Phosphatidylinositol-specific phospholipase C: Conformational changes upon membrane binding

Author: Xiaomeng Shi

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PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C:
CONFORMATIONAL CHANGES UPON MEMBRANE BINDING
dissertation
by
XIAOMENG SHI

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

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Phosphatidylinositol-specific phospholipase C: conformational changes upon membrane binding

Xiaomeng Shi

Under the direction of Dr. Mary F. Roberts

ABSTRACT

Phosphatidylinositol-specific phospholipase C (PI-PLC) from *B. thuringiensis* is activated by phosphatidylcholine (PC) surfaces for both phosphatidylinositol (PI) cleavage to inositol 1,2-(cyclic)-phosphate (cIP) and subsequent hydrolysis of cIP to inositol-1-phosphate. These enzyme kinetics strongly suggest that this PI-PLC has two discrete binding sites for phospholipids – the active site binding PI (or substrate competitors) and an activator site specific for PC. However, it is difficult to determine the orientation and conformation of peripheral membrane proteins when docked to target membranes, let alone where sites for these might be on the protein. In this thesis, various biophysical techniques were applied to this bacterial PI-PLC to obtain structural information in the absence and presence of membranes to characterize specific conformational changes that occur when the protein binds to activating membranes.

The crystal structures of an interfacially impaired double mutant of PI-PLC, W47A/W242A, was solved and showed the protein as a homodimer. The major interactions came from four clustered surface tyrosine residues from each monomer. This structure suggested the possibility of PI-PLC dimerization on membrane surfaces as part of the mechanism for interfacial activation. Mutations of these tyrosines showed a loss of activity and membrane binding. Crystal structures of these mutant proteins showed no significant change in the proteins, consistent with either disruption of a dimerization interface of a specific PC binding motif.
FRET was used to try and monitor oligomerization of PI-PLC, derivatized on a cysteine introduced at residue 280 (W280C) with either a donor or acceptor fluorophore, on vesicle surfaces. The results suggested some specific aggregation could occur on very PC-rich surfaces but not on phospholipid vesicles with at least 50 mol% anionic phospholipids, strongly suggesting that a stable dimer was not forming when the enzyme was bound to vesicles mimicking conditions where enzyme specific activity is high. If dimerization occurs on surfaces, it must be transient.

To examine which portions of the PI-PLC are interacting with membrane and to further explore if there is any evidence for PI-PLC dimerization on membrane surface, deuterium exchange coupled by mass spectrometry experiments were carried out with wild type PI-PLC, W47A/W242A and a covalent dimer formed from W242C that is more active than wild type enzyme. Results showed (i) a stable short helix B (containing an exposed tryptophan thought to insert into membranes) in wild type PI-PLC and its complete destabilization in W47A/W242C, (ii) a flexible surface loop (containing another tryptophan thought to partition into the membrane) that became protected when the protein was bound to vesicles, and (iii) reduced deuterium exchange for the peptide containing the tyrosines that either mediate transient dimerization or form a PC binding site. These observations modify how we envision the protein anchoring to substrate-containing membranes.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>B. cereus</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td>Bacillus thuringiensis</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>cIP</td>
<td>D-myoinositol 1,2-cyclic phosphate</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>CSA</td>
<td>chemical shift anisotropy</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>diC6PC</td>
<td>dihexanoylphosphatidylcholine</td>
</tr>
<tr>
<td>diC7PC</td>
<td>diheptanoylphosphatidylcholine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOPA</td>
<td>1,2-dioleoyl-phosphatidic acid</td>
</tr>
<tr>
<td>DOPG</td>
<td>1,2-dioleoyl-phosphatidylglycerol</td>
</tr>
<tr>
<td>DOPMe</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylmethanol</td>
</tr>
<tr>
<td>DOPMe</td>
<td>1,2-dioleoyl-phosphatidylmethanol</td>
</tr>
<tr>
<td>DOPS</td>
<td>1,2-dioleoyl-phosphatidylserine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EF</td>
<td>elongation factor</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinoethanesulfonic acid</td>
</tr>
<tr>
<td>I-1-P</td>
<td>D-myoinositol 1-phosphate</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio galactoside</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>Listeria monocytogenes</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
</tbody>
</table>
PA  phosphatidic acid
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PC  phosphatidylcholine
PCR  polymerase chain reaction
PE  phosphatidylethanolamine
PEG  polyethylene glycol
PG  phosphatidylglycerol
PH  pleckstrin homology
PI  phosphatidylinositol
PI3K  phosphatidylinositol 3-kinase
PI-PLC  phosphatidylinositol-specific phospholipase C
PIP_n  Phosphoinositide
PKB or AKT  protein kinase B
PKC  protein kinase C
PLA  phospholipase A
PLB  phospholipase B
PLC  phospholipase C
PLD  phospholipase D
PMe  phosphatidylmethanol
POPC  1-palmitoyl-2-oleoyl-phosphatidylcholine
PS  phosphatidylserine
QFF  Q-sepharose fast flow
SDS  sodium dodecyl sulfate
SH2  Src homology 2
SUV  small unilamellar vesicle
TEMED  N,N,N',N'-tetramethylethylenediamide
TIM  triose phosphate isomerase
TX-100  Triton X-100
WT  wild type
X_{PC}  mole fraction of PC
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Chapter 1: Introduction
1. Phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes

