripheral protein can be recruited to the organelle where they will function directly, while the integral membrane protein must be initially inserted into the membrane of the ER and then moved through several compartments to reach the site of action. Moreover, if they are misrecruited, correction for the peripheral protein will be much easier since release from the membrane will place them back in solution. Many peripheral proteins play important roles in membrane trafficking, including the coat proteins which form vesicles, the motors that move vesicles, and the tethers which provide the specificity of vesicle fusion. Several groups of proteins are involved in
helping peripheral membrane proteins get recruited to the membrane, including small GTPases such as the ARF/sar family, Rab GTPases and some lipid species such as the phosphoinositides, and diacylglycerol (DAG)\textsuperscript{9,10}.

Two classes of G proteins, the ARF/sar family and the Rab family, can recruit a large number of peripheral membrane proteins. The ARFs are divided into three classes. ARF1 is the most studied protein in ARF family. The ARF-GDP form shows only weak binding to the lipid bilayer. After being activated by GEF (guanine exchange factor) family, which share a Sec7 domain that is sufficient for GTP exchange, ARF-GTP undergoes a conformational change and can now tether other proteins to the bilayer. ARF1 is required for recruiting lots of proteins to Golgi membranes; proteins recruited include coat protein complex I (COPI) and clathrin coats\textsuperscript{11}. For the other big family of Rab proteins, many of Rab GTPases are only associated with a single organelle or membrane-trafficking step. A number of effector proteins, which are highly organelle-specific, have been identified that are recruited by binding to the GTP form of Rab. Rab can be specifically activated by GEF. But unlike the ARF proteins, the GDP form of Rab can still be membrane-associated because the conformation of the membrane-anchoring part is independent of the nucleotide state. However the GDP form of Rab can be removed from the membrane by Rab GDP-dissociation inhibitor (GDI)\textsuperscript{12}.

Another major way of recruiting peripheral proteins is through the recognition of specific lipids. Different phosphoinositides play different roles in membrane trafficking. several lipid-specific targeting domains can recognize specific
phosphoinositides to mediate the recruitment of peripheral membrane proteins. For example, phosphatidylinositol (4,5)-bisphosphate (PI[4,5]P$_2$) is now recognized as an important plasma membrane signal that establishes sites for vesicular trafficking, membrane movement and actin cytoskeletal assembly$^{13,14}$. PI(4,5)P$_2$-binding domains are needed for a protein to interact with PI(4,5)P$_2$. The most characterized of PI(4,5)P$_2$-binding domains is the pleckstrin homology (PH) domain$^{15}$. Recently many other novel PI(4,5)P$_2$-binding domains have been identified, such as Lys/Arg-rich PI(4,5)P$_2$-binding domains$^{10}$. After recruitment by ARF protein, phosphoinositide kinases and phospholipase D (PLD) promote synthesis of PI(4,5)P$_2$ in specific membrane domains. In mammalian cells, PI(4,5)P$_2$ is thought to be the beginning of maintaining Golgi organization and protein trafficking through the Golgi. GTPases of the ARF and Rho families are key regulators of phosphoinositide kinases.$^{Error! Bookmark not defined.}$ They can recruit and regulate the activity of PI(4)P 5-kinase for the synthesis of PI(4,5)P$_2$ though activation of PLD, which generates PA as an activator of PI(4) 5-kinase. Besides phosphoinositides, other lipids such as diacylglycerol (DAG) and phosphatidylserine (PS) also play an important role in recruiting peripheral membrane proteins$^8$.

**IV. Phospholipases**

A phospholipase is an enzyme that catalyzes the hydrolysis of specific ester bonds in phospholipids to remove or modify phospholipids in the cell membrane. There are three major classes, phospholipase A, C and D that are distinguished by the site of hydrolysis they catalyze (Figure 1-5).
A. Phospholipase A (PLA)

The PLA superfamily catalyzes the hydrolysis of the fatty acyl side chains of membrane phospholipids and has two subtypes - phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂).

PLA₁ is an enzyme that hydrolyzes ester bonds of phospholipids at the sn-1 position and generates free fatty acids (FFA) and 2-acyl-lysophospholipids. PLA₁ activities have been detected in many cells and tissues. There are nine PLA₁ enzymes known in mammals; three are intracellular enzymes and the other six are extracellular enzymes. The intracellular and extracellular PLA₁s share no sequence homologies and apparently have distinct functions. Some PLA₁s have broad substrate scope and hydrolyze phospholipids, triacylglycerol (TG) and galactolipids (a subclass of glycolipids). By contrast, other PLA₁s such as phosphatidylserine (PS)-specific PLA₁ (PS-PLA₁), membrane-associated phosphatidic acid (PA)-selective PLA₁α
(mPA-PLA1α) and mPA-PLA1β show a strict substrate specificity and act specifically on PS and PA. However, there is only limited information available about the structure and molecular function on PLA1 isozymes.

The super family of phospholipase A2 (PLA2) enzymes consists of five distinct types of enzymes, the secreted PLA2s (sPLA2), the cytosolic PLA2s (cPLA2), the Ca2+ independent PLA2s (iPLA2), the platelet-activating factor acetylhydrolases (PAF-AH), and the lysosomal PLA2s. These PLA2s differ from each other in terms of substrate specificity, Ca2+ dependence and lipid modification. In mammalian cells, PLA2s hydrolyze phospholipids at the sn-2 position to generate 1-acyl-lysophospholipids and free fatty acids, notably arachidonic acid (AA). Both products play important roles in signaling pathways. For example, AA can be converted into eicosanoids through the action of a variety of proteins. After binding to specific G-protein coupled receptors, eicosanoids affect a wide range of physiological and pathological processes such as sleep regulation, immune responses, inflammation, and pain perception. The lysophospholipid is a precursor to generate lipid mediators such as lysophosphatidic acid (LPA) or platelet activating factor (PAF). After binding to G-protein coupled receptors, LPA is involved in cell proliferation, survival and migration and PAF is specifically involved in inflammatory processes.

B. Phospholipase D (PLD)

Phospholipase D, which is located in the plasma membrane, hydrolyzes the phosphodiester bond of the polar headgroup to generate phosphatidic acid (PA) and a related base. Many PLD enzymes can also carry out a transphosphatidyllation reaction
to exchange the base esterified to PA. Thus, they can generate a variety of phosphatidyl alcohols. The PA product of PLD can be converted into lyso-PA by a PLA$_2$ or converted into DAG by PA-phosphohydrolase. All known eukaryotic PLD superfamily members contain one or two copies of a conserved HKD motif (HxKxxxxD where x is any amino acid). These PLDs have four conserved regions (regions I–IV), and both regions II and IV contain one catalytic HKD motif. Most mammalian PLDs also have a Phox (PX) and a Pleckstrin (PH) homology domain, while plant PLDs have a C2 domain. PLD is present in plants, bacteria, yeast and mammalian cells and plays roles in signal transduction, vesicular trafficking, cytoskeletal rearrangements, membrane remodeling, and membrane lipid degradation.

C. Phospholipase C (PLC)

Phospholipase C (PLC) enzymes cleave the phosphodiester bond that connects the glycerol backbone and phosphate to release DAG and a phosphate ester. Phospholipase C enzymes play a central role in signal transduction. Phosphatidylinositol-specific PLC (PI-PLC) enzymes exhibit substrate preferences for PI and its phosphorylated derivatives in the case of the mammalian systems.

1. Bacterial PI-PLC

PI-PLCs have been isolated from bacteria, yeast, mold, plants, inserts and mammals. Of the currently characterized PI-PLCs, the bacterial PI-PLCs are secreted while the other enzymes are intracellular. The small bacterial PI-PLCs which can be produced by some pathogens, such as *Bacillus cereus*, *B. thuringiensis*, *Listeria*
monocytogenes, and Staphylococcus aureus are considered to be potential virulence factors.  

Figure 1-6. Two step PI-PLC catalyzed PI hydrolysis.

The catalytic mechanism of Bacterial PI-PLC

PI-PLCs from B. cereus and B. thuringiensis, which are very closely related, have received the most attention. They have been shown to catalyze a two-step reaction (Figure 1-6). In the first step, PI-PLCs work as a phosphotransferase. They cleave phosphatidylinositol and produce DAG and D-myoinositol 1,2-cyclic phosphate (cIP). In the second step, PI-PLCs work as a cyclic phosphodiesterase. The water-soluble intermediate product cIP is hydrolyzed, producing D-myoinositol 1-phosphate, I-1-P. The first reaction proceeds at a rate that is about 10³-times faster than that of the second reaction, which leads to the accumulation of cIP in the reaction mixture. A lot of attention has been focused on the first reaction because it provides information about the interaction of the enzyme with the membrane lipids.

Inositols are cyclohexanes substituted with one OH group at each carbon atom. The most abundant stereoisomer in nature is myo-inositol in which every hydroxyl
group is equatorial except for the 2-position. In PI, the phosphate is bonded to inositol at the 1-position. Bacterial PI-PLCs cannot hydrolyze PI molecules with phosphates on the 4- and 5-OH. Bacterial PI-PLCs are specific for the D-configuration of the esterified myo-inositol. L-myo-inositol is neither a substrate nor an inhibitor; it does not bind to the active site\(^\text{26}\). In contrast to this strict headgroup sensitivity, bacterial PI-PLCs are insensitive to the glycerol backbone or the lipid chains. All of these results suggest that there are multiple specific interactions between the active site residues and the hydroxyls of the myo-inositol, while the lipid part of the substrate either has little contact with the proteins or only binds to the enzyme though non-specific interactions.

Glycosylphosphatidylinositol (GPI) is another important substrate for PI-PLCs. In GPI there is a glycosidic bonds between the 6-OH group of the PI myo-inositol and a linear oligosaccharide. GPI-PLC activity is not found for any of the mammalian PI-PLCs. GPI is a unique substrate of the bacterial PI-PLCs, and the products are DAG and a glycosylated polypeptide terminating in cIP.

Bacterial PI-PLCs have a single polypeptide chain of similar length, about 300 amino acids. The two Bacillus PI-PLCs have very high degree of similarity - 98% since only eight amino acids are different. The Bacillus PI-PLC consists of a single globular domain which fold as a distorted (\(\beta\alpha\))\(_8\)-barrel, also called a TIM-barrel. The TIM-barrel is a conserved protein fold consisting of eight \(\alpha\)-helices and eight parallel \(\beta\)-strands\(^\text{26}\). The parallel \(\beta\)-strands form the inner wall of the barrel, whereas the \(\alpha\)-helices form the outer wall of the barrel. In Bacillus PI-PLC, the TIM-barrel is
disrupted in region IV/V and V/VI. A loop in region V/VI and an anti-parallel strand in region V/VI replace the missing helices\textsuperscript{25}. So the TIM-barrel structure is partially open in Bacillus PI-PLC. The sequence similarity of bacterial PI-PLC from different species is not very high. However, the secondary structures of bacterial PI-PLCs are very similar. The active site, which is near the surface, is between the N- and C-terminal halves. Crystal structures\textsuperscript{26} with or without myo-inositol show there is no obvious conformational change when Bacillus PI-PLC binds to the inositol portion of the substrate. The PI-PLC binds the inositol ring with the 2-, 3-, 4-, and 5- OH groups strongly coordinated with a network of hydrogen bonds. No close contact is found between the protein and 1- or 6-OH group of the myo-inositol ring (the latter interesting because the enzyme does hydrolyze GPI-anchors). There is also a planar stacking interaction between the apolar side of the myo-inositol ring (the face with the axial protons) and the phenol ring of Tyr-200.

The catalytic mechanism is a general base and general acid mechanism, similar to that for ribonuclease\textsuperscript{27} (Figure 1-7 A). The two sequential reactions of PI-PLC are sn2 reactions. Two key histidine residues are conserved in bacterial and mammalian PI-PLCs. In the Bacillus enzymes, these are His32 and His82. His32 loses its proton and forms a hydrogen bond with the 2-OH group of inositol and acts as a general base\textsuperscript{25}. The side chain of Asp274 forms a hydrogen bond with His32 to help stabilize the imidazole of His32 in the correct tautomeric state for base catalysis. Asp274, His32, and the 2-OH group of inositol ring work together to form a catalytic triad and increase the nucleophilicity of oxygen for the attack on phosphorus. At the
same time, the protonated form of His82 begins to donate its hydrogen to the ester bond (Figure 1-7 B). DAG and cIP are produced from the substrate PI after collapse of the transition state. In the reverse reaction, which is much slower, cIP is hydrolyzed to I-1-P. When cIP is generated in the active site, His82 is deprotonated; it must in turn deprotonate water to act as the nucleophile. Protonated His32 can then supply a proton to generate I-1-P. The accessibility to water in the active site (to easily reprotonate His82) and the high $K_m$ for cIP makes this reaction slow.
Figure 1-7. Mechanism of bacterial PI-PLC catalyzed PI hydrolysis (A) and crystal structure of the \textit{B. cereus} enzyme with active site residues shown and myo-inositol bound (B). (A) is adapted from ref (27); the structure in (B) is generated using Pymol.

A

\textbf{1st reaction}

\textbf{2nd reaction}

B
Interfacial activation and inhibition of B. thuringiensis PI-PLC by PC

PI does not dissolve in water but PI-PLC is water soluble, so this hydrolysis reaction takes place at the interface between lipid and solution. PI-PLC enzymes are modulated by interfaces in several ways. For interfacial reactions, the overall rate of catalytic turnover is affected not only by the kinetics of those interfacial catalytic steps but also by the kinetics of binding and desorption of the enzyme at the interface. *Bacillus thuringiensis* PI-PLC exhibits 'interfacial activation,' meaning that the activity is higher when PI is presented in an interface instead of as a monomer in solution.\(^{28}\)

When water-soluble cIP is used as the substrate of this cyclic phosphodiesterase, almost all detergents examined activate the enzyme at least 2-fold. However, phosphatidylcholine (PC) leads to the largest increase in cyclic phosphodiesterase activity\(^ {29}\) (as show in Figure 1-8 A). The enhanced activity generated by this non-substrate phospholipid is thought to represent a specific binding interaction.\(^ {30}\)

For interfacial reaction progress, the local concentration instead of bulk concentration is important for the overall kinetic rate. In diluting the substrate at the interface by adding more detergents or non-substrate lipids while keeping the total concentration of substrate constant, many phospholipases including PI-PLC exhibit a lower specific activity. This phenomenon is termed 'surface dilution inhibition'. The first step of the hydrolysis PI by the bacterial PI-PLC takes place at the interface. In a mixed micellar system, the phosphotransferase activity of PI-PLC toward long-chain PI was increased with PC present.\(^ {2}\) In a vesicle assay
system, when the PC mole fraction is lower than 0.20, the specific activity of *B. thuringiensis* PI-PLC towards PI is significantly enhanced. As the PC mole fraction is increased, the activity decreases because of the surface dilution inhibition (as show in Figure 1-8 B).

These two kinetic characteristics, interfacial activation and surface dilution inhibition, are exhibited by many different PI-PLC enzymes. Molecular mechanisms may vary somewhat, but observing these features is a hallmark of phospholipases.
Figure 1-8 (A) Cyclic phosphodiesterase activity of PI-PLC as a function of inositol cyclic 1,2-monophosphate (cIP) concentration in the absence of detergent \( (\circ) \) or presence of \( (\square) 8 \, \text{mM} \) Triton X-100 or \( (\triangledown) 8 \, \text{mM} \) diC\(_7\)PC. The left axis label is for the points connected by a solid line; the right axis label is for points connected by a dashed line\(^{39}\). (B) Relative activity of \textit{B. thuringiensis} PI-PLC towards covesicles of DMPC and PI and as a function of the mole fraction DMPC. The PI was kept constant at 10 mM while the DMPC varied from 0 to 90 mM. The specific activity is normalized to that for the enzyme acting on pure PI vesicles\(^{31}\).
2. **Mammalian PI-PLC**

PI-PLC isoforms (Figure 1-9) found in eukaryotes play a key role in signal transduction by catalyzing hydrolysis of phosphatidylinositol 4,5-biphosphate (PI(4,5)P$_2$) to yield two second messengers, inositol-1,4,5-trisphosphate(IP$_3$) and DAG. IP$_3$ mediates release of intracellular Ca$^{2+}$ and DAG can activate protein kinase C. The activity of PI-PLCs also affects the concentration of PI(4,5)P$_2$ which directly regulates important biological processes such as the activities of more than 20 distinct ion channels. Mammalian PI-PLCs and bacterial PI-PLCs have different substrate preferences: the mammalian PI-PLCs prefer PIP$_2$ $\geq$ PIP$_{1,4}$ $\geq$ PI; while bacterial PI-PLCs only hydrolyze PI (and GPI, also nonphosphorylated). Since the 1950’s, the PI-PLC enzymes have been studied because of the key role of these enzymes in phosphoinositide metabolism and signaling. Since the late 1980s, cDNA sequences of mammalian PI-PLC isoforms have been determined. Based on the sequence similarities, these isoforms were classified into five families: δ, β, γ, ε and η.
There is a \( \delta \)-like core sequence in all of the isozymes except a sperm specific PLC isoform (PI-PLC\( \zeta \)) which plays important role in calcium mobilization required for fertilization. The \( \delta \)-like core contains four domains: the PH domain, the EF hand domain, catalytic domain and the C2 domain. A unique region in PLC\( \beta \) is present at the C-terminal end. This extension is thought to interact with regulatory proteins. The
sequence unique to PLCη is also located at the C-terminus. Although the domain organization of PLCη and PLCβ are similar, the sequence analysis shows that the PLCη aligns more closely to PLCδ. PLCγ has several added domains: a second PH domain inserted through a flexible loop into the catalytic domain, two SH2 (Src homology 2) regions and one SH3 (Src homology 3) region. PLCε contains one CDC25 domain which has a guanyl-nucleotide exchange factor activity and two RA (Ras association) domains that play roles in binding small GTPases from the Ras family. The crystal structure of PLCδ, obtained in the 1990s, was determined in two parts: the isolated N-terminal PH domain and the remainder of the enzyme. More recently a structure of PLCβ in association with Gαq has been solved.

The PH domain

The PH domain was first described in the protein pleckstrin and subsequently reported to be present in over 100 proteins. PH domains consist of about 120 residues and have a remarkably conserved three-dimensional architecture despite only very limited sequence similarity. There are seven antiparallel β strands and a C-terminal α–helix arranged in a barrel-like structure. Although proteins containing PH domains are capable of membrane association, specificity for a particular phospholipid has been found only in a few PH domains. The PLCδ1-PH domain was the first observed one that had high-affinity for a particular lipid head-group, PI(4,5)P₂. In unstimulated cells, PLCδ1 is associated with the plasma membrane, and after stimulation the PH domain is dislodged from the plasma membrane. Activated PLCδ1s decrease the concentration of PI(4,5)P₂ and increase the concentration of the
IP$_3$ to enable feedback regulation of PLCδ1 and dissociation from the plasma membrane. The crystal structure of PLCδ1-PH domain and IP$_3$ show that IP$_3$ binds the PH domain via a network of hydrogen bonds, involving nine amino acid residues and the 4- and 5-phosphate groups of IP$_3$, and van der Waals connect between the inositol ring and Trp36$^{39}$. The interaction between the IP$_3$ and PLCδ1-PH domain is not conserved among the mammalian PI-PLC classes. Even for the PLCδ isozymes, the residues are not strictly conserved. Thus, the high-affinity of the PLCδ1-PH domain for PI(4,5)P$_2$ may be unique. The PH domain from PLCγ1 has been reported to show high affinity for PI(3,4,5)P$_3$$^{40}$. However, many of the PH domains of the PLC subfamily do not have a high-affinity for PI(4,5)P$_2$ or PI(3,4,5)P$_3$ and instead bind a zwitterionic phospholipid, phosphatidylcholine (PC), tightly$^{41}$.