Phospholipase enzymes hydrolyze the different ester bonds in a variety of phospholipids. They exhibit different specificities and in eukaryotic cells play key roles in signal transduction. Phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes catalyze the hydrolysis of phosphatidylinositol (PI) or glycosyl-PI (GPI) components of membranes to generate diacylglycerol (DAG) and inositol phosphates. In some microorganisms these enzymes are secreted and involved in enhancing infectivity by generating DAG from components of target eukaryotic cells. PI-PLC enzymes belong to the general class of lipolytic enzymes, which includes carboxylesterase, lipase and phospholipase activities (1). Many of these have high biotechnical potential for modification of organic molecules to generate specific modules for synthesis.

a. Sequence homologies and structures of PI-PLC enzymes

The mammalian phospholipase C family has several isozymes including PLC δ, β, γ, ε and ζ. They all exhibit high sequence homologies. Most of the isozymes contain 4 basic domains: a PH-domain (pleckstrin homology domain), EF hands domain, catalytic domain and C2 domain (Figure 1.1). Some isozymes in PLCβ family have an extension at the C-terminus. Members of the PLCγ family have additional PH and SH (src homology) domains between catalytic subdomains X and Y(2). The catalytic domain of mammalian PLC has a TIM-barrel structure. The structure of bacterial PI-PLC shows high similarity to the catalytic domain of mammalian PI-PLC (Figure 1.2).

There are two crystal structures of mammalian PI-PLC enzymes – PI-PLC δ1 (PDB code 1QAS (3)) and PI-PLC β2 (PDB code 2FJU(4)). Both of them consist of the basic domains as
shown in Figure 1.2 A and B. PI-PLC δ1 has 756 amino acids and PI-PLC β2 has 1181 amino acids. Both catalytic domains fold into the large structural superfamily of (βα)₈-barrels (TIM barrels). But the structures are not regular (βα)₈–barrels which fold as a strictly closed circular structure. The bacterial PI-PLC (crystal structures of the enzyme from Bacillus cereus as well as from Listeria monocytogenes have been solved, Figure 1.2C and D) folds as a single domain of 298 residues that is like the catalytic domain in mammalian PI-PLC. The structure also folds to an imperfect TIM-barrel, and has several surface loops that have the same orientation as in mammalian PI-PLC δ1. The PI-PLC δ1 has a C2 domain and is calcium dependent for its activity (Ca²⁺ binds to the active site as well as to noncatalytic domains such as C2 and possibly the EF hands). Bacterial PI-PLC (bPI-PLC) enzymes have no calcium in crystal structures and do not require calcium during catalysis. The PH, C2 and EF hand domains in PI-PLC δ1 are important for membrane recruitment and binding. In the smaller, single domain bPI-PLC, surface hydrophobic residues and charged loops play roles in lipid association.

---

**Figure 1.1** Mammalian PLC isozymes. (2)
**Figure 1.2.** Comparison of the overall structures of different PI-PLC enzymes: (A) PLCδ1 (note the PH domain is missing as is the X/Y linker); (B) PLCβ2 (here the PH domain is better defined and the initial part of the X/Y linker is visible); (C) *B. thuringiensis* PI-PLC with helix B(red) and the 237-244 loop (red) and Tyr strip(orange) highlighted; and (D) *L. monocytogenes* PI-PLC showing the same helix and loop as in (C) but also the high cationic character of the ‘bottom’ side of the protein. Different domains are shown with EF hand domain in magenta, C2 domain in yellow, catalytic domain in cyan and PH domain in green.
b. Crystal structure of *Bacillus cereus* PI-PLC

The *Bacillus cereus* PI-PLC was the first PI-specific phospholipase C for which a structure was determined (PDB code IPTG) (5). The enzyme is a single domain protein containing 298 amino acids with a molecular weight of 34.7 kDa. The protein adopts an imperfect (βα)₈-barrel structure with 8 α-helices (A to H): residues 4-8 (A), 42-48 (B), 55-61 (C), 91-107 (D), 127-139 (E), 204-222 (F), 243-264 (G) and 284-294 (H) and 8 β-sheets (I to VIII): residues 29-34 (I), 64-72 (II), 108-114 (III), 155-163 (IV), 173-176 (V), 193-201 (VI), 226-236 (VII) and 269-274 (VIII) (5). The arrangement of these secondary structure units in the primary sequence is shown in Figure 1.3. Two helices that should connect β-sheet IV-V and β-sheet V-VI are missing and lead to a thinning of the barrel. Inside the barrel, active site residues His32, Arg69, His82, Lys 115, Arg 163, Trp178, Asp198, Tyr200 and Asp274 form a binding pocket that the myo-inositol ring headgroup occupies during catalysis ((5) and Figure 1.4)

![Amino acid sequence](image)

**Figure 1.3.** Amino acid sequence of *B. thuringiensis* PI-PLC with α-helix sequence shown in red and β-sheet sequences shown in blue.
c. Phospholipid – structures and aggregation states

In phospholipids (Figure 1.5), there are two basic parts: the polar head group and the hydrophobic acyl chain(s). Fatty acyl chains are linked to two hydroxyl groups of the glycerol backbone (the sn-1 and sn-2 positions of L-glycerol phosphate). A polar group (R-) is linked to the third hydroxyl group forming a phosphodiester bond. Various R groups will form different types of phospholipids. Some phospholipids are zwitterionic like phosphatidylcholine (PC) and phosphatidylethanolamine (PE), while many are anionic such as phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA).

A phospholipid bilayer consists of an outer leaflet with polar head group facing watery surroundings and an inner leaflet with head groups in interior and fatty acid long chains in contact with those from outer layers. The major force for the formation of the lipid bilayers is hydrophobic interactions. The polar head groups prefer polar aqueous environment and the van der Waals force will increase the interactions between non-polar lipid tails in both outer and
inner leaflets. Also the hydrogen bonds and electrostatic forces will help stabilize the formation of bilayer.

Liposomes, or bilayer vesicles, composed of phospholipids, can be made in several size ranges. These SUVs (small unilamellar vesicles) and LUVs (large unilamellar vesicles) are good

**Figure 1.5** Structure of common phospholipids.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>R</th>
<th>net charge, pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>phosphatidic acid (PA)</td>
<td>– H</td>
<td>-1 or -2 (depends on matrix)</td>
</tr>
<tr>
<td>phosphatidylcholine (PC)</td>
<td>– CH$_2$CH$_3$N(CH$_3$)$_3$</td>
<td>0</td>
</tr>
<tr>
<td>phosphatidylethanolamine (PE)</td>
<td>– CH$_2$CH$_2$NH$_3$</td>
<td></td>
</tr>
<tr>
<td>phosphatidylglycerol (PG)</td>
<td>– CH$_2$CH(OH)CH$_2$OH</td>
<td>-1</td>
</tr>
<tr>
<td>phosphatidylinositol (PI)</td>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>phosphatidymethanol (PMe)</td>
<td>– CH$_3$</td>
<td>-1</td>
</tr>
<tr>
<td>phosphatidylserine (PS)</td>
<td>– CH$_2$CHCOO$^-$NH$_3$</td>
<td>-1</td>
</tr>
</tbody>
</table>
models to study membrane protein function, folding and assembly (Figure 1.6). SUVs have a size range from 20 to 50 nm and can be prepared by sonication or extrusion. SUVs are very asymmetric due to their large curvature. For PC SUVs around 22 nm diameter, there are about 1900 and 1100 molecules in the outer leaflet and inner leaflet of the vesicle (6). SUVs are metastable and will eventually fuse to form larger unilamellar vesicles with lower curvature or even multilamellar structures. LUVs are larger with a range from 50 to 10,000 nm; these are prepared by multiple passage through filters with proper sized pores. Due to the larger sizes and usual method of preparation (extrusion), LUVs have a heterogeneous size distribution (6).