**The EF-hand domain**

The EF-hand domain contains four helix-loop-helix motifs and forms a flexible tether between PH domain and the rest of the enzyme. Like the PH domain, the EF-hand domain also has a remarkably conserved three-dimensional architecture despite only very limited sequence similarity. In PLCs, the first two motifs appear to be much more flexible than the last two and are only partially visible in the crystal structures of the catalytic core of PLCδ1. The last two motifs make interactions with the C-terminal domain of PLCδ1. Although there are some residues that would be required for calcium binding in the first two motifs, there is no evidence that the PLCδ1 domain does indeed bind calcium ions. A long, well-ordered linker sequence connects the EF-hand domain to the catalytic domain.
The catalytic domain

The catalytic domain of PLCδ1 consists of a distorted TIM barrel with very similar secondary structure to the bacterial PLCs. The mammalian PLCs and bacterial PLCs have similar catalytic residues, notably the two catalytic histidines, providing evidence of an evolutionary relationship between the enzymes. The residues which form the two halves of the barrel are connected by insertion of a linker sequence. The sequences of the two halves are usually called as the X and Y region. The X region shows the greatest sequence conservation among the mammalian PLCs. Because of the salt bridge between the 4- and 5-phosphoryl groups and the basic residues in the active site, the substrate preference of the mammalian PI-PLCs is PI(4,5)P₂ > PI(4)P or PI. The mammalian PLCs catalyze the cleavage of PI(4,5)P₂ via a two-step mechanism similar to that for the bacterial enzymes but with the help of a Ca²⁺ cofactor. There is a direct interaction between the Ca²⁺ cofactor and 2-OH of the substrate (Figure 1-10). The role of the cofactor is to lower the pKa of the 2-OH and aid in the nucleophilic attack on the 1-phosphate and to neutralize the negative charge developed in the transition state of the reaction. The Ca²⁺ cofactor is also proposed to make a direct interaction with the transition state and the intermediate of the reaction, which may explain why the mammalian enzyme tends to retain the cyclic phosphate intermediate in the active site and complete the second step of the reaction to generate the acyclic product. Residues His311 and His356 in PLCδ1 have been considered for the role of His32 and His82 in the bacterial PI-PLC. Besides the two general acid/base catalytic residues, Glu341 and Glu390 not only bind the calcium but also
form hydrogen bonds with the 2-OH group of the inositol (Figure 1-10 B) and are thought to help the proton transfer of the 2-OH group.

**The C2 domain**

The C-terminal domain of PLCδ1 is present in all of the isozymes and consists of an eight-stranded anti-parallel β-stand. These domains have high sequence similarity and are similar to the second conserved domain from protein kinase C, the so called C2 domain. The C2 domain is a calcium-dependent phospholipid binding domain, and the crystal structure of the PLCδ1 shows three Ca²⁺ binding sites in this domain⁴⁴. This suggests that the C2 domain may be involved in membrane binding, perhaps through Ca²⁺ binding to PS⁴⁵.

Mechanism of bacterial PI-PLC catalyzed PI hydrolysis (A) and crystal structure of the *B. cereus* enzyme with active site residues shown and myo-inositol bound (B). A are adapted from ref (27); the structure in B is generated using Pymol.
Figure 1-10. (A) Mechanism of mammalian PLC catalyzed PI hydrolysis. (B) Crystal structure of mammalian PLCδ1 active site showing substrate and cofactor Ca\(^{2+}\) interactions with the protein. (A) is adapted from ref (27); the structure in (B) is generated using Pymol.
V. Thesis directions

The aim of this dissertation is to understand on a molecular level the activation mechanism of *B. thuringiensis* PI-PLC, and the kinetic activation and inhibition mechanism of the catalytic domain of the multiple-domain peripheral membrane protein, rat PLCδ1.

The first part of my project was to investigate the mechanism by which the catalytic activity of *B. thuringiensis* PI-PLC is activated by binding to surfaces. Previous studies from the group have underlined the importance of Trp47 in helix B. Removal of this residue reduced activation by phosphatidylcholine (PC) and also reduced the affinity of the protein for PC interfaces. Later on, the crystal structure of an interfacially impaired *B. thuringiensis* PI-PLC, the W47A/W242A mutant, was solved and shown to be a dimer. In this structure, a dramatic conformational change of the protein was observed: helix B no longer exists and these residues adopt a loop conformation. In order to explore what conformation of the protein can stabilize the membrane activation, a series of mutations of helix B residues were constructed and characterized. In these studies, CD was used for detecting any changes in protein secondary structure, NMR was used for analyzing PC activation of PI cleavage and cIP hydrolysis, and fluorescence was used to probe the affinity of the altered proteins for different interfaces. Although there was no direct evidence for the dimer form of the wild type protein as the conformation upon membrane binding, my studies suggested this could occur.

Besides the helix B residues, a number of hydrophobic residues along the rim of
the αβ-barrel and close to both helix B and the active site were also altered to assess their contribution to membrane binding and kinetic activation. My studies clearly show that several of these residues do contribute to the protein binding to phospholipid vesicles. Tyr86 and Tyr88, but not Tyr118, contribute to the protein binding to PC vesicles. These residues are capable of cation-π interactions with the choline headgroup of the activating phospholipid PC.

The second part of my project focused on the studies of mammalian PLCδ1. Although previous studies have revealed the important role of PH domain and C2 domain in the membrane binding of the enzyme\textsuperscript{45}, there has not been much research focused on how the catalytic domain contributes to membrane binding. Studies of the \textit{B. thuringiensis} PI-PLC as a model, however, pointed to the fact that rim residues in the catalytic domain should play some role in membrane activation and interaction. In my studies of the rat PLCδ1, the X/Y linker region – which covers the active site and is full of negatively charge residues – was removed from the protein. The interfacial binding and the corresponding enzyme activity of this mutant against WT were measured in both micelles and large unilamellar vesicles (LUVs). The mutant protein had much higher activity than WT in most assay systems. In order to monitor the binding in multi-component LUVs, fluorescence resonance energy transfer (FRET) using moderate protein concentrations and fluorescence correlation spectroscopy (FCS) using nM protein were used. At µM protein concentration there was no large difference between the PLCδ1 and the deletion mutant in terms of vesicle binding. However, the deletion mutant showed much higher membrane binding affinity at nM
concentrations. These results shed some light on the activation or inhibition role of the catalytic domain and pointed to a possible direction of future studies, for example examining specific mutant enzymes in the interfacial loop region.

**References:**


Opin Cell Biol, 12, 475-482.


34 Suh, B. C. and Hille, B. (2005) Regulation of ion channels by phosphatidylinositol


Chapter 2

Materials and Methods

I. Materials

A. Phospholipids

Most of the lipid powders or solutions in organic solvent were purchased from Avanti Polar lipids, Inc., and used without further purification. These include the synthetic short-chain phospholipid diheptanoylphosphatidylcholine (diC\textsubscript{7}PC) and long-chain phospholipids 1-palmitoyl-2-oleoyl-phosphatidic acid (POPA), 1-palmitoyl-2-oleoyl-phosphatidylinerine (POPS), L-\(\alpha\)-phosphatidylinositol (PI) from bovine liver, 1,2-dioleoyl-sn-glycero-3-phosphatidymethanol (DOPMe), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG). Crude PI from Sigma Chemical Inc. was used for the enzymatic generation of cIP by PI-PLC\textsuperscript{1}.

B. Resins

Q Sepharose Fast Flow and Glutathione Sepharose 4B were purchased from GE Healthcare. Phenyl Sepharose CL-4B was purchased from Sigma Chemical Inc.

C. Other chemicals

Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs. Most other chemicals, such as isopropanol (iPrOH), chloroform, ethanol, D\textsubscript{2}O, Tris, HEPES, glycerol and Triton X-100, were purchased from Sigma. All competent cells used in mutagenesis (Super blue) and overexpression (BL21 Codonplus) were obtained from Stratagene.
II Methods

A Molecular biology techniques

1. Cloning and site-directed mutagenesis

Figure 2-1 Plasmid map of the *B. thuringiensis* PI-PLC gene inserted into the pHN 1403 vector. The map shows the PI-PLC coding region, the promoter and other important features of the vector (adapted from Jianwen Feng, Ph.D. thesis, Chemistry Department, Boston College, 2004).

A plasmid containing the wild type *B. thuringiensis* PI-PLC gene was obtained as a gift from Dr Ming-Daw Tsai at Ohio State University. The plasmid map is shown in Figure 2-1. The plasmid containing the wild type mammalian (rat) PLC δ1 gene was a gift from Dr. Matilda Katan at the Cancer Research UK Centre for Cell and
Molecular Biology, Chester Beatty Laboratories, the Institute of Cancer Research, London, U.K. The plasmid map of this gene is shown in Figure 2-2.

**Figure 2-2**  Plasmid map of mammalian PI-PLCδ1 gene inserting into the PGEX-2T vector. The map shows the PI-PLC coding region and other important features of the vector.

All *B. thuringiensis* PI-PLC mutants were constructed using the Quik-Change™ Site-Directed Mutagenesis Kit. We also used the Quik-Change™ Site-Directed Mutagenesis Kit to delete 11 amino acids in the X-Y linker loop of the mammalian PI-PLC. An overview of the QuikChange site-direct mutagenesis method is shown in Figure 2-3. Primer design followed the instruction manual with the following constraints. (a) Both primers must have the desired mutation. (b) Primer length should be between 25 and 45 bases, and the desired mutation (deletion) should
be in the middle. (c) The melting temperature ($T_m$) should be over 78 °C. (d) The primer needs to be at least 40% GC. The HPLC purified mutagenic primers were purchased from Operon. The identities of the mutations in the altered genes were confirmed by sequencing performed by GENEWIZ. Several double mutants or triple mutants were made by introducing additional mutations in the gene coding for a single mutant or double mutants. The list of mutagenic primers is show in Table 2-1.
Figure 2-3. QuikChange site-directed mutagenesis method. The annealing
temperature was set as 68°C for *B. thuringiensis* PI-PLC.

**Step 1**
Plasmid Preparation

Gene in plasmid with target site for mutation.

**Step 2**
Temperature Cycling

For *B. thuringiensis* PI-PLC:
- Denaturation (95°C for 30 s)
- Annealing (68°C for 1 min)
- Extension (68°C for 12 min)

For mammalian PI-PLCδ1:
- Denaturation (95°C for 30 s)
- Annealing (68°C for 1 min)
- Extension (68°C for 15 min)

12-18 cycles

Denature the plasmid and anneal the oligonucleotide primer containing the desired mutations.

Using the *Pfu Turbo* DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands.

Digest the methylated, nonmutated parental DNA template with *DpnI*.

**Step 3**
Digestion

Mutated plasmid

**Step 4**
Transformation

Transform the circular, nicked ds DNA into XL1-Blue supercompetent cells.

After transformation, the XL1-Blue cells repair the nicks in the mutated plasmid.
Table 2-1. List of mutagenic primers corresponding to site specific mutagenesis of PI-PLC.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Complimentary Mutagenic Primers (from 5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>B. thuringiensis</strong> PI-PLC</td>
</tr>
<tr>
<td>L39A</td>
<td>TAGTGGGACGTTCAAG-GCG-CAAAATCCGATTAAGGCG</td>
</tr>
<tr>
<td></td>
<td>GCTTAATCGGATTGTGGC-CTTGAAACGTCCCACCTAG</td>
</tr>
<tr>
<td>V46A</td>
<td>AATCCGATTAAGCAA-GCG-TGGGGAAATGACGCAAGGCG</td>
</tr>
<tr>
<td></td>
<td>CTTCGCTATTCCCCACACCTTG-GTC-CCTCCAATCCACCTAG</td>
</tr>
<tr>
<td>G48A</td>
<td>CGATTAAGCAAGTGTTGG-GCA-ATGACGCAAGGATATG</td>
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<tr>
<td></td>
<td>CATATTCTTGCGCAT-TGC-CCACACTTGGCTTTAATCG</td>
</tr>
<tr>
<td>K44E</td>
<td>TGGAAAAATCCGATT-GAG-CAAGTGTTGGAATGACGCG</td>
</tr>
<tr>
<td></td>
<td>GCGTCATTCCCCACACCTTG-GTC-CCTCCAATCCACCTAG</td>
</tr>
<tr>
<td>P42G</td>
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<td></td>
<td>GGG-CTTTAATCCGATTAAGGCG</td>
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<td></td>
<td>CCCCAACTTGGCTTTAAT-CCTTCCAATCCAAGGACGTTC</td>
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<td>Q45A</td>
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<td></td>
<td>GCGTCATTCCCCACAC-TGC-CTTTAATCCGCTTTAATCG</td>
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<td>Y88A</td>
<td>CATCATGGGCCATTA-GCT-CTTTACGTAACACTG</td>
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<td>CAGTGGTTACGTAAG-AGC-TAATGGCCCATGATG</td>
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<td>L85A</td>
<td>TCTTCATCATGGGGCCA-GCA-TATCTTTACGTAACACTG</td>
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<td></td>
<td>GCAGTGGTTACGTAAGAT-GTA-TGGGGCCCATGAGA</td>
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<td></td>
<td>GCAGTGGTTACGTAAGAT-GTA-TGGGGCCCATGAGA</td>
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<td>Y86A</td>
<td>CATCATGGGCCATTA-GCT-CTTTACGTAACACTG</td>
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<td>Y88A</td>
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<td>CATGCAGTGGTTAC-GGC-AAGATATAATGGC</td>
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<td>Y86AY88A</td>
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<td>Y88C</td>
<td>GCCATTATATCTT-TGC-GTAACACTGCAATG</td>
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<td>Y88W</td>
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<tr>
<td>H81A</td>
<td>ATACGATAGTTCTT-GCT-CATGGGCTATTATC</td>
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<tr>
<td>Rat PI-PLCδ1</td>
<td></td>
</tr>
<tr>
<td>D11</td>
<td>GCCACTGACGTGTCT-GCTGTGCGCAGCCAA</td>
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<td>H311</td>
<td>CTTAGTGCTCTTTCC-GCC-AACACCTACTGCTGG</td>
</tr>
<tr>
<td>H356A</td>
<td>GAACCCATCATCTAC-GCC-GGCTACACTTTTACC</td>
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</table>

2.2 Over-expression

The plasmid was transformed into *E. coli* BL21-Codonplus (DE3)-RIL cells by the heat-shock method. A clone was picked and incubated overnight in 5 ml LB media containing 34 μg/ml chloroamphenicol and 100 μg/ml ampicillin. Then the 5 ml LB media was added to 2 L LB media with the same concentrate of antibiotics and incubated at 37 °C. When the OD$_{600}$ reached 0.8, IPTG was added to a final concentration of 0.6 mM; the cell suspension was then incubated at 16 °C. After 16-20 hours, the cells were harvested by centrifugation at 5000 rpm for 15 min; the cell pellets were frozen at -20°C. Using this protocol, both PI-PLC and rat PLCδ1 have
been expressed previously.

The cell pellets of *B. thuringiensis* PLC were dissolved in Tris buffer (20 mM Tris HCl, pH 8.0) and the cell pellets of mammalian PI-PLCδ1 were dissolved in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.3). The cells were lysed by sonication on ice (30 s pulses at moderate power repeated 10 times). After centrifugation (13,000 rpm, 30 min), the supernatant with enzyme was collected for purification steps.

3. Purification

(a) *B. thuringiensis* PI-PLC

The protocol for purification of recombinant PI-PLC follows previously published methods in the laboratory\(^1\). Supernatant with enzyme was dialyzed overnight against 20 mM Tris HCl, pH 8.0, at 4 °C. A Q-sepharose fast flow column (1.5 cm x 12 cm) was equilibrated with Tris buffer (Tris HCl, pH 8.0). The supernatant containing the protein was loaded onto the QFF column at a rate of 2 ml/min. The enzyme was eluted with a NaCl gradient ranging from 0 to 0.6 M in the Tris buffer at rate of 2 ml/min. The enzyme was checked using SDS-PAGE and was further purified using a phenyl-sepharose column. After adjusting the NaCl concentration to 0.6 M, we loaded the enzyme onto the phenyl-sepharose column (1 cm x 10 cm) at rate of 1 ml/min. The enzyme was eluted with a decreasing NaCl gradient ranging from 0.6 M to 0 mM in Tris buffer at rate of 1 ml/min. The purity of PI-PLC enzymes was above 95% as monitored by SDS-PAGE (see Figure 2-4). Protein solutions were concentrated using Millipore Centraplus 10 filters;
concentrations were determined by Lowry assays\(^2\).

(b) Mammalian PI-PLC\(\delta_1\)

Purification of the GST-tagged mammalian PI-PLC followed procedures developed by the Katan laboratory\(^3\). The supernatant containing enzyme was incubated overnight with glutathione Sepharose 4B (2 ml) in PBS buffer (pH 7.3). The column with enzyme was washed three times with PBS buffer, and then incubated with thrombin (50 unit). Eluted protein was concentrated using Millipore Centraplus 10 filters. Concentrations were determined by UV using calculated molar extinction coefficients. The purity of mPLC enzyme was above 85% as monitored by SDS-PAGE (example shown in Figure 2-4).

Figure 2-4. SDS-PAGE of purified B. thuringiensis PI-PLC (btPLC) and rat PLC\(\delta_1\) and the XY-linker deletion mutant protein (\(\Delta\)11).

<table>
<thead>
<tr>
<th>Marker</th>
<th>btPLC</th>
<th>PLC(\delta_1)</th>
<th>(\Delta)11</th>
<th>marker</th>
</tr>
</thead>
</table>

4. Modification of PI-PLC

(a) Covalent dimer formation

Several mutants of B. thuringiensis PI-PLC were made to introduce a single cysteine at different positions for spin-labeling the protein\(^4\). This bacterial PI-PLC has
no cysteine normally. In the absence of dithiothreitol (DTT), *B. thuringiensis* PI-PLC Cys mutants (Y88C, L85C), stored at a high concentration (~10 mg/ml), formed some disulfide-linked homodimers as monitored by non-reducing SDS-PAGE. The yield was over 90% dimers after one week. These covalent dimers were fairly stable and could be stored up to 20 days prior to use without loss of activity (measured after adding DTT). Protein monomers were generated and maintained in solution by the storage of the PI-PLC solutions with 5 mM DTT or by adding 5 mM DTT 20 min before use in kinetics or binding studies.

(b) PI-PLC site-directed spin labeling

*B. thuringiensis* PI-PLC Cys mutants Y88C and L85C were specifically spin labeled on the Cys with 1-oxyl-2,2,5,5-tetramethylpyrrole-3-methyl-methanethiosulfonate (MTSL). The reaction scheme is shown below:

$$\text{MTSL} \quad \text{Protein-SH} + \text{RS-S-CH}_3 \quad \rightarrow \quad \text{Protein-S-SR} + \text{H-S-CH}_3$$

MTSL, obtained from Toronto Research Chemicals Inc., was prepared as a 10 mg/ml stock solution in acetone and stored in the freezer. The purified PI-PLC variants (concentrated to 20 mg/ml in 20 mM Tris, pH 8.0) were first incubated with 5 mM DTT for 20 min at room temperature or overnight at 4 °C for full reduction of any intermolecular disulfide bonds, monitored by non-reducing SDS-PAGE. DTT was then removed by two Micro Bio-spin 6 columns (up to 75 µl solution could be loaded on each spin column). The protein was then diluted to 5 mg/ml in 20 mM Tris, pH 7.5,
and 3-fold DTT was added to make sure all the Cys were reduced to free sulfhydryls. Then a 10-fold MTSL over total –SH was then added and the resulting solution incubated for 1 h at room temperature, followed by overnight incubation at 4 °C. The excess spin label reagent was removed by three successive Micro Bio-Spin 6 columns. The labeling percentage was > 95%, monitored by non-reducing SDS-PAGE.