Phospholipids can also be aggregated in other structures as well. A monolayer of phospholipid spontaneously forms at the air-water interface with the acyl chains pointing into the air (mostly N₂ and hydrophobic) and the polar headgroup in the aqueous phase. If the acyl chains of the phospholipid are short (e.g., diheptanoyl-PC), very asymmetric (in the extreme 1-acyl-PC or lyso-PC which has a single chain), or the headgroup very large compared to the acyl chain volume (e.g., ganglioside GM1), the lipid may form micelles. Any phospholipid mixed with a detergent will form detergent mixed micelles. Each of these can serve as an assay system for phospholipases.
d. PI-PLC cleavage and mechanism

Different classes of phospholipases exist for the four ester bonds in a phospholipid (Figure 1.7). Phospholipase A₁ cleaves the sn-1 ester bond to generate a free fatty acid and 2-acyl-phospholipid. Phospholipase A₂ enzymes hydrolyze the sn-2 ester bond and generate the 1-acyl-phospholipid as well as free fatty acid. Phospholipase D enzymes catalyze the hydrolysis of
phosphodiester bond to release the alcohol esterified to the phosphate as well as phosphatidic acid. Phospholipase C enzymes cleave at the phosphodiester bond that connects the glycerol backbone and phosphate to release diacylglycerol (DAG) and a phosphate ester (Figure 1.7). PI-specific PLC enzymes exhibit substrate preferences for PI and various forms of phosphorylated PI in the case of the mammalian enzymes.

![Diagram](image)

**Figure 1.7.** Classification of phospholipases based on ester bond cleavage sites.

Manmlalian PI-PLC (mPI-PLC) enzymes typically prefer PI(4,5)P$_2$ over PI(4)P or PI(5), and these monophosphorylated PIs are usually better substrates than PI. mPI-PLC plays a key role in signal transduction pathways by releasing two second messengers: I(1,4,5)P$_3$ and DAG (7-9). Bacterial PI-PLC (bPI-PLC) can only hydrolyze PI or glycosyl-PI anchors, but not PIP or PIP$_2$.

bPI-PLC catalyzes the hydrolysis of PI in two steps (Figure 1.8) via a general acid-base mechanism (5). In the first step, His32 acts as a general base to remove the proton on the 2'-OH of inositol. The deprotonated inositol oxygen than carries out an intramolecular attack on the PI
phosphorus. The transition state is stabilized by an arginine at the active site. His82 acts as general acid and provides the proton for the DAG. This series of steps generates a stable, water-soluble cyclic intermediate, inositol-1,2-cyclic-phosphate (cIP), along with the very hydrophobic DAG. In the second phase of the overall reaction, His82 acts as general base to deprotonate a water molecule, which then attacks the cIP phosphorus to generate the second product, inositol-1-phosphate (I-1-P). A very similar mechanism is used by mPI-PLCs, except that Ca$^{2+}$ is used to stabilize the transition state as opposed to an arginine in bPI-PLC. The first step is much faster than the second step and occurs optimally on an aggregated surface. Because cIP is soluble its hydrolysis can be studied separately from PI cleavage to cIP and DAG.

**Figure 1.8.** Catalytic mechanism of bacterial PI-PLC (adapted from (5)).

2. Interfacial activation of bPI-PLC

Phospholipases must have the ability to bind to lipid surfaces because their substrates exist in membrane phase. One of the most important properties of this class of enzymes is
interfacial activation – catalytic activity is enhanced by the enzyme binding to aggregated substrates as opposed to monomeric ones (10). This was first shown with short-chain phospholipid substrates that can exist in solution in the mM range as monomers but which aggregate to form micelles over a critical micelle concentration (CMC). However, other forms of interfacial activation exist for PI-PLC enzymes. For *B. thuringiensis* (and *B. cereus*) PI-PLC, a non-substrate PC surface can increase the $k_{cat}$ and decrease the $K_m$ towards both PI cleavage to cIP and cIP hydrolysis to I-1-P (11-13).

A large fraction of the PC activation of that bPI-PLC derives from enhanced binding of the protein to substrate-containing aggregates. Studies have shown that two tryptophan residues in two discrete surface structural features, one in short helix B (Trp47) and another in a longer surface loop (Trp242), are needed for the enzyme to bind to activating PC surfaces and for optimal catalytic activity. Removal of either one or both of these tryptophan residues greatly decreases the membrane binding affinity and catalytic activity of the enzyme (14, 15).