(c) PI-PLC specific N-terminal labeling with Alexa Fluor 488

Mammalian PLCδ1 enzymes (WT and ∆11) were specifically labeled on the N-terminal amino group with the succinimidyl ester of the Alexa Fluor 488 carboxylic acid according to the manufacturer's protocol. The AF488 dye was prepared as a 5 mg/ml (7.8 mM) stock solution in anhydrous DMSO and stored at -20 °C. The protein concentration in the reaction was in the range of 5-20 mg/ml (concentrations lower than 2 mg/ml are said to greatly decrease the efficiency of the reaction). Critical to the labeling protocol is to ensure that the solutions is free of any amine-containing substances such as Tris, glycine, ammonium ions, or stabilizing proteins such as bovine serum albumin. This reagent reacts with non-protonated aliphatic amine groups, including the N-terminus of the protein and the ε-amino group of lysines. The ε-amino group has a \( pK_a \sim 10.5 \), while the N-terminal amino group has a lower \( pK_a \), typically around 8.5-9. To preferentially label the N-terminal amine, the reaction is done in a buffer with a slightly basic pH. Prior to labeling, 1 µL of 2 M sodium phosphate buffer (pH 8.5) was added for each 50 µL solution of protein. A 2-fold molar excess of AF488 dye was added to the protein and the solution incubated on ice for 20 min to 1 hour. The AF488 labeled protein was separated from free AF488
using three Bio-Spin 6 columns. Less than 50 µL sample was loaded onto each spin column for efficient separation. All variants had a labeling ratio of 90±20%, determined by comparing the absorption of the protein at 280 nm to that of the probe at 495 nm. The equation used for calculating the labeling ratio was: \[ \frac{[\text{Dye}]}{[P]} = \frac{(92665 \text{ cm}^{-1}\text{M}^{-1}/71000 \text{ cm}^{-1}\text{M}^{-1}) \times A_{495}/(A_{280}-0.11A_{495})}{(92665 \text{ cm}^{-1}\text{M}^{-1}/71000 \text{ cm}^{-1}\text{M}^{-1})} \times \frac{A_{465}}{(A_{280}-0.072A_{465})} \] where 92665 cm\(^{-1}\)M\(^{-1}\) is the extinction coefficient of PLC\(\delta\) at 280 nm, 71000 cm\(^{-1}\) M\(^{-1}\) is the extinction coefficient of AF488 at 495 nm and 0.11 A\(_{495}\) is the correction for the absorbance of the dye at 280 nm.

(d) PI-PLC specific N-terminal labeling with NBD

Mammalian PI-PLC\(\delta\) proteins (WT and \(\Delta\)11) were also specifically labeled on the N-terminal amino group with the succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate (NBD-X, succinimidyl ester) according to the manufacturer's protocol (described above for Alexa labeling. The NBD dye was prepared as a 25 mg/ml (32 mM) stock solution in anhydrous DMSO and stored at -20 °C. A 1-fold molar excess of NBD-X dye was added to the protein and the solution incubated on ice for 20-40 min. The NBD-X labeled protein was separated from free NBD-X using three Bio-Spin 6 columns. All variants had a labeling ratio of 90±20%, determined by comparing the absorption of the protein at 280 nm to that of the probe at 465 nm. The equation used for calculating the labeling ratio was: \[ \frac{[\text{Dye}]}{[P]} = (92665 \text{ cm}^{-1}\text{M}^{-1}/24000 \text{ cm}^{-1}\text{M}^{-1}) \times A_{465}/(A_{280}-0.072A_{465}) \] where 92665 cm\(^{-1}\)M\(^{-1}\) is the extinction coefficient of PI-PLC\(\delta\) at 280 nm, 24000 cm\(^{-1}\) M\(^{-1}\) is the extinction coefficient of NBD-X at 465 nm and 0.072 A\(_{465}\) is the correction for the
absorbance of the dye at 280 nm.

D. Spectroscopic techniques

1. CD spectroscopy

Circular Dichroism (CD) is the differential absorption of left- and right- circularly polarized light. Electromagnetic radiation is plane polarized when the electric and magnetic fields each oscillate in a single plane. Circular polarization occurs when the electric and magnetic fields rotate around the direction of propagation in either a clockwise or a counterclockwise sense but still remain perpendicular to each other. When circularly polarized light passes through a sample, the absorption between right and left polarizations can differ. CD spectroscopy can determine secondary structure in the far-UV spectral region (190-250 nm). In the far-UV region the peptide bond is the main chromophore, and the signal changes when it is located in different chiral environments. α-helix, β-sheet, and random coil structures each give a characteristic CD spectrum. The CD signal reflects an average of the entire protein secondary structure. Analyzing the far-UV CD spectrum as a linear combination of the different secondary structure motifs provides us with the approximate fraction of each secondary structure type in the protein. CD spectroscopy can also be used to monitor the thermal denaturation of proteins.5

Recombinant PI-PLC secondary structure was measured using an AVIV 202 CD spectrophotometer (Figure 2-5). Comparison of secondary structure for recombinant wild type and mutant B. thuringiensis PI-PLC used wavelength scans from 195 to 300 nm with protein (0.3–0.4 mg/ml) in a 0.1 cm cell at 25 °C; the program CDNN.678
Figure 2-5. (A) CD spectrum of WT PI-PLC (0.4 mg/ml) in 10 mM borate pH 8.0. (B) Temperature dependence of CD signal of *B. thuringiensis* PI-PLC at 222 nm (adapted from Jianwen Feng, Ph.D. thesis, Chemistry Department, Boston College, 2004).
was used to estimate fractions of secondary structure. The $T_m$, or temperature for loss of 50% of the secondary structure, can be used to assess overall folding of proteins. In the case of PI-PLC the transition is irreversible. $T_m$ for PI-PLC was monitored at 222 nm (which is dominated by $\alpha$-helix) by increasing the temperature from 25 to 90 °C (1° per min); the protein, 0.03–0.04 mg/ml, was contained in a 1 cm cell.

2. **NMR spectroscopy**

(a) $^{31}$P NMR spectroscopy

The specific activity of PI-PLC mutants was measured by monitoring cIP or I-1-P generation using $^{31}$P NMR (202.3 MHz) spectra obtained on a Varian INOVA 500. NMR parameters were based on those previously reported$^9,^{10}$. Phosphorylated product formation for either fixed time points or continuous assays was analyzed$^{11}$. For *B. thuringiensis* PLC, the buffer was 50 mM HEPES, pH 7.5, 1 mM EDTA, and for PLC$\delta$1, the buffer was 50 mM HEPES, pH 7.5, 0.5 mM Ca$^{2+}$ and 100 mM NaCl. The amount of enzyme added in each kinetic run (50 ng to 3 µg depending on whether the phosphotransferase or the cyclic phosphodiesterase was monitored, respectively) was adjusted to generate less than 20% product within the desired reaction time. PI cleavage and cIP hydrolysis rates were measured from the integrated intensity of cIP and I-1-P resonances (in the absence of $^1$H decoupling), respectively, as a function of incubation time, using the $^{31}$P resonance of glucose-6-phosphate or KH$_2$PO$_4$ of known concentrations (2 or 4 mM) as an internal reference (Figure 2-6). The PI concentration was 5, 6 or 8 mM; diC$_7$PC was usually 24 or 32 mM but was also increased for surface dilution studies. The assay temperature for *B. thuringiensis*
PLC was 22 °C and 37 °C for PLCδ1. The hydrolysis of cIP (20 mM) was monitored in the absence or presence of 5 or 8 mM diC₃PC. For PI-PLC activity toward PI presented in small unilamellar vesicles (prepared by sonication), the PI concentration was 8 mM with 2 mM POPC.

Figure 2-6. NMR spectra for the activity assays of (A) the bacterial PI-PLC phosphotransferase reaction, (B) the bacterial PI-PLC cyclic phosphodiesterase reaction, and (C) the mammalian PLCδ1 reaction (at a fixed time point).
(b) High resolution field cycling $^{31}$P NMR (fc-P-NMR)

High resolution field cycling $^{31}$P NMR (fc-P-NMR) experiments were run at Brandeis University using a unique system devised by Prof. Alfred G. Redfield. The basic experiment is to measure the spin-lattice relaxation rate, $R_1 = 1/T_1$, over a wide range of magnetic fields. For my studies, $R_1$ experiments were obtained at 25°C on a Varian Unity$^{\text{plus}}$ 500 spectrometer using a standard 10-mm Varian probe in a custom-built device that moves the sample between the probe and a higher position within, or just above, the magnet, where the magnetic field is between 0.06 and 11.7 T. To access lower fields (0.005 up to 0.07 T), the sample was shuttled to a region outside and above the magnet and into the middle of an extra coil, where the current was adjusted to the desired field. The samples for field cycling were sealed in a 10 mm tube with a minimum amount of head space to avoid bubble formation as the tube is rapidly shuttled up and down the magnetic bore. Experiments to cover a field range of 0.005 up to 11.7 T typically took a span of 24-36 h.

The fc-P-NMR technique to measure relaxation rates is extremely useful for $^{31}$P nuclei (in particular phospholipids in different aggregates but also for small phosphorylated molecules). At the high fields of modern spectrometers, the bulk of the $^{31}$P relaxation is caused by chemical shift anisotropy (CSA). Dynamics in molecules are best assessed through dipolar relaxation, and for phospholipids in vesicles, field strengths of less than 2 Tesla (T) are needed to have relaxation rates dominated by dipolar interactions. For comparison, the field of a 500 MHz spectrometer is 11.7 T. Sensitivity and resolution are provided by the high field.
spectrometer – chemical shifts for different head group phospholipids are nicely resolved – but interpretation of $R_1$ values is nearly impossible. fc-P-NMR allows us to measure $R_1$ over a very large range and then use standard NMR theory to describe the relaxation as a function of field strength. There are three easily observed dispersions at different time scales (each characterized by a correlation time $\tau$) in these field dependence profiles: (i) a 50-300 ps dispersion (above 2 T) that dominates high field $R_1$ and is from CSA and fast motions of nonspherical electron clouds around the $^{31}\text{P}$ (not very useful for exploring protein/ligand binding dynamics), (ii) a 5-20 ns dipolar dispersion around 0.08-1 T that has been proposed to reflect phospholipid wobble motion, and (iii) a 1 $\mu$s or so dipolar dispersion only observed at very low fields (below 0.02 T) that has contributions from vesicle tumbling and lateral diffusion of phospholipids in the bilayer leaflet. Extracting dynamic parameters for phospholipids in vesicles in each of these regions has been described in detail\(^\text{12}\). However, for my work, the correlation times and maximum extrapolated relaxation rates in each region are of minor importance. I introduce a spin-label at a specific site on the \textit{B. thuringiensis} PI-PLC and then look at how the very large electron dipole perturbs $^{31}\text{P}$ nuclear relaxation in the very low field region. The difference in $R_1$ profiles for a vesicle sample with unmodified and spin-labeled protein provides a direct measure of the paramagnetic relaxation enhancement for the $^{31}\text{P}$. The larger this is, the closer the phospholipid is to the spin-label site on the protein. Also, if an effect is seen in this region it means a discrete complex must exist where the spin-label/phospholipid orientation is maintained for at least 1 $\mu$s but less than 1 ms
(otherwise the bound phospholipid is in slow exchange and not detectable in the high resolution experiment because its concentration is so low). More details on how an averaged distance between a $^{31}$P of a phospholipid and the electron on the spin-label attached to the protein is obtained are provided in Chapter 4.

3. **Fluorescence spectroscopy**

The electrons in molecules of certain compounds are excited by a beam of light. Return of the system back to the ground electronic state can occur with the emission of light of a lower energy. The emission or fluorescence can be used to monitor environmental and conformational changes of macromolecules by looking for changes in maximum emission wavelength or altered emission intensity$^{14}$.

(a) **Intrinsic fluorescence of PI-PLC**

Fluorescence measurements of *B. thuringiensis* PI-PLC (2 µM) were carried out on Fluorolog R-3 spectrofluorometers. The excitation wavelength was 282 nm (to excite Trp and Tyr residues). Changes in the fluorescence intensity at 337 nm (the emission maximum and characteristic of Trp emission) upon the addition of lipids (either diC$_7$PC or POPC SUVs) were expressed as $(I-I_o)/I_o$, where $I_o$ is the emission intensity of protein alone, and $I$ is the intensity in the presence of an additive. An apparent binding constant to POPC vesicles was obtained by fitting the change in intensity with a hyperbolic function to extract a bulk concentration of PC for half the maximum change.

(b) **Fluorescence resonance energy transfer (FRET)**

FRET is a distance-dependent interaction whereby one chromophore can
transfer energy to another. First, the donor chromophore absorbs light to the electronic excited state. The excited donor chromophore then transfers its energy to an acceptor chromophore through nonradiative dipole-dipole coupling (Figure 2-7). For efficient FRET, the distance between donor and acceptor should be close, although the range of distances sensed varies with the FRET pair of chromophores (10-100 Å). There must be overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor. For most efficient FRET, the donor and acceptor transition dipole orientations should be approximately parallel. The interaction of PLCδ1 with the membrane was evaluated using NBD-labeled mammalian PLCδ1 serving as donors and rhodamine-labeled lipids as energy acceptors (Figure 2-5). A solution of NBD-PLCδ1 (donor) in 20 mM Tris, pH 7.5, with 100 mM NaCl and 1 mg/ml BSA, was prepared. Then SUVs with a few % rhodamine-labeled lipid (acceptor) were added to the protein solution in several sequential doses. The mixture was incubated for 2 min at 25 °C, and the fluorescence spectra were obtained before and after adding the acceptor. The excitation monochromator was set at 465 nm with a 2 mm slit width and the fluorescence emission was measured at 535 nm with a slit width of 5 nm. The percent of energy transfer (E) was determined by measuring the decrease in the quantum yield of the donor (NBD-PLCδ1) as a result of the presence of the acceptor (rhodamine-labeled lipids). The % FRET was determined experimentally by the ratio of the fluorescence intensities of the donor (NBD-PLCδ1) in the presence (F_{da}) and absence (F_{d}) of the acceptor (rhodamine-labeled lipids), at the donor’s maximum emission wavelength (535 nm). Monitoring the donor emission is usually easier than
the acceptor emission, since the acceptor can be quenched by other interactions when bound to the protein. The extent of FRET is given by

\[
\%\text{FRET} = (1 - \frac{F_a}{F_d}) \times 100
\]

**Figure 2-7** Excitation (dashed line) and emission spectra of NBD (green) and rhodamine (blue).

(c) Fluorescence Correlation Spectroscopy (FCS).

FCS is an experimental technique which uses statistical analysis of the fluctuations of fluorescence in a system in order to decipher dynamic molecular events, such as diffusion or conformational fluctuations of biomolecules. We used FCS to analyze the binding of fluorescently labeled mammalian PLCδ1 to lipid vesicles. FCS experiments were performed using a home-built confocal setup (in the Gershenson laboratory at the University of Massachusetts, Amherst) based on an IX-70 inverted microscope (Olympus) as shown in Figure 2-8\(^\text{15}\).

Briefly, the 488 nm line of an air-cooled argon-krypton laser was used to excite the sample, and a 500 drlp dichroic mirror reflected the laser light into a 60X water objective mounted on the inverted microscope. The same dichroic passed the
fluorescence emission, and any remaining scattered laser light was blocked by a filter. A 30 µm confocal pinhole was used to define the observation volume and to block out of focus fluorescence. The fluorescence was collimated, split by a non-polarizing 50-50 beam splitter and focused onto two photodiodes. For AF488 labeled protein, HQ535/50 bandpass filters before the focusing lenses blocked the Raman scattering. Rhodamine-labeled SUVs were excited using the 520 nm laser line with a change in all the different filters. The photon counts from the photodiodes were collected by a 2-channel data acquisition card, and associated software was used to calculate and analyze the auto- and cross-correlations (ISS).

FCS experiments were carried out at 22 °C on 300 µL samples in phosphate buffered saline (PBS), pH 7.3, plus 1 mg/ml bovine serum albumin, to stabilize PI-PLC. The samples were placed in chambered coverglass wells (LabTek). Prior to use, the chambers were coated with 10 mg/ml bovine serum albumin for 10 min and rinsed with PBS buffer to prevent PLCδ1 protein adhesion on the sides of the wells. For PLCδ1 vesicle binding experiments, 8 nM labeled protein was titrated with unlabeled vesicles. The substrate, PI was not used for FCS experiments because PI cleavage by PI-PLC produces DAG leading to vesicle fusion\textsuperscript{16}. Thus, the anionic phospholipids DOPMe, POPG, DOPA and DOPS were used as substrate analogues.
Figure 2-8 FCS experimental schematic. Individual laser lines from an air-cooled argon-krypton, multi-line laser are separated using a quartz prism, P. The 488 nm laser line is picked off, expanded 5 times using two lenses, L1 & L2, in order to overfill the back aperture of the microscope objective and reflected into the sample using a 500drlp dichroic, D1. In the IX-70 inverted microscope, the 60X water objective, Obj, with a numerical aperture of 1.2 focuses the laser light into the sample and collects the emission. The emission passes through D1 and any remaining scattered laser light is blocked by a HQ505lp long pass filter, F1. The emission is then focused onto a 30 μm pinhole by the lens, L3, in the microscope to limit the detection region and collimated by lens L4. To calculate the cross-correlation, the fluorescence signal is split by a 50-50 beam splitter D2, and passed through a HQ535/50 bandpass filter, F2, F3 and focused on two avalanche photodiodes, APD1, APD2, by lenses L5 and L6, respectively. The rhodamine-labeled LUVs were excited using the 520 nm laser line with a 535drlp dichroic mirror (D1) and HQ450lp longpass filter (F1) in the microscope, and the filters in front of the photodiodes (F2 & F3) were replaced by HQ645/75 bandpass filters (Chroma Technology). (Adapted from Mingming Pu, Ph.D. thesis, Chemistry Department, Boston College, (2009)).
References:


of a bacterial phosphatidylinositol-specific phospholipase C, *J. Biol. Chem.* 278, 24651-24657


Chapter 3
Role of Helix B Residues in Phosphatidylcholine Activation of a Bacterial PI-PLC

I. Introduction

The *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) exhibits a very specific interfacial activation towards monomer and aggregated substrates upon binding to phosphatidylcholine interfaces.\(^1\) Although the \((\beta\alpha)_8\)-barrel core of closely related PI-PLC appears relatively rigid, hydrophobic regions at the rim of the barrel in helix B (Ile43-Gly48) and the 237-244 loop have significant mobility as measured by B factors.\(^2\) Previous work has clearly pointed to the importance of Trp47 and Trp242 in binding to activating PC interfaces (and at least one of them crucial for the protein to bind to anionic phospholipids surfaces).\(^3\) The rim Trp residues appear to be part of a binding site for PC molecules.\(^4\) Removal of either Trp reduces activation by PC; removal of both renders the enzyme incapable of binding to PC interfaces.\(^3a\) Indeed, for the rim loop interfacial mutants of PI-PLC examined thus far (shown are Trp242 alterations), the specific activity (in both phosphotransferase and cyclic phosphodiesterase assay systems) correlates with how tightly the PI-PLC binds to activating PC SUVs (Figure 3-1). However, helix B mutations do not follow this trend. Introduction of a tryptophan at other positions in helix B in the PI-PLC mutant W47A generated proteins that could bind well to PC vesicles but usually were much less active than wild type PI-PLC.\(^3c\).
Figure 3-1. Correlation of apparent $K_d$, which measures the partitioning of PI-PLC and mutants to POPC SUVs, with enzymatic activity toward (A) 8 mM PI solubilized in 24 mM diC$_7$PC or towards (B) 5 mM cIP with 5 mM diC$_7$PC added. Squares represent W242A (in the rim loop) and rescue mutants, the open triangle is wild type PI-PLC, and the filled circles are helix B W47A and rescue mutants (data from reference (3c)).

Furthermore, the single helix B mutant that regained significant kinetic activation by PC compared to W47A, I43W/W47A, did not bind tightly to PC vesicles.$^{3c}$ I43W/W47A has a tryptophan on the same side of the two-turn helix B as Trp47. The lower activity of I43W/W47A as well as the other helix B mutants, which bind PC well but are relatively inactive,$^{3c}$ suggest most of the mutations cannot retain the
unusual conformation that is critical for interfacial activity.

Recently, the crystal structure of the W47A/W242A mutant was solved. This interfacially impaired PI-PLC is a dimer in contrast to the highly homologous B. cereus PI-PLC, which crystallizes as a monomer. The dimer interface has a hydrophobic core of tyrosine residues that are quite removed from the active site. In the dimer, residues Ile43 to Gly48 no longer form a helix but instead adopt an irregular loop with an increased distance between residues 43 and 46 (Figure 3-2). In the B. cereus PI-PLC, helix B has the Ile43, Val46 and Trp47 side chains packed together to form a plug extending into solution. Such a hydrophobic plug could aid in initiating and stabilizing membrane binding. In the W47A/W242A double mutant, the Ile43 side chain is reoriented towards the protein core. If attachment of the plug is a part of the initial binding of the protein to a PC interface, then mutations that destabilize the helix in solution would be expected to reduce PC affinity and lead to lower PC activation of cIP hydrolysis. However, there are interactions of residues in and around helix B (e.g., Pro42 and Gln45) that appear to stabilize the dimer interface, and these cannot be accommodated with a helical structure for this segment of the protein. To explore in more depth the contribution of helix B residues to enzyme activity and phospholipid aggregate binding, I systematically altered individual residues in helix B (an a few neighboring residues) to Ala. The original idea was to see if we could find support for the existence of a dimeric protein similar to that seen in the W47A/W242A crystal structure.
II. Study of Helix B by mutagenesis

In order to explore what is needed to stabilize the ‘membrane active’ conformation of the protein, a series of PI-PLC mutants of helix B residues (as well as regions around this structural feature) were constructed and characterized. Alanine mutants of each position in helix B (43-48) as well as Leu39 were constructed and characterized for any changes in secondary structure (CD spectroscopy), PC...
activation of PI cleavage and cIP hydrolysis, and the ability to bind to PC interfaces (comparing both protein intrinsic fluorescence in the presence of PC vesicles and a vesicle filtration assay). The mutant that replaces Lys44, the only charged residue in helix B, with an anionic group (Glu) was also examined. Pro42, the residue immediately before the start of helix B, was altered to P42G, replacing a conformationally restricted residue with a very flexible one.

All these mutants except K44A exhibited lower activity for one or both steps of PI hydrolysis under standard assay conditions. However, there were differences among the mutants that allow us to propose a mechanism for PI-PLC activation by PC and to assess the formation of a membrane-induced dimer as a key step for optimal activity. The results are consistent with (i) formation of helix B in solution to prevent dimerization of the protein in solution, (ii) initiation of binding to PC membranes by the hydrophobic helix B ‘plug’, and (iii) possible disruption of helix B once the protein is anchored in preparation for either dimer formation with another membrane bound PI-PLC to generate the ‘membrane active’ form of PI-PLC or interaction of the surface Tyr occluded by the plug with PC headgroups enhancing protein binding to interfaces.

A. Secondary structure of helix B alanine mutants

In the crystal structure of monomeric wild type B. cereus PI-PLC, helix B points the hydrophobic side chains of Trp47 and Ile43 out into solution. In the structure of the interfacially impaired dimeric B. thuringiensis W47A/W242A, helix B no longer exists – rather the residues adopt a loop that is closer to the rim loop that
normally contains Trp242. Pro42 contributes to dimer formation through hydrophobic interactions with both Tyr247 and Tyr248 of the opposite chain. Gln45 participates in a hydrogen bond with Gly239 of the opposite chain. Since the dimer is observed in an interfacially impaired mutant and residues in the helix B region appear to stabilize the dimer, there are two key questions: (i) is the membrane active structure of this PI-PLC a dimer, and (ii) is helix B intact when the protein binds to interfaces. Recent mutagenesis of a cluster of tyrosines that form the hydrophobic core of the dimer interface are consistent with a dimeric structure of PI-PLC for optimum activity (although participation of the Tyr in π-cation interactions with PC molecules can not be excluded). To assess the contribution of helix B to activity and binding of activator phospholipids, we have constructed alanine mutants at each position in helix B as well as at Leu39 and introduced a Gly in place of Pro42, the residue that forms a cap over helix B. All mutants overexpressed well in E. coli and had similar CD spectra and T_m (as monitored by loss of negative ellipticity at 222 nm) as wild type recombinant PI-PLC indicating that they were well-folded with the same overall structure. I43A, K44A, W47A had the same secondary structure as PI-PLC. However, L39A, P42G, Q45A, V46A, and G48A exhibited a reduced α-helix content (as much as 3% loss compared to the wild type recombinant protein in solution) in low ionic strength buffers when compared by CD (Figure 3-3). For all of these but Q45A, the addition of 2 mM diC7PC caused the protein to regain α-helix structure to different degrees. These results illustrate a structural maleability in this region of the protein when it is in solution and several of the residues are altered.
Figure 3-3. Difference in α-helix content for each mutant compared to recombinant PI-PLC: protein (~ 0.4 mg/ml in 10 mM borate buffer, pH 8.0, in 1 mm cuvette) in the absence (○) and presence (///) of 2 mM diC7PC. For comparison, the secondary structure of the native protein from CD appears to be 25.5% α-helix, 24.4% β-sheet, 17.8% β-turn, and 32.3 % random.3b
B. Mutant phosphotransferase and cyclic phosphodiesterase activities

A comparison of the cIP hydrolysis rates in the absence and presence of diC\textsubscript{7}PC is shown in Figure 3-4A for these helix B mutants. In the absence of any interfaces, all the mutants had cIP cleavage rates were 58 to 96% of recombinant PI-PLC enzyme activity (except for L39A and Q45A which exhibited 10% and 39%). Thus, monomer activity was more or less unaltered in this series of mutants. Addition of diC\textsubscript{7}PC micelles increased all activities with the activation-fold (ratio of the activity plus PC compared to that in its absence) comparable to native protein for L39A, I43A and K44A; V46A was reasonably activated as well although not quite to the same extent (Figure 3-4B). P42G, Q45A, W47A and G48A were all much less sensitive to kinetic activation by this nonsubstrate phospholipid suggesting either a reduction in diC\textsubscript{7}PC affinity or an altered binding of the protein to these micelles.

While the cyclic phosphodiesterase rates were very sensitive to modification of helix B residues, the phosphotransferase activities only varied at most three-fold (Figure 3-4C). Poor PC activation of cIP hydrolysis did not necessarily imply poor phosphotransferase activity (e.g., P42G and G48A show the opposite behavior). Conversely, for some enzymes with high cIP activation, phosphotransferase activity could be low (L39A, I43A).
Figure 3-4 Activities of PI-PLC helix B mutants with Ala or Gly (for P42G) substituted at the position indicated. (A) Specific activity for cleavage of 20 mM cIP in the absence (○) and presence (●) of 8 mM diC₂₇PC. (B) DiC₇PC activation presented as the ratio of cIP hydrolysis activity in the presence or absence of diC₇PC. (C) Specific activity for cleavage of PI (8 mM) solubilized in 32 mM diC₇PC. Errors in specific activities were less than 15% of the indicated values.
C. Binding of mutants to PC SUVs

Recombinant PI-PLC bound moderately tightly to POPC SUVs with an apparent 

\[ K_d = 0.067 \pm 0.015 \text{ mM} \]. Of these helix B region mutants, only K44A and G48A exhibited affinities for PC SUVs comparable to the native PI-PLC protein (Figure 3-5). Cationic Lys44 does not insert into membranes and the structural change from Gly to Ala would not alter the \( K_d \) for G48A significantly even if this group were positioned in a membrane. L39A and W47A had the weakest affinity for PC, with apparent \( K_d \) values roughly 50-70-fold more than that of native PI-PLC. The estimated \( K_d \) values for each mutant in Figure 3-5 is based on free energy differences for side chains in membranes from the Wimley-White scale. The increased apparent \( K_d \) for W47A is clearly consistent with the indole ring partitioning into the membrane. However, the apparent \( K_d \) for the Leu39 substitution is far greater than expected solely on the basis of membrane partitioning. Although the Leu side chain could localize in the membrane, it is likely that substitution at this position alters the protein conformation and disposition of other residues involved in membrane partitioning (possibly Trp47). Alanine substitutions at the other positions in helix B exhibited a 6-12-fold loss of affinity for PC. Here, the loss of binding affinity does not correlate with changing the identity of a group that inserts into the membrane. Rather, these residues must be involved in other interactions that modulate PC binding.

In the crystal structure of \( B. \ cereus \) PI-PLC, helix B side chains do not interact significantly with the rest of the protein (Figure 3-2, C and D). If helix B remains intact and acts as a hydrophobic plug for interface binding, only the removal of
residues poised on the hydrophobic helix face, Ile43, Val46 and Trp47, should reduce membrane binding. However, in the W47A/W242A dimer, both Pro42 and Gln45 make intersubunit contacts (Figure 3-2, E and F). Therefore, the significantly weakened affinities of P42G and Q45A (compared to native PI-PLC) for PC could reflect impaired dimerization on the membrane surface. Reduced dimerization could weaken the binding of the enzyme to the PC surface by increasing the off rate of the protein.

Figure 3-5 Apparent dissociation constants for helix B region mutants binding to POPC SUVs in 10 mM Tris HCl, pH 7.5. The binding was monitored by both changes in the intrinsic Trp fluorescence as a function of added POPC SUVs as well as a filtration/centrifugation binding assay. The $K_d$ in the hatched bars for the nonpolar side chains is what would be predicted for the $K_d$ if that particular residue partitioned into the bilayer. The apparent $K_d$ for native PI-PLC is 0.06±0.02 mM.
D. Positional mutants and charge reversal mutants of helix B

If a helical hydrophobic plug (residues Ile43-Gly48) is needed for initial interaction of PI-PLC with PC, it should be stabilized by hydrophobic residues facing outwards on the same side of helix B. Indeed, this is consistent with the observation that only I43W/W47A and no other Trp rescue mutant, could significantly recover the PC interfacial activation of cIP hydrolysis lost in W47A. However, full PI cleavage activity was not observed with I43W/W47A, nor was it observed in I43A (Figure 3-4C). This might suggest a specific positional arrangement of hydrophobic residues is critical in the helix B region of the protein.

Two additional mutants were examined to check for the importance of where the helix B Trp was placed. The swap mutant I43W/W47I should be equivalent to native PI-PLC kinetically and structurally if the position of Trp on helix B matters less than its orientation in solution and its stabilization by another hydrophobic side chain. I43W, which maintains the critical Trp47 residue in helix B was made to see if the addition of a larger hydrophobic side chain had any effect on PI-PLC kinetic behavior. Both proteins had comparable secondary structure and similar phosphotransferase and cyclic phosphodiesterase specific activities (Table 3-1) to native PI-PLC. Specific activities were also determined for cleavage of PI solubilized in 30% iPrOH, PI in Triton X-100 micelles, and PI in vesicles with 20 mol% PC as well as for the PI/diC7PC mixed micelle system (Table 3-1).

The swap mutant had similar activity to native PI-PLC in all but the iPrOH assay system. I43W was not quite as active as native PI-PLC with the additional Trp
residue in this region. Nonetheless, these results suggest that the position of the Trp in the helix B region is less important that its packing with another hydrophobic residue (presumably to stabilize helix B in solution).

The swap mutant also had about the same affinity for POPC SUVs as native protein (Table 3-2). I43A, where the Ile that stabilizes outward facing Trp47 in PI-PLC has been removed, had a weakened affinity for PC vesicles that correlates with its reduced phosphotransferase and PC activation of cIP hydrolysis rates. Changing Ile to Trp (I43W) enhanced enzyme partitioning to SUVs, but not enough to imply that both Trp in helix B are inserted into the PC membrane (the predicted $K_d$ if both Trp insert would be 0.005 mM versus the experimental value of 0.026 mM). Since the specific activities for this mutant were always lower than native PI-PLC, it is possible that packing two Trp together in that helix is either difficult or makes the helix a bit more hydrophobic and alters its orientation.

We also examined in greater detail changes at residue 44. Lys44 represents the only charged residue in the helix B region. Replacement of the cationic side chain by a methyl group had no significant effect on enzyme activity in assay systems with 5 mM substrate in either diC$_7$PC micelles or in a water-iPrOH cosolvent or PI/PC vesicles.$^{3b}$ Specific phosphotransferase activity was only reduced with PI dispersed in Triton X-100. If Lys44 does contribute significantly to enzyme binding to substrate-containing PI/PC, we must be sufficiently above the apparent $K_d$ such that there is little effect on the specific activity with the assay conditions used.
Table 3-1 Relative phosphotransferase (towards PI in different assay systems) and cyclic phosphodiesterase (cIP as substrate) activities of rPI-PLC and selected helix B mutants.\(^a\)

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>PI (5)</th>
<th>(5)</th>
<th>(5)</th>
<th>(8)</th>
<th>cIP (5)</th>
<th>Activation (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay system</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% iPrOH</td>
<td>PI/diC(_7)PC (1:4)</td>
<td>PI/TX100 (1:2)</td>
<td>PI/POPC (4:1) SUVs</td>
<td>diC(_7)PC (5 mM)</td>
<td>+diC(_7)PC/diC(_7)PC</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.72 (^b)</td>
<td>1.00</td>
<td>0.67 (^b)</td>
<td>0.017</td>
<td>0.19</td>
<td>46</td>
</tr>
<tr>
<td>I43A</td>
<td>0.32</td>
<td>0.36</td>
<td>0.31</td>
<td>0.006</td>
<td>0.15</td>
<td>36</td>
</tr>
<tr>
<td>I43W/W47A</td>
<td>0.13 (^b)</td>
<td>0.54 (^b)</td>
<td>0.42 (^b)</td>
<td>0.008</td>
<td>0.056</td>
<td>47</td>
</tr>
<tr>
<td>I43W/W47I</td>
<td>0.39</td>
<td>0.90</td>
<td>0.60</td>
<td>0.016</td>
<td>0.09</td>
<td>33</td>
</tr>
<tr>
<td>I43W</td>
<td>0.45</td>
<td>0.82</td>
<td>0.54</td>
<td>0.014</td>
<td>0.11</td>
<td>55</td>
</tr>
<tr>
<td>K44A</td>
<td>0.68</td>
<td>0.98</td>
<td>0.36</td>
<td>0.018</td>
<td>0.16</td>
<td>33</td>
</tr>
<tr>
<td>K44E</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.024</td>
<td>4.5</td>
</tr>
</tbody>
</table>

\(^a\) All specific activities, except for K44E, were obtained at 22\(^{\circ}\)C, and are referenced to cleavage of 5 mM PI solubilized in 20 mM diC\(_7\)PC (955 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) at 22\(^{\circ}\)C). Values are averages of fixed time point assays done in duplicate; errors were < 15\% of the indicated value.

\(^b\) From Feng et al. (2002).\(^3\)

\(^c\) Values for cIP in the absence of activating PC were lower than reported previously,\(^3\)\(^a\)\(^c\) which were obtained at a higher temperature (28 versus 22 \(^{\circ}\)C). The value for native PI-PLC under these conditions was 2.8 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\). The ratio shown is for hydrolysis of 5 mM cIP in the presence of 5 mM diC\(_7\)PC.

\(^d\) The extent of PC activation of the enzyme for cIP hydrolysis was estimated by comparing specific activity in the presence of diC\(_7\)PC to the activity towards cIP alone.
However, replacing Lys44 with an anionic residue (Glu) reverses the net charge on helix B, and leads to an enzyme, K44E, with both reduced PI cleavage and cIP hydrolysis in the presence of activating PC (Table 3-1) and reduced affinity for POPC vesicles (Table 3-2). Introduction of a negative charge at this position weakens binding of the enzyme to PC interfaces. The recombinant K44E also had an unusual CD spectrum in that it had 7.4% less α-helix than native PI-PLC, suggesting less stable helical conformations in solution. However, the addition of 2 mM diC₇PC led to an increase in α-helix at the expense of β-sheet and random coil (similar to what was observed for P42G). The selective loss of PC activation with the charge reversal of the lone charged group in helix B could suggest that it may be very close to a PC binding site, possibly near the phosphate rather than the trimethylammonium moiety. Alternatively, it may indicate that changing the charge on helix B will repel it from any negatively charged lipids and this reduces both specific activities and also affects the ability of PC to bind and act synergistically with substrate to anchor the enzyme.
Table 3-2 Apparent binding constants for recombinant PI-PLC and selected helix B mutants interacting with PC aggregates.

<table>
<thead>
<tr>
<th>PI-PLC</th>
<th>app $K_a$ (mM)</th>
<th>calc. $K_a$ (mM)</th>
<th>$K_{0.5}$ (mM)$^a$</th>
<th>$K_{0.5-CMC}$ (mM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POPC SUVs</td>
<td>diC$_7$PC</td>
<td>micelles</td>
<td></td>
</tr>
<tr>
<td>native</td>
<td>0.067±0.010</td>
<td>1.86±0.08</td>
<td>0.36±0.06</td>
<td></td>
</tr>
<tr>
<td>I43A</td>
<td>0.37±0.07</td>
<td>2.28±0.10</td>
<td>0.75±0.06</td>
<td></td>
</tr>
<tr>
<td>I43W/W47A</td>
<td>0.85±0.17$^d$</td>
<td>2.48±0.39$^d$</td>
<td>1.08±0.33$^d$</td>
<td></td>
</tr>
<tr>
<td>I43W/W47I</td>
<td>0.064±0.008</td>
<td>1.67±0.07</td>
<td>0.18±0.07</td>
<td></td>
</tr>
<tr>
<td>I43W</td>
<td>0.026±0.06</td>
<td>1.65±0.07</td>
<td>0.17±0.09</td>
<td></td>
</tr>
<tr>
<td>K44A</td>
<td>0.087±0.01</td>
<td>1.59±0.07</td>
<td>0.08±0.04</td>
<td></td>
</tr>
<tr>
<td>K44E</td>
<td>0.50±0.20$^e$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $K_{0.5}$ is estimated as the bulk concentration of diC$_7$PC that leads to 50% of the maximum change in fluorescence intensity upon binding to that short-chain PC; the value for WT PI-PLC is the average of three separate experiments.

$^b$ $K_{0.5-CMC}$ is the value extracted for 50% of the fluorescence increase if only data above the CMC is used and fit with a hyperbolic function that reflects micellar diC$_7$PC only. Micellar PC is calculated as the concentration of diC$_7$PC minus the CMC (1.5mM).

$^c$ Extracted from data presented in (3a).

$^d$ Extracted from Feng et al.$^3c$

$^e$ Estimated by using the increase in intrinsic fluorescence of the protein when it binds to PC vesicles.$^9$

Previous work suggested that Trp242 is the main fluorophore responsible for the increased intrinsic fluorescence intensity when PI-PLC binds to PC activator micelles.$^3a$ This can be exploited to compare the affinities of the different helix B mutants for diC$_7$PC micelles. As an example (shown in Figure 3-6A), the change in fluorescence intensity of native PI-PLC upon diC$_7$PC micelle formation increased at lower diC$_7$PC concentrations than that of I43A, consistent with the weaker binding of I43A to POPC surfaces. The slight lag in the fluorescence change for I43A suggests more PC molecules are needed to drive micelles binding.
Figure 3-6 Increase in fluorescence intensities of *B. thuringiensis* PI-PLC proteins (WT (●), I43A (□), I43W (○), I43W/W47I (▲), K44A (X)) at 337 nm as a function of diC₇PC (A) total diC₇PC concentration and (B) micellar diC₇PC.

The mutants I43W/W47I and I43W exhibited fluorescence behavior comparable to native PI-PLC/ The *K₀.₅* values, which represent total diC₇PC, ranged from 1.59 to 2.48 mM. If the CMC for pure diC₇PC (1.5 mM) is subtracted from the bulk PC concentrations, and the resultant curves fit (except for I43A) with a
hyperbolic function (Figure 6B), we obtain apparent micelle $K_d$ values that show a more striking change in micellar PC affinity (Table 3-2). Removal of the hydrophobic side chain of Ile43 or placing a Trp at this position and an Ala at 47 generates enzyme with weaker affinity for diC$_7$PC micelles. The swap mutant (I43W/W47I), I43W, and K44A all bind slightly more tightly to PC micelles than native PI-PLC protein (Table 3-2). Interestingly, K44A shows less than half the fluorescence change of native PI-PLC (Figure 3-6), indicating a difference in the environment of the fluorophore in this mutant. In a monomeric protein structure residue 44 is not close to Trp242. However, in the W47A/W242A dimer, the helix B residues are much closer to the Trp242 in the rim loop.

E. PI-PLC induced aggregation of diC$_7$PC

PI-PLC binding to diC$_7$PC micelles can also be monitored by the increase in the $^{31}$P linewidth of diC$_7$PC when the protein is present. The width at half height, $\Delta \nu_{1/2}$, for the phosphorus resonance is broad in the presence of PI-PLC protein as soon as the diC$_7$PC concentration is greater than the CMC (Figure 3-7). The very large increase in $^{31}$P linewidth when protein binds to micellar diC$_7$PC reflects a combination of aggregation of the protein/diC$_7$PC system and slower exchange between monomer, micelle, and enzyme-bound diC$_7$PC such that the exchange is intermediate on the NMR time-scale. At high concentrations of diC$_7$PC (~15-20 mM), the limiting linewidth of the phosphorus in the presence of rPI-PLC is much larger than that for pure diC$_7$PC micelles. At 20 mM diC$_7$PC, if the diC$_7$PC molecules were in fast exchange between monomer, micelles, and enzyme-sites, the weight-averaged
linewidth should approach the linewidth of pure diC₇PC. That it does not for native PI-PLC is consistent with a PI-PLC/diC₇PC complex that is considerably larger than pure diC₇PC micelles.

The same titration experiments carried out for I43W and I43W/W47I show much smaller increases in ³¹P linewidth right above the CMC and much smaller limiting linewidths (Figure 3-7). Both mutants exhibit K₀.₅ values for diC₇PC similar to native PI-PLC as measured by intrinsic fluorescence changes. From the difference in diC₇PC ³¹P linewidths in the absence and presence of protein at PC concentrations above the CMC, a ‘bound’ linewidth, Δb, (extrapolated broadening of one diC₇PC molecule caused by one PLC) can be estimated as well as an excess limiting particle linewidth, which must reflect increases in the average protein/lipid mixed micelle particle size. For native PI-PLC, that value, Δb, is 373±41 Hz; this ‘bound’ linewidth is reduced to 36.1±5.7 Hz for I43W/W47I and 14.1±6.6 Hz for I43W. The limiting excess particle linewidth is 5.9±1.6 Hz for native PI-PLC, 3.2±0.2 Hz for the double mutant and essentially zero (0.30±0.27 Hz) for I43W. Since all three species are quite active, this suggests that the aggregate structure per se is not responsible for diC₇PC activation of cIP hydrolysis. Rather the aggregation upon interaction with diC₇PC micelles is likely to reflect placement of the hydrophobic Trp residues at the rim of the PI-PLC barrel and the size and orientation of the hydrophobic plug this produces.
Figure 3-7 (A) $^{31}$P linewidth (Hz) of the diC$_7$PC phosphorus resonance in the presence of 3 mg/ml WT (●), I43W (○), or I43W/W47I (■) PI-PLC. For comparison the linewidth for diC$_7$PC as a function of concentration without enzyme is shown (bold line). (B) Difference in diC$_7$PC line width ($\Delta\Delta\upsilon_{1/2}$) in the presence of PI-PLC for the three PIPLC enzymes.
III. Discussion

Previous studies of the crystal structure of the interfacially impaired *B. thuringiensis* PI-PLC W47A/W242A established that there is a major conformational change in two regions of the protein. Based on the structure of the monomeric *B. cereus* PI-PLC,\(^2\) the helix B region (Ile43 to Gly48) in wild-type PI-PLC, whose Trp residue has been proposed to partially insert into the membrane,\(^3\) orients the side chains of Ile43 and Trp47 (and possibly Val46 as well) so that they pack together and form a hydrophobic protrusion that extends outwards toward solvent. In the W47A/W242A mutant structure,\(^5\) the helical secondary structure of this segment has been disrupted to form an extended loop. The rim loop that would contain Trp242 has also been displaced. The removal of the two Trp residues thought to insert into membranes could explain the loss of PC binding and low phosphotransferase and cyclic phosphodiesterase activities. However, an unexpected and very interesting feature of this double mutant was that PI-PLC W47A/W242A exists as a homodimer. Residues in the helix B region, notably Pro42 and Gln45, play important roles in stabilizing this dimer. The key question is whether a dimer forms with native PI-PLC and, if so, how close the structure of W47A/W242A is to the ‘active’ dimer. Formation of a dimer at the membrane surface is appealing because it could provide the protein with an easier means of processive catalysis – release of water-soluble products by one subunit could occur with the other still tightly anchored to the membrane. Mutation of three or more of the tyrosine residues that form the hydrophobic core of the dimer interface in W47A/W242A dramatically reduce
PI-PLC phosphotransferase and phosphodiesterase activities. Those results could support a dimer as the membrane-bound form of this PI-PLC. However, whether or not helix B is intact in the membrane-bound form still needs to be addressed.

The results in this study of mutating residues in the helix B region of PI-PLC are consistent with dimer formation, although they do not prove that dimers form. Q45A has reduced affinity for PC interfaces. Yet if helix B is intact, the residue at position 45 should not contribute to membrane binding (or dimer formation) since it is on the opposite face of helix B (Figure 3-2 D). With an intact helix B, both Ile43 and Trp47 should dominate binding of the helix to the membrane. The reduced PC activation of Q45A indicates that the membrane-active conformation of the enzyme is less favorable with this mutation, supporting the hypothesis that helix B is unlikely to remain intact at the membrane surface. In the W47A/W242A dimer, Gln45 forms a hydrogen bond with Gly239 of the other subunit (Figure 3-2E). If this contributes to dimer stabilization at the membrane, Gln45 removal would be expected to reduce PC affinity and reduce enzyme activities, and indeed both cIP and PI cleavage were reduced with the Q45A mutation. Pro42 is also involved in dimer stabilization in W47A/W242A. However, P42G could also alter helix B formation and orientation – the introduction of a very flexible residue allows the enzyme to have much greater variability in the helix B region. Interestingly, diC₇PC activation of cIP hydrolysis is affected with only small affects on PI cleavage. This could suggest that the presence of acyl chains on the bound substrate also contribute to dimerization of PI-PLC.

Leu39 provides an interesting wrinkle on membrane binding and dimer
formation of *B. thuringiensis* PI-PLC. Like W47A, L39A binds very poorly to PC vesicles. Furthermore, the magnitude of the drop in affinity is much greater than would be predicted if an alanine rather than a leucine side chain partitioned in the membrane. In the crystal structure of *B. cereus* PI-PLC as well as the W47A/W242A dimer, Leu39 is embedded inside the enzyme and should not have a significant effect on PC binding. The significantly weaker binding of L39A to POPC vesicles suggests a membrane-active dimer is not exactly the same as the W47A/W242A dimer and that there is a conformational change in this region of the barrel rim that either orients Leu39 outward so that it can partition directly into the PC surface or positions this residue to interact with the initial portions of the hydrophobic acyl chains of the substrate.

A proposed model for activation of *B. thuringiensis* PI-PLC is shown in Figure 3-8. PI-PLC in solution has an intact helix B (shown in green) that keeps the enzyme as a monomer and prevents premature dimerization (conformation A in Figure 3-8). Solution dimerization, were it to occur, might prevent tight binding of the protein to membranes since the resultant dimer (based on W47A/W242A) has a highly concave surface. An intact helix B also provides the hydrophobic surface for the initial binding of the protein to a PC membrane (or a PC-enriched region in mixed substrate/PC bilayers). Once contact occurs, Trp47 is inserted into the bilayer/interface. The insertion of Trp47 could trigger a rearrangement of helix B residues altering both the position and structure to an extended loop that could provide several points of contact, Ile43 and Val46 as well as Trp47, with the
membrane (conformation B in Figure 3-8). Membrane anchored protein monomers can now dimerize on the PC-containing membrane surface via Tyr246, Tyr247, Tyr248, and Tyr251 residues\textsuperscript{6} with both Pro42 and Gln45 part of the dimerization network. Enhanced PI cleavage to cIP would in part reflect the increased lifetime of the protein dimer on the membrane. However, other changes must occur for cIP hydrolysis to also be activated by PC interfaces. Work by us\textsuperscript{1,3,4,11} and others\textsuperscript{12,10} has suggested there is a conformational change in the Bacillus protein (as also suggested in a similar PI-PLC from \textit{Listeria monocytogenes}\textsuperscript{13}) when it binds to activating interfaces that increase $k_{\text{cat}}$ and decrease the apparent $K_d$ for membranes. This is reflected in a tighter affinity for activating interfaces\textsuperscript{3a,c} and an increased inhibition of cIP hydrolysis by substrate analogues such as PA or PMe\textsuperscript{11}. In a W47A/W242A-like dimer, the accessibility of active site residues is altered to present a more extended surface\textsuperscript{34} that could alter how the activator and substrate molecules bind to PI-PLC.

**Figure 3-8. Model for the role of helix B residues in PI-PLC activation by PC interfaces.**

There are also likely to be more subtle changes in the active sites of the dimer that modulate the catalytic step. The transformation of PI to cIP occurs by His32
acting as a general base to deprotonate the inositol C(2)-OH so it can attack the phosphate group. His82 then acts as the general acid to protonate the DAG leaving group. In the second step (cIP hydrolysis to I-1-P) water, deprotonated by His82 acting as a general base, acts as a nucleophile to attack the phosphorus while protonated His32 contributes a proton to the inositol C2 oxygen. It has been suggested that the catalytic triad of His82-Asp33-Arg69 is not optimally arranged to catalyze the hydrolysis of cIP.

Rather, hydrophobic interactions of the rim and perhaps the hydrocarbon chains of the PI substrate facilitate the changes necessary for optimal catalysis (hence PI cleavage will be much faster than cIP hydrolysis). For cIP in solution, both His32 and His82 would have the ‘wrong’ initial ionization states. Enhancing catalysis, particularly for the second step, would be provided by an allostERIC pathway between helix B residues and catalytic residues that shifts local pKa values so that enhanced bond cleavage occurs. Binding to a PC interface provides this local change while not binding to the active site in place of cIP. Gly48, at the C-terminal end of helix B, would appear to be a key residue in this process. In structures of the B. cereus PI-PLC and W47A/W242A, Gly48 is close to His82 (< 4 Å separation). While the binding of G48A to PC is very similar to native PI-PLC, the activities for both PI cleavage and cIP hydrolysis are lower than WT. What’s more, the activation by PC for cIP hydrolysis is dramatically decreased for this mutant relative to native PI-PLC (3-fold versus 22-fold). Since binding to PC induces conformational changes of the enzyme, Gly48 might be a very important site for communicating with the active site His82 about the conformational change of the
helix B region.

References:


Chapter 4

Mutagenesis Studies of Rim Hydrophobic Residues of Bacillus thuringiensis PI-PLC

I. Introduction

In order to better understand the function and regulation of PI-PLCs, which are peripheral membrane proteins, it is very important to investigate the interaction between the enzyme and the lipid bilayer. The B. thuringiensis PI-PLC, as a model system of the catalytic domain of mammalian PLCs that play important roles in signal transduction, has been shown to interact with zwitterionic surfaces (e.g., phosphatidylcholine (PC)) via two tryptophan residues (Trp47 in two-turn helix B and Trp242 in a disordered loop) at the rim of the barrel structure. The association of PI-PLC and the lipid bilayers may also cause conformational changes, but the result of this interaction is activation of the catalytic activity of the enzyme. A crystal structure of an interfacially impaired mutant W47A/W242A was solved, and this enzyme forms a dimer with significant conformational rearrangement of the loop and helix B residues. Whether the active form of btPI-PLC is a monomer or dimer is not known.

Figure 4-1 shows a fragment of the surface of this bacterial PI-PLC. In addition to the Helix B region (highlighted in green) that has been shown to have a strong influence on the binding of the enzyme to the membrane, a string of residues forming the separate hydrophobic core of this interface (highlighted in blue) are also very close to the active site (highlighted in red), and therefore could have significant
effects on the interfacial binding of the enzyme. There is also one residue that can be positively charged in the region. Among the interesting residues in this region are His$^{81}$, Leu$^{85}$, Tyr$^{86}$, Tyr$^{88}$, and Tyr$^{118}$. The three tyrosines, in particular, are right above the active site and might be involved in direct interaction with the membrane. Since it is difficult to tell which ones contribute to the interaction of the protein with the membrane surface from the crystal structure that was obtained in the absence of an interface, mutation studies of these residues individually as well as in combination were carried out and their effect on activity of the enzyme and PC binding will be the focus of this chapter.

Figure 4-1. Surface representation of *B. thuringiensis* PI-PLC with active site in red, Helix B in green and rim residues in blue).
II. Techniques to characterize protein/membrane interactions

There are several techniques available to study the interaction of proteins with membranes including centrifugation, fluorescence and NMR. Here we used intrinsic fluorescence of the PI-PLC and high resolution field cycling $^{31}$P NMR (fc-P-NMR) to study the protein-lipid interaction.\(^3\) Intrinsic fluorescence is a method that senses the interactions based on the quenching of intrinsic fluorophores Trp, Tyr and Phe. Because most of the emissions are due to excitation of Trp residues based on the quantum yields and lifetimes, the fluorescence spectrum of a protein containing these residues usually resembles that of Trp. The intensity, quantum yield and wavelength of maximum fluorescence emission of Trp are highly solvent dependent with the intensity of the fluorescence increased when the polarity of the solvent surrounding the Trp residues decreases. Trp fluorescence can be quenched by a neighboring quencher such as a dibromo- or nitroxide group. One shortcoming of the method is that the intrinsic fluorescence only shows the overall protein-lipid binding but not the details.

$^{31}$P NMR is an alternative choice for studying the interaction because PI-PLC can recognize the head groups of substrate, analogue, and activator phospholipids and these have different $^{31}$P chemical shifts. A traditional method is to monitor the increase of the line width of the $^{31}$P resonance upon protein binding, but the limitation for this method is poor resolution and large line widths of resonances for phospholipids in vesicles at the high fields of modern instruments. An alternative method is the high resolution field cycling $^{31}$P NMR (fc-P-NMR) spin-lattice
relaxation technique, which resolves the dynamics of each phospholipid component in mixed phospholipid vesicles through monitoring relaxation rates over a wide range of magnetic fields. Over most of the accessible high field range chemical shift anisotropy (CSA) has more contribution to $^{31}$P relaxation than the dipole-dipole interactions, even when small phosphorus-containing molecules are free in solution. If the small phosphorus-containing molecules are bound to a large complex such as an enzyme, their observed relaxation rate, $R_1$, becomes much larger. The extent of the $R_1$ change is related to the binding affinity and size of the transient aggregate. If there is an excess of ligand molecules compared to the enzyme and bound and free ligand are in fast exchange on the NMR timescale, then the field-dependent $R_1$ depends on the ratio of ligand/protein and the proximity of the $^{31}$P to the nearby proton dipoles that relax it. Adding an electron-spin label to the protein near a suspected phospholipid binding site can enhance the effect on the $^{31}$P. For this nucleus, the much larger magnetic dipole of the electron is much more effective than nearby protons, even with a high ligand/protein ratio. To vary the magnetic field, the sample is mechanically shuttled between the center high field region of the commercial NMR magnet's probe, and a substantially lower magnetic field located above the probe before, and after, the delay times normally used in spin-lattice relaxation rates. This allows us to obtain relaxation rates over a wide field range (0.002 to 11.7 T). The shape of the $R_1$ versus field curve provides several correlation times ($\tau$) for each phospholipid molecule on timescales ranging from ps to $\mu$s. This method can provide information on the location of any discrete phospholipid sites on the protein.
The binding energy of *B. thuringiensis* PI-PLC to a phospholipid interface is a sum of different energies, most of which are from van der Waals contacts and electrostatic interactions. Dr. Nathalie Reuter at the Department of Molecular Biology in the University of Bergen, Norway, carried out explicit molecular dynamics simulations adding this PI-PLC into a pre-equilibrated dimyristoyl-PC (DMPC) bilayer with solvent present. A protein was added to each side of the bilayer. In these simulations (20 ns in total), four distinct interactions of a PC with the protein molecule were observed with two PC molecules binding to each monomer (Figure 4-2). Interestingly, the interactions on each side were not the same – different regions of the protein formed what appeared to be discrete complexes with a PC headgroup. The choline headgroup can be involved in π-cation interactions with aromatic residues, and *B. thuringiensis* PI-PLC has many tyrosines located in regions that are likely to be in the proximity of the membrane surface. The simulation suggested that Tyr86, Tyr 88, Tyr204, Tyr246, and Tyr251 along with Trp242 could participate in formation of these transient complexes,

Of the many Tyr residues in the rim region above the active site of the protein, replacement with alanine at several sites (close to helix B as well as the active site) was carried out to investigate the interactions between enzyme and membrane. A loss in PC vesicle binding affinity suggests that the interaction seen in the simulations may indeed contribute to transient anchoring of the protein to the surface.
Figure 4-2. Snapshots of the main $\pi$-cation interactions observed in the trajectory of $Bt$PI-PLC binding a DMPC bilayer. This explicit membrane molecular dynamics simulation was carried out by Nathalie Reuter, University of Bergen, Norway. (a) Positions of the aromatic residues involved in $\pi$-cation interactions with the choline group. (b) Pocket formed by Tyr204 and Tyr251, and double partner of Tyr251. (b) Pocket formed by Tyr251 and Trp248. (d) $\pi$-cation interaction of Trp47. (e) Interaction of Tyr86 and Tyr88.
Also generated were mutations where His81 and Leu85 were changed to alanine since these residues are also in the region around the top of the active site and might contribute to electrostatic binding to phospholipid surfaces – the lysine supplies a cation to interact with any negatively charged phospholipids, while the histidine may be partially cationic. We would expect these two mutant proteins to have only moderately changed affinities for PC but a possible reduction in binding to vesicles of an anionic phospholipid that mimics the substrate (e.g. phosphatidylglycerol, PG, or phosphatidylmethanol, PMe).

III. Results

A. Secondary structure and thermal stability of mutated PI-PLCs

Mutations of His81, Le85, Tyr86, Tyr88 or Tyr118 to alanine were prepared, together with double and triple mutants of these positions. CD spectra of WT and mutant PI-PLCs were used to check for the overall conservation of the secondary structure of the enzymes. The thermostability of the enzymes was measured by monitoring the loss of secondary structure at 222 nM with temperature. The proteins denature irreversibly so this is not an equilibrium experiment. However, it allows us to see if there are any many changes in protein structure.

Table 4-1 summarizes the estimates of native PI-PLC and mutant secondary structure calculated from the CD wavelength spectra by CDNN. While L85A, Y86A, and Y118A had the same secondary structure as native protein, H81A, Y88A, double mutant Y86A/Y88A and triple mutant Y86A/Y88A/Y118A exhibited reduced α-helix content and increases in β-sheet and random coil structure. All mutants had similar Tm
values to that for WT protein – values were 54-57°C compared to the 57°C for the native (WT) protein.

Table 4-1. Secondary structure and $T_m$ of mutated PI-PLC enzymes.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>Secondary Structure (%)</th>
<th>$T_m$ °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Helix</td>
<td>β-Sheet</td>
</tr>
<tr>
<td>WT</td>
<td>26.3</td>
<td>20.6</td>
</tr>
<tr>
<td>H81A</td>
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</tr>
<tr>
<td>L85A</td>
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</tr>
<tr>
<td>Y86A</td>
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</tr>
<tr>
<td>Y88A</td>
<td>20.3</td>
<td>23.8</td>
</tr>
<tr>
<td>Y118A</td>
<td>28.7</td>
<td>19.4</td>
</tr>
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<td>Y86A/Y88A</td>
<td>23.3</td>
<td>22.0</td>
</tr>
<tr>
<td>Y86A/Y88A/Y118A</td>
<td>20.5</td>
<td>23.5</td>
</tr>
</tbody>
</table>

B. Phosphotransferase and cyclic phosphodiesterase activity

The enzymatic activities of these enzymes bearing surface mutations were measured using a detergent (diC$_7$PC) mixed micelle system to monitor PI cleavage (the phosphotransferase step) and cIP hydrolysis in the absence and presence of diC$_7$PC to monitor the phosphodiesterase activity and ability of a PC interface to activate the enzyme (Table 4.2).

Two of the altered enzymes exhibited a reduced phosphotransferase activity compared with native PI-PLC. H81A and L85A had PI cleavage rates of 40% and 51% of what WT enzyme showed. Y86A and the double and triple mutants were comparable to WT. Interestingly, Y88A and Y118A were significantly more active than WT.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PI (8 mM)</th>
<th>cIP (20 mM)</th>
<th>cIP/diC&lt;sub&gt;7&lt;/sub&gt;PC</th>
<th>Ratio of cIP activity ± diC&lt;sub&gt;7&lt;/sub&gt;PC</th>
</tr>
</thead>
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<tr>
<td></td>
<td>µmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>µmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>µmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>955</td>
<td>2.80</td>
<td>62.5</td>
<td>22.3</td>
</tr>
<tr>
<td>H81A</td>
<td>386</td>
<td>0.1</td>
<td>4.56</td>
<td>44.1</td>
</tr>
<tr>
<td>L85A</td>
<td>484</td>
<td>1.14</td>
<td>7.41</td>
<td>6.5</td>
</tr>
<tr>
<td>Y86A</td>
<td>1022</td>
<td>1.01</td>
<td>29.9</td>
<td>29.7</td>
</tr>
<tr>
<td>Y88A</td>
<td>2790</td>
<td>1.50</td>
<td>41.6</td>
<td>27.8</td>
</tr>
<tr>
<td>Y118A</td>
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<td>Y86A/Y88A/Y118A</td>
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<td>0.5</td>
<td>5.1</td>
<td>10.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The PI is solubilized in 32 mM diC<sub>7</sub>PC. Specific activity values are ± 20%

<sup>b</sup> 5 mM diC<sub>7</sub>PC was added to the 20 mM cIP sample to test PC activation. Specific activities are <20% of the value tabulated.

A comparison of the cIP hydrolysis rates in the absence and presence of diC<sub>7</sub>PC is shown in Table 4-2 for these rim hydrophobic residue mutants. In the absence of any interfaces, all the mutants had cIP cleavage rates of 32 to 83% of WT enzyme activity except for H81A, and Y86A/Y88A/Y118A, which exhibited 4% and 18% of the rate of WT. Addition of diC<sub>7</sub>PC micelles increased all the activities with the activation-fold (ratio of the activity in the presence of PC compared to that in its absence) comparable to WT for Y86A, Y88A, and Y118A. L85A had the least activation by diC<sub>7</sub>PC (an increase of only 6.5-fold). H81A was significantly activated, although the final specific activity was still much less than WT (Table 4-2).
Y86A/Y88A and Y86A/Y88A/Y118A were both much less sensitive to kinetic activation by this non-substrate phospholipid suggesting either a reduction in diC₇PC affinity or an altered binding of the protein to these micelles.

C. Binding of PI-PLC enzymes to PC vesicles

Previous studies have shown that the intrinsic fluorescence intensity of *B. thuringiensis* PI-PLC increases with binding of the protein to PC activator micelles or vesicles, while fluorescence intensity decreases with ligands such as PA (phosphatidic acid) or PG (phosphatidylglycerol) binding to the active site. Out of the seven tryptophans that all contribute to the intrinsic fluorescence emission, two of them (Trp47 and Trp242) located at the binding surface, in particular, were responsible for most of the spectral change upon interfacial binding. The rim hydrophobic residues I mutated are spatially close to these two tryptophans according to the *B. cereus* PI-PLC crystal structure. The structural or conformational changes caused by mutations of these residues, therefore, may affect the fluorescence intensity. To assess this, the fluorescence intensities of H81A, L85A, Y86A, Y88A, Y118A, Y86A/Y88A and Y86A/Y88A/Y118A were examined at 337 nm, the maximum emission wavelength, as POPC SUVs were added. Fitting the increase in fluorescence as a function of PC concentration provides a measure of the binding affinity of the enzyme for the vesicles. Figure 4-3 shows a typical binding profile for Y88A binding to PC SUVs. The apparent $K_d$ value is the bulk PC concentration for half the maximum fluorescence change.
Figure 4-3. (A) Change in intrinsic fluorescence for Y88A (0.02 µM) as a function of added POPC SUVs. (B) Titration curves for Y88A binding to POPC SUVs. The lines through the data represent the best fit.
The apparent $K_d$ values for the different proteins are summarized in Table 4-3. *B. thuringiensis* PI-PLC binds tightly to POPC SUVs with an apparent $K_d = 0.078\pm0.009$ mM. Of these rim hydrophobic residue mutations, only Y118A was close to WT (2-fold weaker). H81A, L85A, and Y86A were 5-6-fold weaker. For the leucine and tyrosine substitutions this loss of binding affinity is about what one would predict based on free energies of transfer of leucine and tyrosine side chains versus alanine.\(^6\) Y88A had a larger change for the PC $K_d$ than expected based on membrane insertion. The double and triple mutant proteins showed weaker binding to PC, but not quite as large a chain if these side chains partitioned into the bilayer. However, partial insertion, as might occur for $\pi$-cation interactions with the choline group, could easily account for the successive increases in $K_d$.

**Table 4-3. Apparent dissociation constants for mutated PI-PLC enzymes to PC vesicles.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Apparent $K_d$ (mM)</th>
<th>$K_d$ mutant/$K_d$ WT</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>$0.078\pm0.009$</td>
<td>1</td>
</tr>
<tr>
<td>H81A</td>
<td>$0.50\pm0.13$</td>
<td>6</td>
</tr>
<tr>
<td>L85A</td>
<td>$0.46\pm0.13$</td>
<td>6</td>
</tr>
<tr>
<td>Y86A</td>
<td>$0.37\pm0.11$</td>
<td>5</td>
</tr>
<tr>
<td>Y88A</td>
<td>$0.94\pm0.07$</td>
<td>12</td>
</tr>
<tr>
<td>Y118A</td>
<td>$0.15\pm0.03$</td>
<td>2</td>
</tr>
<tr>
<td>Y86A/Y88A</td>
<td>$0.68\pm0.25$</td>
<td>9</td>
</tr>
<tr>
<td>Y86A/Y88A/Y118A</td>
<td>$1.77\pm0.33$</td>
<td>23</td>
</tr>
</tbody>
</table>

What is striking about the vesicle binding data is that tight binding and high specific activities are not necessarily correlated. Y118A has about twice the specific
activity of WT for PI cleavage and within a factor of two for the PC $K_d$. However, Y88A has the highest PI cleavage rate but the weakest binding to PC SUVs. The other notable observation is that removing Tyr86 and then looking at the double and triple Tyr mutant proteins, one sees that PI cleavage is only moderately affected while there is a much more pronounced loss of cIP hydrolytic activity, particularly in the presence of diC$_7$PC. For the triple mutant, the lack of a large PC activation is not caused by poor PC binding, because in the assay the diC$_7$PC concentration is 8 mM, significantly above the 1.77 mM $K_d$. There must be some changes affecting productive binding of substrate in the active site that are not important if the substrate has acyl chains but are critical for cIP hydrolysis. It is as if the PC acyl chains cannot bind in a specific manner that usually enhances cIP hydrolysis.

**D. Searching for specific phospholipid binding sites on PI-PLC by field cycling $^{31}$P NMR**

Spin-lattice relaxation fc-P-NMR can provide more details about the location of spin labels relative to vesicle components. When the spin-label is close to the phospholipid and a complex with a lifetime of at least a microsecond is formed, the major contributor to $^{31}$P dipolar relaxation is the $^{31}$P electron interaction. Thus, these types of experiments provide two types of information: (i) if a transient phospholipid/protein complex does indeed form, and (ii) what the averaged nitroxide to $^{31}$P distance is for that complex. Since multiple PC molecules might bind to the protein, effects may be averaged. However, previous work with the *B. thuringiensis* PI-PLC showed that for PMe/PC (1:1) vesicles, a spin label on D205S was close to a
PC molecule, while the label on W47C or H82C was closer to PMe, which binds at the active site.\(^4\)

These experiments used 0.5 mg/ml (0.014 µM) protein bound to total 10 mM 1:1 PC/PMe mixture SUVs, so the mole ratio of protein/phospholipid molecules is ~1/700. At high field, most of the \(^{31}\)P relaxation is not dipolar, which makes it impossible to see effects of the spin-labeled protein unless the protein concentration is very high. At that point the SUV surface is covered with spin-labeled protein and electron spin-spin interactions can occur that complicate interpretation of any observed effects. At low fields (<0.02 T), the \(^{31}\)P relaxation, in the absence of spin-labels, is affected by a few nearby protons that results in a clear and easy-to-see dispersion. In this region a nearby electron spin-label has a very large and easily detected effect on the nuclear spin. What is important is that the effect of each phospholipid varies and therefore the patterns for PC and PMe are different, indicating they have distinct apatral locations when bound to the protein. Spin-lattice relaxation rates (\(R_1 = 1/T_1\)) can be obtained over a wide range of magnetic field strengths. The field dependent of \(R_1\) can then be analyzed to obtain correlation times for the electron-\(^{31}\)P interaction and the magnitude of the relaxation enhancement.

To explore the binding of rim hydrophobic residue mutants to SUVs, we used mutant proteins where Leu85 or Tyr88 was altered to cysteine. They were chosen based on their activity and fluorescence results. L85A has reduced activity while Y88A has much higher activity than native PI-PLC (Table 4-2), and they both have lower PC SUV binding affinity compared with WT (Table 4-3). The \(K_d\) for L85A for
pure SUVs is higher than WT, but there is no information available for PMe binding, which prompted us to carry out the measurements with fc-P-NMR. In order to introduce a much larger dipole in the place where the phospholipids may bind, a nitroxide spin-label, MTSL, was covalently linked to the cysteine.

The phosphotransferase activities of L85C and Y88C were checked before the fc-P-NMR measurements. Table 4-4 lists the results for the dimeric (disulfide-linked), monomeric (with excess reducing agent) as well as the spin-labeled enzymes. A PI/diC₇PC assay system was chosen so that only active site binding might be reflected. It is interesting to note that the change in activity is more dramatic for L85C than Y88C. The activity of the L85C monomer is close to that of L85A, already a poorer enzyme than WT. A dimer formed at this position really reduces activity – possibly by reducing substrate accessibility to the active site. The activity of L85C with spin-label attached is about a factor of two lower than that of unlabeled enzyme, again probably because the steric size of the attached label makes it more difficult for the substrate to enter the active site. In contrast, the spin label on Y88C has little effect on phosphotransferase activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dimer</th>
<th>Monomer</th>
<th>Spin-labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>N168C</td>
<td>1500</td>
<td>1630</td>
<td>-</td>
</tr>
<tr>
<td>L85C</td>
<td>85</td>
<td>532</td>
<td>279</td>
</tr>
<tr>
<td>Y88C</td>
<td>641</td>
<td>979</td>
<td>812</td>
</tr>
</tbody>
</table>

Table 4-4 Phosphotransferase activities (PI(8 mM)/diC₇PC(32 mM)) of PI-PLC enzymes with a cysteine substitution in the proposed membrane binding region.ᵃ

ᵃErrors in specific activities were typically <15% of the tabulated value.
Previous assay of the modified protein by M. Pu indicated there was no significant change in the activity of the modified protein.

The field dependence profiles for spin-labeled PI-PLC (0.5 mg/ml, 14.4 μM) in the 10 mM PC/PMe (1:1) SUVs are summarized in Figure 4-4. The fit in the mid to high field region provides information on protein binding and general membrane proximity and not a specific phospholipid/protein complex since the correlation time characterizing this region is ~5 ns in the absence of of protein⁷ and 10-15 ns if unlabeled protein is present.⁸

As shown in Figure 4-4A, τₑ for PMe, 3.9±0.9 ns, is similar to the values for the same vesicles without protein, 5.0±1.1 ns, so it is unlikely that PMe is binding well. In contrast, there is a pronounced relaxation effect of the spin-label on PC in the same SUVs. The curve is shifted to the left indicating a longer correlation time consistent with protein restricting motion of the PC. The τₑ extracted for PC, 13.2±3.6 ns, is noticeably greater than for SUVs without protein (5.5±0.9 ns). Effects of the spin-labeled protein on the magnitude of the relaxation rate are small in this region.

The low field region is a better indicator of phospholipid/spin-labeled protein proximity. As shown in Figure 4-4B, both relaxation rates increase at lower field. To assess how much of this behavior is caused by proximity to the spin-label on the protein, we subtract the control for unlabeled protein at this concentration binding to the vesicles (Figure 4-4C). The residual or excess relaxation rate, ΔR₁, due to the spin-labeled protein varies with magnetic field and is related to on average how close the spin-label site is to a particular phospholipid when it is bound to the protein.
Figure 4-4. Field dependence of 5 mM each PMe (©) and PC (©) $^{31}$P relaxation rates in the presence of spin-labeled L85C (14 μM): (A) mid-to-high field data; (B) low field data; (C) $R_1$ due to spin-label(control $R_1$ with unlabeled protein subtracted).

We fit the data with a simple expression for just the dipolar relaxation of the $^{31}$P
by the electron:

\[ \Delta R_1 = \Delta R_{P-e}(0)/(1 + \omega_P^2 \tau_{P-e}^2) \]

where \( \Delta R_{P-e}(0) \) is the limiting relaxation at zero field, and \( \tau_{P-e} \) is the correlation time for this interaction. The parameter \( \omega_P \) is the magnetic field strength \( H \) (in Tesla) times the magnetogyric ratio for \(^{31}\)P: \( \omega_P = \gamma_P H \). The distance of the \(^{31}\)P to the spin-label, \( r_{P-e} \), is derived from the following:

\[ r_{P-e}^6 = ([\text{PI-PLC}]_o/(2/3)[\text{lipid}]_o) \times (S^2 \tau_{P-e} / \Delta R_{P-e}(0)) \times (0.3 \mu^2(h/2\pi)^2 \gamma_P^2 \gamma_e^2) \]

This assumes in these SUVs that 2/3 of the total phospholipid is in the outer leaflet. The effect measured depends on fast exchange between phospholipid bound to the protein at a discrete site and the bulk pool of phospholipids in the vesicles.

For the analysis, \( \tau_{P-e} \) was assumed to be 1 \( \mu s \) (based on extracting this parameter for spin-labeled PI-PLC variants that were closer to the phospholipid binding sites\(^4\)). While it was expected that PMe in the active site should be relaxed by this label, little effect was observed, implying that the binding of PMe to the site is much weaker than assumed. No significant PMe complex exists with a lifetime \( \sim 1 \mu s \). This is consistent with the low phosphotransferase specific activity of spin-labeled L85C. The excess PC \( R_1 \) in the low field region that is due to the spin-labeled protein allows us to extrapolate \( \Delta R_{P-e} \) from Figure 4-4C and estimate the distance of the tight PC site to the spin-label at L85C as 16.5\( \AA \).
Figure 4-5. Comparison of $R_1$ as a function of field for (A) 5 mM PMe and (B) 5 mM PC in 1:1 SUVs with 14 µM spin-labeled L85C. The dotted lines show profiles for the controls (individual control $R_1$ shown as +). The solid line for PC represents the fit to the points shown.

To further emphasize that there is a small effect of spin-label on L85C on PC and at most a similar small effect on PMe, we show the field dependence profile for PC and PMe but compared to the unlabeled control in Figure 4-5. Leu85 is right above the active site residues so it was expected that a spin-label positioned here would have a strong relaxing effect on PMe. The $K_d$ for L85C binding to PC SUVs is ~0.5 mM, so at 10 mM total lipid in the NMR experiment we expect all the enzyme to partition
onto the SUVs.

There appears to be a small rise above the control at 0.01T but the values are very close to the control. From the crystal structure one would have expected much higher $R_1$ values for PMe – to values greater than $10 \text{ s}^{-1}$ at 0.06 T. Since there is little effect of this spin-label on PMe, this again emphasizes that the PMe-protein complex must have a very short lifetime and/or a very high two-dimensional $K_d$. The $R_1$ values for PC are also fairly close to the control, although the change is a little larger. The PC site is thought to be ~15 Å from the active site, so this is about the amount of relaxation one might expect with this ratio of labeled L85C to phospholipid.

All of these results point to the fact that L85A mutant protein might be an example of poor substrate binding but moderately reasonable PC binding.

For spin-labeled Y88C PI-PLC, the relaxation profile was also interesting. This is a position where the tyrosine interacts with a phosphocholine headgroup in the MD simulation. However, in this mutant protein we have replaced the aromatic group with a cysteine and added a spin-label to it. Nonetheless it is still in the region around the active site, so we expected some effect of the spin-label at this position on the PMe $^{31}$P relaxation rate at low field. Figure 4-6 shows the field dependence of $R_1$ for PC and PMe in the presence of the spin-labeled Y88C. What is striking is that in the mid-field region (Figure 4-6A), the profile for both PMe and PC have significantly increased $R_c(0)$ values – this value is typically ~ $1 \text{ s}^{-1}$ for pure PMe/PC vesicles and increases a small amount with unlabeled protein (at 1 mg/ml) added. Here the value for the limiting $R(0)$ for this dispersion is $1.7-1.8 \text{ s}^{-1}$. This indicates the position of the
spin-label is certainly in the vicinity of the membrane.

Figure 4-6. (A) Mid to high field region and (B) low field region showing the variation of $^3$P $R_1$ with magnetic field dependence for PMe (5 mM)/PC (5 mM) SUVs with 14 µM Y88C PI-PLC: (ymbol PMe; (ymbol PC.
The low field region, which reports on the phospholipids binding to a specific site, does not show a large effect for either phospholipid (Figure 4-6B). There are small effects on each, but nothing suggests a long-lived complex of the protein with PMe or close proximity of the spin-label to a PC site (although there is a small effect of the spin-label on PC relaxation). This is better illustrated in Figure 4-7 where the profile for each phospholipid is compared to the control (a comparable concentration of non-spin labeled protein). The $\Delta R_1$ due to the spin-label is shown as a function of field in Figure 4-7C. Clearly there is no discernible effect of PMe but there is a small effect on the PC. Fitting the excess relaxation data for PC shown in (C) provides an estimate of $r_{p-e}$ as 17.5 Å. This would indicate there is still a tight PC site, but off on the periphery of the protein, not near the active site in this protein.

Y88C is a reasonable enzyme, perhaps half as active as the cysteine control (and not quite as active as Y88A), but PMe does not stick long in the active site. We know both PMe and PC in the bilayer are close to the spin-label site from the excess relaxation in the 0.1 T dispersion. However, in the low field region there is no significant effect on PMe so when it binds to the active site it is there only for a brief period of time. Previous FCS studies of Y88A$^3$ showed a $K_d$ of 0.3 mM for Y88A compared to 0.01 mM for the native PI-PLC control binding to 1:1 PG/PC bilayers. In light of the fc-P-NMR results we would interpret this weaker binding as being primarily due to a shorter $k_{off}$ of PMe in the active site.
Figure 4-7. Comparison of the low field dependence of $R_1$ for (A) PMe and (B) PC in Figure 4-6 compared to the control with unlabeled protein. The (+) symbol indicates the behavior of the control. In (C) is shown the excess $R_1$ due to the spin-label for PMe (⊗) and PC (⊙).
IV. Summary

The rim hydrophobic residues have very special interfacial locations that are close to both the active site and the membrane binding region, helix B, so we expected these residues would play a role in enzyme interfacial activation. A series of mutants of these residues (H81A, L85A, Y86A, Y88A, Y118A, Y86A/Y88A, Y86A/Y88A/Y118A) were constructed and characterized. All of the mutants had altered behavior compared with the wild type PI-PLC, especially L85A and Y88A.

L85A had lower activity and more importantly this mutant exhibited only small activation by PC. PC activation is thought to involve some conformational change of the protein around the active site when PC binds and anchors the protein at a site distant from this region.\(^9\) PC binding is weakened in L85C, but at the concentrations used in assays, the PC interactions should be saturated. Clearly, removal of the leucine at this position disrupts the ability of PC to enhance activity, even though the protein can still bind to PC vesicles. Field cycling experiments by Pu et al.\(^4\), suggested a discrete PC site existed on the periphery of the protein in the vicinity of Tyr204, Tyr246 and the other tyrosine residues in that region. That site may still be intact, since the field cycling does show a long-lived PC effect ~15-16 Å from the label on L85C. MD simulations suggest other π-cation interactions between the protein aromatic residues in PI-PLC and the PC headgroup, and it is possible that one of these is disrupted in L85C. However, the fc-P-NMR also shows that PMe does not bind well to spin-labeled L85C. The lower PI cleavage activity could result from misaligned substrate in a conformation where the allosteric effects of PC in enhancing
activity are less reduced.

Y88A had really high phosphotransferase activity (3 fold higher than wild type in a PI/diC₇PC mixed micelle assay) and lower interfacial binding affinity based on the results of intrinsic fluorescence and FCS. This tyrosine was observed to form a PC complex with Tyr86 in the MD simulations. Its removal does indeed weaken PC binding but the intriguing result is that activity is higher. If this π-cation interaction were absolutely critical to membrane binding, one would have expected a large decrease in activity. The high activity of mutant, spin-labeled or not, suggests such an interaction of Tyr88 with PC is transient at best and not really critical to activity under normal conditions. It may be that this interaction is what leads to surface dilution inhibition by PC of PI cleavage in vesicle systems.

Previous NMR and FCS results with native PI-PLC indicated that at high mole fraction of PC, the protein was sequestered in a PC-rich region and exchange of substrate and product into and out of the active site was reduced and that this was likely the cause of the observed surface dilution inhibition (loss of activity at fixed substrate concentration with increasing non-substrate – in this case PC). The observation that Y88A had weaker binding to PC and reduced surface dilution inhibition may, in fact, indicate that it is Tyr88 that is a major contributor to protein sequestration on a PC-rich vesicle.

In summary, these initial studies suggest that with the more sensitive NMR technology we can explore the function of specific rim hydrophobic residues with respect to changes in substrate analog or activator PC binding.
References:


Chapter 5
Role of X/Y Linker in Interfacial Activation of a Mammalian PI-PLC

I. Introduction

Mammalian PI-PLC isozymes play an important role in signal transduction pathway by catalyzing the hydrolysis of phosphatidylinositol 4,5-biphosphate (PI(4,5)P$_2$) into the two secondary messengers of diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$)$^1$ (Figure 5-1). The level of the substrate, PI(4,5)P$_2$ can directly regulate important biological processes, for example modulating activities of many ion channels.$^2$ For the two products, DAG is an activator of protein kinase C (PKC) and IP$_3$ functions as a universal calcium (Ca$^{2+}$) mobilizing messenger. These processes are essential for various cellular processes including fertilization, division, differentiation, platelet shape change, aggregation, muscle contraction and hormone secretion. PI-PLCs can be activated by numerous hormones, growth factors, neurotransmitters, antigens, and other external stimuli.$^3$

Figure 5-1. PI-PLC catalyzed hydrolysis of PI(4,5)P$_2$ generates DAG and IP$_3$. 
At least thirteen PI-PLC isozymes are expressed in humans: PLC-β1 to β4, PLC-γ1 and γ2, PLC-δ1, δ3 and δ4, PLC-ε, PLC-ζ, and PLC-η1 and η2. All of the PI-PLC isozymes are multi-domain proteins and share the same backbone that includes, from N-terminus to C-terminus, the PH domain, EF hand domain, X-Y catalytic domain and C2 domain (with the exception of PLC-ζ that lacks the PH domain). The N-terminal PH domain possesses a high affinity for PIP₂. The EF hand domain is critical for catalytic activity, although its function is unclear. Unlike most other EF hands, those in PLC do not bind Ca²⁺ tightly as deduced from the crystal structure where no Ca²⁺ ions were detected in this domain. The catalytic domain is composed of the X and Y domains separated by a linker region.

The difference in the regulation of PLC-β, -γ, -ε isoforms can be partly explained by the specific regions beyond the common backbone (Figure 5-2). PLC-β isoforms contain a long C-terminal extension and can be activated by a wide range of stimuli from tiny odorant molecules to full-sized proteins. Activation by the latter requires specific combinations of Gα and Gβγ subunits to couple their effectors. Different regions of PLC-β interact with Gα subunit and Gβγ subunits which are released from heterotrimeric G proteins by activation of G protein-coupled receptors. The α subunit has high affinity for the extensive C-terminal tail of PLC-β isoforms, while the Gβγ subunit binds to the N-terminal PH domain. Different PLC-β isoforms have different affinity for the G protein subunits: PLC-β1 and PLC-β3 exhibit high affinity for the Gα subunit, while PLC-β2 and PLC-β3 are sensitive to the Gβγ subunit. Recently, Hicks et al. reported that a loop (X-Y linker) separating the two halves of the catalytic
TIM barrel occludes the active site of PLC-β2. Removal of X-Y linker constitutively activates PLC-β2 without ablating its capacity to be further stimulated by classical G protein modulators, indicating that PLC-β2 may have an autoinhibitory regulation mechanism.\textsuperscript{10}

PLC-γ isozymes (PLC-γ1, PLC-γ2) have an insertion, which is a split PH domain containing two Src homology (SH2) domains and an SH3 domain, between the two halves of the TIM-barrel catalytic domain. The PLC-γ isozymes are primarily regulated by various receptor tyrosine kinases.\textsuperscript{11} After growth factor binds to the receptor tyrosine kinases, the SH2 domains of PLC-γ1 mediate binding to auto-phosphorylated tyrosine residues and the Tyr\textsuperscript{783} of the PLC-γ1 is phosphorylated.\textsuperscript{12} PLC-γ isoforms can also be activated by a variety of receptors that lack intrinsic tyrosine kinase activity and by various non-receptor tyrosine kinases.\textsuperscript{ref}

As the largest PLC isoform, PLC-ε has two special domains: (1) an RA domain located at the C-terminus of PLC-ε mediates the interaction with Ras family small G-proteins; (2) a CDC25 homology domain, located at the N-terminus of PLC-ε, also mediates the interaction with Ras family small G-proteins.\textsuperscript{13} PLC-ε can also be stimulated by Rho family small G-proteins; this PLC plays an important role in cell proliferation.\textsuperscript{14}

Recently, two isoforms of PLC-η were independently identified: PLC-η1 and PLC-η2.\textsuperscript{15} PLC-η contains a long extended region at its C-terminus that shows no homology with any other protein. Both PLC-η1 and PLC-η2 isoforms exhibit Ca\textsuperscript{2+}-dependent PI(4,5)P\textsubscript{2} hydrolysis, while PLC-η2 can be activated by the Gβγ
subunit. There are no well-established protein activators for sperm-specific PLC-ζ which is uniquely sensitive to the concentrations of calcium.

Figure 5-2. Domain organization of mammalian PLC isoforms (adapted from Katan, 2005).

The regulation mechanism of PLCδ is not very clear. Previous work showed that PLCδ is uniquely sensitive to physiologically relevant concentrations of calcium, but there are other possible regulation mechanisms. Feng et al. reported that the high-molecular-weight GTP-binding protein Gh can stimulate PLCδ activity. Homma et al. reported that a protein similar to Rho-GAP can stimulate PLCδ activity. More recently, Ranjinder et al. reported PLCδ was inhibited after binding to the calcium-signaling molecule calmodulin (CaM), while addition of Ral reversed this inhibition.

The PH domain and C2 domains of PLC play roles in protein / membrane
interactions. Several PLC PH domains bind phosphoinositides tightly, and this aids in anchoring the enzyme to target membranes. The C2 domain binds Ca\(^{2+}\) and it is thought that this domain also contributes to membrane binding via forming a Ca\(^{2+}\) complex with anionic phospholipids.

There is no direct report of interfacial activation of the catalytic domain of mammalian PI-PLC. In contrast, the *Bacillus thuringiensis* PI-PLC was shown to exhibit very specific activation upon binding to phosphatidylcholine-containing interfaces.\(^{20}\) Bacterial PI-PLC and the catalytic domain of the mammalian PI-PLC enzymes share very similar structures.\(^ {21}\) Studies also show that their catalytic mechanisms are also very similar. Therefore, bacterial PI-PLC is largely used as a model for mammalian PI-PLCs. In addition, Hicks et al. reported that the X-Y linker in the PLC-δ1 catalytic domain might also play an auto-inhibitory role.\(^ {10}\) The X/Y linker is located on the protein surface above the active site. However, the X/Y linker region is very flexible and not observed (except for a few residues) in the PLC-δ1 crystal structure (Figure 5-3).\(^ {22}\) The details of how the X/Y linker region regulates interfacial binding and catalytic activity have not been investigated. The basic structure of PLC-δ1, which is one of the simplest mammalian PLC isoforms, makes it a good system for exploring this effect.

In order to investigate the role of the X/Y linker region in interfacial binding and activation, a mutated enzyme with the deletion of residues 461-471 (Δ11) that are located at the center of the X/Y linker region and full of negatively charged residues was constructed and characterized for kinetic activation. Micelles and large
unilamellar vesicles (LUVs) were used to measure interfacial binding and the corresponding enzyme activity. In order to study the different effects of substrate and nonsubstrate lipids, we monitored the binding in multi-component LUVs using fluorescence resonance energy transfer (FRET) at higher protein concentration and fluorescence correlation spectroscopy (FCS) at much lower protein concentration. The PI-PLC activity in micelles and LUVs was monitored using $^{31}\text{P}$ NMR. The results indicate that the 11 residues in X/Y linker region play auto-inhibitory role in membrane binding and have an effect on protein activity.

Figure 5-3. Crystal structure of mammalian PLCδ1 (X/Y linker shown in blue and active site shown in red).
II. Results

A. Rat PLCδ1 activity towards PI in micelles

The mammalian PLCs have similar catalytic residues, to the bacterial PLCs and catalyze the cleavage of substrates via a two-step mechanism. Ca$^{2+}$, a cofactor in the mammalian enzyme, binds at the active site, and lowers the pKa of the inositol 2-OH. It is proposed to make direct contact with the transition state and the intermediate of the reaction, cIP. The interaction with cIP keeps this initial product bound in the active site and explains why the mammalian enzymes generate two water-soluble products at the same time (cIP and I-1-P in Figure 5-4 or their phosphorylated analogs).

Figure 5-4 $^{31}$P spectrum of a reaction mixture of PI(8 mM)/Triton X-100(16 mM) incubated with PLCδ1.

In our kinetic assays, PI was chosen as the substrate (even though it is a poorer substrate than PI(4,5)P$_2$, because it is considerably cheaper and more available than phosphorylated PI species. PI can be presented to PI-PLC in a range of different
matrices: micelles, small unilamellar vesicles, large unilamellar vesicles or water/organic solvent co-mixtures. Differences in PLC activity towards substrate in different types of interfaces can often provide insights into what factors control the activity of this enzyme. We monitored enzyme activity (µmol min\(^{-1}\)mg\(^{-1}\)) towards PI/TX-100 mixed micelles. PI hydrolysis was kept between 8-25% in order to obtain specific activities with errors < 20%. TX-100 is a nonionic detergent that solubilizes PI bilayers at TX-100/PI > 2.\(^{23}\)

As shown in Table 5-1, the phosphotransferase activity of the deletion mutant PLC-δ1 Δ11 towards PI (8 mM) in TX-100 (16 mM) mixed micelles was increased significantly (4-fold) compared with the wild type (WT) enzyme. Increasing TX-100 at fixed PI concentration (8 mM) will eventually “inhibit” the enzyme if the surface concentration of PI is important for rat PLCδ1 activity. This phenomenon, termed ‘surface dilution inhibition,’ has been documented for the *B. thuringiensis* PI-PLC.\(^{26}\) Increasing TX-100 concentration to 32 mM led to a drop in the PLC specific activity for both Δ11 and WT. However, the relative specific activity of Δ11 compared to WT was essentially the same. Keeping the ratio of PI/TX-100 fixed but decreasing the total concentration of PI (4 mM) in TX-100 (8 mM) again led to a drop in the specific activity with the decrease in activity larger for WT than for Δ11 - a result that might suggest better substrate binding characteristics for the deletion mutant. The consistent increase in activity with the removal of the linker region rich in acidic residues is in keeping with this section of the protein acting as an autoinhibitor.
Table 5-1. Specific activities ($\mu$mol min$^{-1}$ mg$^{-1}$) towards PI solubilized in Triton X-100 micelles and the ratio of PLC$\delta$1 wild type and $\Delta 11$ deletion mutant activities (errors in values are within 20%).

<table>
<thead>
<tr>
<th>assay sample</th>
<th>WT</th>
<th>$\Delta 11$</th>
<th>$\Delta 11$/WT</th>
</tr>
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</tr>
</tbody>
</table>

B. Activation effect of non-substrate phospholipids

The structure of the catalytic domain of PLC$\delta$1 is very similar to the *B. thuringiensis* PI-PLC. The *B. thuringiensis* PI-PLC is activated by binding to zwitterionic surfaces, particularly phosphatidycholine (PC). This increases enzyme binding to substrate-containing surfaces (bilayers, micelles, etc.) and also appears to enhance the catalytic step as well. PLC$\delta$1 could also exhibit activation by other phospholipids. The substrate PI(4,5)P$_2$ also binds to the PH domain and helps anchor the catalytic domain. There are reports that PS also activates the enzyme, although whether or not this anionic lipid affects activity in all assay systems or enhances surface binding has not been examined in a systematic fashion. With this in mind, we examined the effect of several non-substrate phospholipids (PA, PC, PG, PMe, PS) on PLC$\delta$1 cleavage of PI in TX-100 mixed micelles. The concentration of enzyme (5 $\mu$g/ml for WT and 1 $\mu$g/ml for $\Delta 11$) and detergent (16 mM TX-100) were kept constant. A PI concentration of 8 mM was used as a standard for comparison to samples with half of the substrate (4 mM) replaced by non-substrate phospholipids (PA, PC, PG, PMe, PS) to determine if the presence of these nonsubstrates alters the enzyme specific activity. Because half of the substrate is replaced by non-substrate
phospholipids, the activity will decrease if non-substrate phospholipids do not have any activation effect. This is because the substrate surface concentration has been decreased by a factor of two.

The specific activities of Δ11 in every condition were higher than those of WT, consistent with the X/Y linker acting as an autoinhibitor. For the WT, the activity decreased dramatically if half of the substrate was replaced by zwitterionic phospholipids (PC). If half of the substrate was replaced by negatively charged phospholipids (PA, PG, PMe, PS), the activity was similar to the standard, with slightly decreased activity when the PI was replaced by PS and slightly higher activity when replaced by PA, PG or PMe. This means that for initial rates, it is the charge of the anionic phospholipids that is critical for specific activity. The results for Δ11 were similar to those of WT but not exactly the same. Trends are shown in Figure 5-5 where the activity for each protein is normalized to the value at 8 mM PI.

For Δ11, the biggest difference was caused by the replacement of PI with PA. The negatively charged PA obviously replaced PI in terms of activity for WT enzyme (where specific activity towards 8 mM PI and 4 mM PI/4 mM PA were essentially the same). However, substitution of half the PI with PA led to a decrease in activity for the Δ11 enzyme. Removal of the anionic linker appeared to enhance the inhibitory potency of PA selectively. PS was also less effective in replacing PI for Δ11 than for WT. These two anionic phospholipids may have a higher affinity for the active site than PMe or PG and access is enhanced in Δ11.
C. Binding of PLCδ1 to interfaces

Previous studies showed that the PH domain and C₂ domain of rat PI-PLC-δ1 play an important role in membrane binding. However, the catalytic domain could also contribute to binding. This domain has a number of hydrophobic residues around the barrel rim that are likely to partition into membranes. There are several established methods for testing peripheral protein binding to membranes. These include centrifugation and gel filtration assays, fluorescence (including intrinsic, FRET, FCS etc.) and NMR methods. We used FRET and FCS to study the binding of the WT and Δ11 PLCδ1 to vesicles in the absence of PIP₂ to see if changes in the catalytic domain altered protein partitioning to interfaces in parallel to changes in enzyme activity.
1. **PI-PLC activity toward PI in PI/PC LUVs**

The specific activities ($\mu$mol min$^{-1}$ mg$^{-1}$) of PLC$\delta$1 towards PI/PC mixed LUVs are shown in Figure 4-6. The substrate concentration (4 mM) was fixed and the mole fraction of PC was gradually increased. When different batches of protein were used there was considerable error, but the trends remained. In the pure PI LUVs, WT and Δ11 showed similar activity towards PI. As the mole fraction of PC, $X_{PC}$, increased, the enzyme activity increased; the activation was highest for WT around $X_{PC}=0.25$. Increasing $X_{PC}$ further led to a decrease in WT PLC$\delta$1 specific activity towards PI. As $X_{PC}$ increases with the bulk PI concentration kept constant, the surface concentration of PI is decreased and surface dilution inhibition, as detected in the mixed micelle system, is seen. Another change is that the surface charge becomes less negative as more PC is added to LUVs. The behavior of WT indicates this enzyme is sensitive to either the substrate surface concentration or the surface charge or possibly both effects. For Δ11 the maximum specific activity was reached at $X_{PC} \sim 0.5$. At this composition of PI/PC, the linker deletion mutant enzyme was ~2.5 times more active than WT at the same $X_{PC}$. PLC specific activity is affected by a wide variety of factors, such as overall vesicle binding of the protein, binding of non-substrate lipid in the active site or altered lipid dynamics that change the substrate affinity for the vesicle-bound protein. Except for pure PI LUVS or when $X_{PC}=0.9$, the specific activity of Δ11 was higher than that of WT with the difference most pronounced in PC-rich vesicles (Figure 5-6). These results might suggest that Δ11 binds more tightly to the PC/PI mixed vesicles, an explanation that could be tested by measuring protein binding to
PC/anionic substrate analog LUVs.

**Figure 5-6. Specific activity of PLCδ1 WT and Δ11 towards PI(4 mM)/PC LUVs as a function of mole fraction PC (PC): WT (---♦---) and Δ11(▬■▬). The ratio of Δ11/WT activities at a given X_{PC} is also shown (…▲…).**

2. **FRET as a probe of PLCδ1 membrane binding**

Fluorescence resonance energy transfer (FRET) was used to monitor binding of PLCδ1 to vesicles. For these experiments, we prepared WT and Δ11 labeled at the N-terminus with a fluorescence dye (NBD) to serve as the donor to the rhodamine-PE (where the rhodamine dye is attached to the amino group on the phospholipid headgroup) in LUVS. Structures of NBD and rhodamine-PE and excitation and emission spectra of NBD and rhodamine are show in Figure 5-7. Cysteine is usually used for protein labeling, but PLC-δ1 has 25 cysteines in total and more than 10 of them are located at the surface of the protein. Thus, the N-terminal amino group of the protein was chosen for attachment of the NBD group. The Förster distance ($R_0$), which defines the distance when FRET efficiency between the NBD/Rho pair is 50%, is ~56 Å.27 The crystal structure of PLCδ1 was obtained in two parts – the PH domain was not
observed in the structure for full length protein and was crystallized separately. Therefore, the relation of the N-terminus of the full length protein to the active site is not known. However, since the PH domain can contribute to membrane binding via its high affinity for PI(4,5)P$_2$, it most likely does get within $R_0$ to the membrane surface.

The labeled donor, NBD-PLCδ1, was kept at a fixed concentration (typically 0.5 µM) and vesicles containing the phospholipids PC and PMe with ~ 0.5 mol% Rho-PE were titrated into the protein solution. The NBD fluorescence at 535 nm was then monitored. PMe was chosen as the PI analog because it is a good inhibitor and should have no interaction with the PH domain. When the NBD-protein binds to the vesicle, FRET occurs from the NBD group on the protein to the Rho group on the lipids and the intensity of the NBD fluorescence decreases. After subtracting the control, which is the sum of intensity of NBD-PLCδ1 titrated with unlabeled vesicles and intensity of unlabeled PLCδ1 titrated with Rho-labeled vesicles, a dramatic decrease in the fluorescence intensity at 535 nm ($\Delta I_{535}$) was clearly observed (Figure 5-8). The lines through the data shown in Part A of Figure 5-8 represent the best fit assuming a simple model for protein binding to the LUVs (a hyperbolic fit was used to extract an apparent $K_d$). The calculated $K_d$ values are summarized in Figure 4-7B. For both WT and Δ11, as the fraction of PC increased (from $X_{PC} = 0.25$ to 0.90), efficient FRET required more vesicles added consistent with an increasing $K_d$, or weaker binding.
Figure 5-7. Structures of NBD and rhodamine-PE and excitation and emission spectra of NBD and rhodamine: (A) NBD; (B) rhodamine-PE; (C) excitation (dashed line) and emission (solid line) spectra of NBD (green) and rhodamine (blue).

(A) succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate NBD-X, SE

(B) 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)

(C)
Figure 5-8. Titration curves for (A) WT and (B) Δ11 NBD-PLCδ1 binding to PC/PMe (0.5 mol% Rho-PE) mixed LUVs at varied $X_{PC}$: $X_{PC} = 0.25$ (---○---), $X_{PC} = 0.5$ (---□---), $X_{PC} = 0.7$ (...◊...). The lines through the data represent the best fit. In (C) is shown the extracted $K_d$ for partitioning of the proteins to these LUVS.

<table>
<thead>
<tr>
<th>PLC</th>
<th>$K_d$ (mM)</th>
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<tbody>
<tr>
<td>WT</td>
<td>$X_{PC} = 0.25$</td>
</tr>
<tr>
<td>Δ11</td>
<td>$X_{PC} = 0.5$</td>
</tr>
<tr>
<td></td>
<td>$X_{PC} = 0.7$</td>
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All of the data point to the fact that the binding of protein becomes weaker as less anionic phospholipid is present. PLCδ1 clearly prefers to bind to a negatively charged surface as has been showed in activity studies. Δ11 appears to bind to the vesicles slightly more tightly than WT but the differences are small and not statistically robust. Thus, removal of the anionic 11-amino acid stretch in the XY-linker region does not have a dramatic effect on partitioning of the protein, at least at this concentration, onto vesicles.

3. PI-PLC binding to vesicles measured by FCS

FCS (Fluorescence Correlation Spectroscopy) is an experimental technique which can monitor protein translational diffusion – a parameter that is dramatically affected when a protein binds to a large vesicle. The significant advantages of FCS are that a low concentration (<1-50 nM) of dye-labeled protein is sufficient for the measurement. This concentration range is typically what is used in kinetic assays. We used FCS for screening different phospholipid compositions to investigate how protein binding was affected by phospholipid compositions.

For these experiments, PLCδ1 was labeled with AF488 at its N-terminus. This Alexa dye has high stability (i.e., does not photobleach readily) and a high quantum yield, so is ideal for FCS. In most FCS studies of peptide and protein association with vesicles, large unilamellar vesicles (LUVs) were generally used due to their higher thermodynamic stability and relatively uniform size. The rat PLCδ1 preferentially binds to LUVs necessitating the use of LUVs for these binding experiments. LUVs composed of different ratios of PC to anionic phospholipids were prepared with a
mini-extruder and had an average diameter of 1000 Å as monitored by light scattering.\textsuperscript{30c}

**Mutant Δ11 binding to mixed component LUVs**

From the activity results, we might expect that the composition of the vesicles should affect the PLCδ1 binding to the LUVs, although this was not seen in the FRET experiments. Since FCS allows us to look at much lower protein concentrations, and since it has been suggested the PLCδ1 aggregates at higher concentrations, we felt it would be useful to measure vesicle binding using FCS. Therefore, the binding of the enzyme to PC/PX (where PX is an anionic phospholipid, either PA, PMe or PS) LUVs. The titration curves for AF488 PLCδ1 Δ11 binding to PC/PX vesicles at $X_{PC} = 0.5$ are shown in Figure 5-9. The binding curves were sigmoidal. The fit to these sigmoidal curves used the expression $f_b = f_{max}^* [PL]^n / (K_d^n + [PL]^n)$, where $f_b$ is the fraction protein bound, $f_{max}$ is the maximum fraction bound (usually 0.7-0.9), and $n$ is a coefficient to account for the sigmoidal nature of the curve. This $n$ is not the Hill coefficient. Rather it is an empirical parameter that accounts for the sigmoidal behavior of the profiles. The apparent $K_d$ is the total phospholipid concentration at which half the maximum binding of protein is observed.

In these mixed vesicles, PLCδ1 Δ11 protein had a lower affinity for PA and PS (the weakest) compared with PMe. These results are perhaps opposite what one would expect from the results of kinetic inhibition using mixed micelles, where PA was the most potent inhibitor, followed by PS with little effect by PMe. However. Binding to micelles and LUVs can be significantly different.
Figure 5-9. Titration curves for AF488-PLCΔ11 binding to PC/PX (where PX is an anionic phospholipid) vesicles at fixed $X_{PC} = 0.5$. The LUVs contained POPC along with POPA (○), DOPMe (□), or DOPS (◊).
Figure 5-10. Titration curves for AF488-PLC-Δ11 binding to PC/PMe vesicles as a function of X_{PC}: 0 (○), 0.25 (□), 0.5 (◊), 0.7 (x) and 0.9 (Δ). The lines through the data represent the best fit.
**WT binding to mixed component LUVs**

We performed the same FCS measurements on AF488-labeled WT protein in order to test its binding to vesicles containing different anionic phospholipids and to PC/PMe mixed LUVs where $X_{PC}$ was varied. The results showed that the enzyme binds to the vesicles with lower affinity as PC content increased, but fitting the data assuming only two species present – free and vesicle bound protein – was problematic.\(^\text{30}\) When we re-examined the protein-only data, which should have only one dye-labeled species present, we found some large particles were present as well as a species whose diffusion constant was consistent with a PLC monomer. The protein in the FCS experiments (28 nM) is present in buffer (100 mM NaCl, Tris pH 8.0) with BSA (1 mg/ml) present to aid in stabilization. We used dimer, trimer, tetramer and so on to try to fit two species equation for the protein alone but none of them fit well. At the very low protein concentration used in the FCS, most WT exists as monomers but there is still some aggregated protein complicating analysis of the data. Interestingly, this aggregation problem did not exist with the deletion mutant.

With LUVs added, we extracted $K_d$ values for labeled WT. Although the absolute values are likely not correct given the presence of aggregated protein, the trends in terms of how $K_d$ varies with $X_{PC}$ are valid. As shown by the results in Figure 5-11, at low protein concentration, the apparent $K_d$ of WT is larger than that for $\Delta 11$. While this difference may not be real, the trend – increased $K_d$ with increasing $X_{PC}$ – is similar to the deletion mutant. It is more pronounced for $\Delta 11$ in FCS binding experiments than in the FRET experiments at considerably higher protein.
concentrations. This may suggest that protein aggregation (perhaps dimer formation) aids in vesicle binding.

**Figure 5-11. Dependence of the apparent PI-PLC dissociation constant, $K_d$, for mixed PC/PMe LUVs on $X_{PC}$: WT (○...○), Δ11(□...□). The ratio of $K_d$ for WT compared to the $K_d$ for Δ11 is also shown (◊...◊).**

The apparent $K_d$ difference between WT and Δ11 is small at lower PC percentage. With increasing $X_{PC}$, the apparent $K_d$ difference between two proteins becomes larger. Since in the FRET experiments the two proteins show little difference in affinity for different $X_{PC}$ LUVs, the difference perceived in the FCS experiments most likely reflects changes in the protein aggregation state at low concentrations that...
destabilize the WT but not Δ11. For example, when $X_{PC}$ equals 0.9, the apparent $K_d$ for Δ11 was 881 µM; with the same vesicles, WT did not show any appreciable binding to the mixed vesicles even when the phospholipids concentration was increased to 5 mM. Since assays are done at concentrations more consistent with the FCS experiments, it suggests that removal of the XY-linker 11-residue peptide aids in stabilizing the form that binds well to vesicles. Furthermore, it strongly suggests that the dramatic loss of affinity in the PC-rich region is the reason for the lower specific activity observed for Δ11.

4. **PLC-δ1 activation at constant $X_{PC}$**

Based on the FCS results, the mutant Δ11 has much lower $K_d$ values than WT in the presence of high levels of PC. This difference in affinity for membrane should result in different specific activities when the lipid concentration is below or above the apparent $K_d$. Therefore, we compared the catalytic activities of WT and Δ11 at $X_{PC} = 0.7$ with the total phospholipid concentration was equal to 20, 8, or 3 mM. The results are shown in Figure 5-12.
The apparent $K_d$ values for WT and Δ11 were 10 mM (again, not too accurate for WT due to difficult in fitting) and 0.23 mM, respectively. Above the dissociation constant, the enzyme should bind well to the lipids. When the total lipid concentration is below $K_d$, on the other hand, the binding should become weaker. Indeed, the specific activities corresponded well with the binding. The specific activity of WT decreased as the total phospholipid concentration decreased, while the activities of Δ11 were similar at 20 and 8 mM with a small decrease in specific activity at the 3 mM phospholipid concentration. Although errors in measuring this activity are moderately large, there is a difference in the two enzymes for 3 mM substrate that is statistically significant. These kinetics confirmed that PMe is a good substrate analogue in this PC-rich region and indicated that vesicle binding controls the enzyme activity at this region. It also suggests the lower $K_d$ values in this region detected in the FRET assay are not directly applicable to enzyme activity. The apparent higher
affinity in the FRET assays likely reflects aggregated protein, which aids in keeping the enzyme anchored on the vesicle surface.

III. Discussion

Interfacial activation localized to the PLCδ1 catalytic domain has not been reported previously, although mutation of individual hydrophobic residues above the barrel rim did lead to reduced catalytic activity\(^{31}\). By measuring the enzyme activity towards different types of interfaces, we have tried to determine what factors control the catalytic activity of this enzyme.

Δ11, a mutant with 11 negatively charged residues located in the X/Y linker region deleted, showed much higher activity than WT. The results obtained with this mutant are consistent with the proposed auto-inhibition role for the X/Y linker. Anionic phospholipids are critical for the protein to bind to interfaces, and in the absence of PI(4,5)P\(_2\), the catalytic domain is likely to contribute to anchoring the protein to the bilayer. The negatively charged linker peptide could occlude the active site and need a strongly negatively charged surface to displace it and allow substrate to enter the active site.

All anionic phospholipids except PA and to a lesser extent PS prevent surface dilution inhibition of PI cleavage (Figure 5-5), but only in Δ11 and not WT. PA, with the smallest head group, prevents inhibition of WT by supplying the correct negative charge, but the activity with Δ11 was decreased, similar to the value for ‘surface dilution inhibition’. PA also can have a -2 charge, depending on the surface charge, so that it may behave more like a competitive inhibitor of PA than the other anionic
phospholipids (see Figure 5-5). PS may also have a slightly higher affinity for the Δ11 active site, and thus, while it supplies the needed surface charge, it also can occupy the active site to some extent. Alternatively for this phospholipid, the pKa of the PS amino group may be shifted to a less basic value and the contribution of PS to surface charge is less than expected.

Removing the linker region also allows the protein to bind to vesicles containing higher PC content. Even though we cannot obtain an accurate $K_d$ for WT, when $X_{PC}$ equals 0.9, Δ11 can bind to PC/PMe mixed vesicles weakly ($K_d = 0.88$ mM) while PLC-δ1 WT does not bind to the vesicles at all.

FCS experiments with the better behaved Δ11 clearly showed that the enzyme binds to the vesicles more weakly as the fraction of PC increased. FRET experiment results showed the same trend, although the loss of affinity was not as dramatic as in the FRET studies. The WT FCS results are not well fit because of multiple mixed forms of protein present in solution. At low protein concentrations, most of the protein exists as a monomer and more and more protein becomes aggregated as the concentration increases. Removing the linker region may also stabilize the protein monomer in solution, suggesting that the aggregation could be related with electrostatics of the catalytic domain and the linker region above this αβ-barrel.

The specific activities in the PLCδ1 assays at constant $X_{PC}$ (Figure 5-12) showed differences between WT and Δ11 in the PC-rich region that are consistent with the FCS results; the WT $K_m$ for substrate in a PC-rich vesicle must be significantly higher than that for Δ11 towards the same vesicles since WT activity decreased significantly
as the total phospholipid concentration was decreased. One possible explanation is that the PLCδ1 exhibits some aggregation at low protein concentration and electrostatic interactions of the X/Y linker region modulates this behavior. Increasing the protein concentration leads to more aggregation, and in the protein aggregate the electrostatic interaction at the X/Y linker region is reduced. Therefore, the apparent $K_d$ of WT and Δ11 are almost same in the FRET experiment where much higher protein concentrations were used to estimate the apparent $K_d$ values.

A proposed model for activation of PLC-δ1 is shown in Figure 5-13. PLC-δ1 WT exists in mixed forms in solution. At low protein concentration, most of the protein exists as monomer but nevertheless there are some aggregated forms. Aggregation in solution almost completely disappears after deletion of the eleven negatively charged residues in the X/Y linker region to generate Δ11. Thus, Δ11 represents a constitutively active enzyme since the active site is not blocked. It is also more prone to inhibition by other anionic phospholipids with PA the most effective. PA may present an interfacial phosphomonoester similar to PIP$_2$ for binding to the active site. The highly negatively charged X/Y linker provides an important control of PLCδ1 activity on negatively charged membranes such as the inner leaflet of the plasma membrane (where the PI(4,5)P$_2$ substrate for the enzyme resides). The negatively charged X/Y linkers are sterically and electrostatically repelled from phospholipid membranes upon recruitment and orientation of PLCδ1 at these negatively charged surfaces. Substrate PI as well as non-substrate anionic phospholipids (PA, PG, PMe, PS) can active the enzyme by displacing the X/Y linker.
Figure 5-13. Proposed model for activation of PLCδ1: each blue oval represents a protein and suggests protein aggregates form from PLCδ1 in dilute solution.

References:


7 Grobler, J.A. and Hurley, J.H. (1998) Catalysis by phospholipase C-δ1 requires that Ca2. bind to the catalytic domain, but not to the C2 domain, *Biochemistry* 37, 5020-5028.


Chapter 6

Future Directions

The work in this thesis was directed towards a better understanding of the activation mechanism of *B. thuringiensis* PI-PLC, as well as the kinetic activation and inhibition mechanism of the catalytic domain of the multiple-domain peripheral membrane protein, rat PI-PLCδ1. The simple single-domain protein was studied as a model for the mammalian catalytic domain, mainly because it has a very similar 3D-structure and catalytic mechanism to the catalytic domain of PLCδ1, and it is much easier to purify. Different approaches were adopted to investigate the interaction between these peripheral membrane proteins and phospholipid vesicles composed of substrate lipid (or analogues) and/or the kinetic activator phospholipid PC.

For the *B. thuringiensis* PI-PLC, the results of the helix B mutant proteins provided support for a model for the binding and activation of the enzyme (Figure 3-8). PI-PLC exists as a monomer in solution and the intact helix B region is stabilized by the rigid residue Pro42 that ensures the correct orientation of Lys44 and Trp47. Following the initial Lys44-mediated attraction to the anionic membrane, the helix B region could lose its structure allowing the substrate PI to bind to the active site pocket. During the binding event, several tyrosine residues (including Tyr86 and Tyr88) in the rim above the active site contribute to the interaction of the protein with PC in bilayers, possibly though π-cation interactions. My results indicate that the association of these residues with PC are transient – no long-lived complex is
observed with this two residues. Such transient interactions are difficult to follow in
detail with the binding methods I used. More sensitive techniques, such as FCS,
which monitors minute spontaneous fluorescence intensity fluctuations rather than
just the steady-state emission intensity, may provide more details about these
interactions. For example, FCS could be used to measure the binding of the
interesting mutant proteins as a function of the mole fraction PC ($X_{PC}$). In particular, it
would be useful to have a complete binding profile for hydrophobic rim residue
mutants including L85A, Y86A, Y86A/Y88A and Y86A/Y88A/Y118A. I showed that
these proteins had a lower affinity for PC, but it would be useful to assess how
binding to the PI analog PG and mixed PG./PC bilayers is affected. The dependence
of $K_d$ on $X_{PC}$ as well as measurement of specific SUV kinetics at different $X_{PC}$ may
provide useful information on which of these mutations also has defects in substrate
analog binding in the active site.

The information on the interaction between the *B. thuringiensis* PI-PLC and the
vesicle interface sheds some light on the binding affinity of the catalytic domain of
mammalian rat PLCδ1. There are several reports on binding affinity of PH domain
and C2 domain of the enzyme, but we are the first to focus on the study of the
catalytic domain. The results presented in Chapter 5 of this dissertation showed that
the linker region between X and Y domains played an auto-inhibition role and
removal of the linker region rendered high substrate activity of the protein as well as
binding to the vesicles under assay conditions. However, WT PLCδ1 aggregation
proved a serious hurdle in our FCS studies at low protein concentrations, which made
the data analysis extremely difficult. Future efforts should focus on identifying a way
to control this behavior. One possible approach is to make and test a PH domain
deletion mutant. There are multiple advantages for studying this mutant. First, the PH
domain plays a role in membrane binding as well (high specificity for PI(4,5)P$_2$) and
may interfere with the measurement of alterations of the active site. The deletion
mutant would avoid this complication. Second, the surface charge of the enzyme is
believed to be an important factor in protein aggregation, and the deletion of the PH
domain may change that attribute dramatically.

Another approach is to make and test a different hydrophobic mutant at the loop
region of the catalytic domain. Preliminary studies along these lines have been carried
out. The residue Trp555 was chosen because it resides at a very similar position in the
crystal structure of the enzyme to the position of Trp242 in the $B. thuringiensis$
PI-PLC. The results showed that replacement of Trp555 with Ala reduced the cIP
hydrolysis activity significantly but showed little effect on binding of the protein to
sphingomyelin and DOPMe single component vesicles through intrinsic fluorescence
or FRET. To better understand the role of Trp555, more kinetics and binding details
are needed. For kinetics, one could measure the PI hydrolysis activity in a micelle as
well as binary (PC/PI) component vesicle system. For measuring the membrane
protein interaction, a more sensitive assay for quantitative surface binding of
mammalian PLCδ1 is needed, and FCS or fc-NMR may be such techniques.