Although the binding of peripheral proteins to membranes often has a strong electrostatic component, tryptophan insertion into the membrane is also frequently observed, particularly for interfacial binding to PC surfaces (16-18). Tryptophan residues are particularly prominent in the region of the bilayer interfaces in membrane proteins as well (19-21). Tryptophan insertion in turn helps anchor the PI-PLC to vesicles and is ultimately connected to enzymatic activation. A variety of biophysical techniques have suggested that binding of the protein to the interface may not only involve tryptophan insertion in a bilayer but may also change the enzyme conformation and shift the enzyme to an ‘activated’ form (11-13, 22). However, no specifics were available on the structure of that ‘activated’ form.
While lots of very specific conformational changes could occur, one possibility explored in my thesis is protein dimerization induced by the interface. Protein oligomerization is one way of regulating enzymatic activity and has been examined for a wide number of soluble proteins. There are also many integral membrane proteins that are purified as monomers but are believed to function as dimers or oligomers (23). Because the long chain substrates exist in membrane phase, it is thought that protein aggregation on this two dimensional surface could help anchor the protein for processive catalysis. However, exploring membrane-induced oligomerization of peripheral membrane proteins, particularly where such aggregation might not be necessary for enzymatic activity but rather enhance catalysis, is very difficult.

Another unique property seen in the kinetics of phospholipases is ‘surface dilution inhibition’ (24-27). How this could affect enzyme activity is suggested in the scheme shown below. In the scheme, E is the enzyme, A is the vesicle, B is a specific phospholipid in the vesicle and Q presents the product.

The binding of membrane proteins can be described in two steps, the bulk adsorption step and the specific surface step. In the bulk step, the enzyme binds to the vesicle bulk surface and forms an enzyme-vesicle complex. If the enzyme binds to vesicle nonspecifically, the association will be a function of both the enzyme and the total lipid concentration. If the enzyme binds specifically to its lipid substrate, the association will be a function of molar concentration of the enzyme and the substrate, regardless of the presence of non-substrate lipids (or detergents in many assay systems). In the second ‘surface step’, the enzyme will specifically bind a substrate in its active site while it is adsorbed on the surface. The catalysis process then occurs to generate
products. If the enzyme-vesicle complex has a sufficiently long lifetime, the enzyme will be able to process one substrate, and release the product, and bind another substrate, etc. – processive catalysis. If the enzyme cannot bind to the vesicle surface very tightly, the enzyme may dissociate from the vesicle. If non-substrate molecules are in the vesicle and these bind the enzyme, the relative substrate surface concentration decreases as more non-substrate is added and the specific activity will decrease. This is termed ‘surface dilution’ behavior and it is often used to test for enzyme surface binding affinity for the substrate. If the binding affinity of an enzyme is high for substrate, increasing the bulk surface will have little effect on enzyme vesicle binding and specific activity. If the binding of enzyme to substrate is similar in magnitude to interactions with non-substrates forming the vesicle matrix, then decreasing the substrate surface concentration means lowering the molar fraction of substrate at fixed substrate concentration.

3. Methods used to study peripheral membrane protein binding to phospholipid aggregates

Since peripheral membrane protein function depends on the ability of these proteins to bind to surfaces, an effective and direct method for monitoring binding is critical. One commonly used method for assessing protein partitioning onto vesicles is to use filtration/centrifugation to separate vesicle-bound protein from soluble proteins (8, 15). SUVs are so much larger than most soluble phospholipases that the basic methodology is applicable. The drawbacks of these methods are the requirement for moderately large amounts of protein (certainly not as dilute as what is used in enzyme assays) and the observation that the filtration (often done with a concentrating filter that retains the vesicles but allows unbound protein to be eluted) or centrifugation in a sucrose gradient may disturb the binding equilibrium since the protein is concentrated during the separation of free and vesicle-bound material.
Fluorometry is also widely used for monitoring protein binding to vesicles (11, 28). When protein binds to lipids, the intrinsic fluorescence may increase or decrease with increasing lipid concentration depending on the proximity of tryptophan residues to the membrane surface. While the mechanism for a given change in fluorescence intensity is not always clear (particularly if the protein has more than one tryptophan), monitoring change in intrinsic fluorescence does provide a way to measure protein binding, as long as light scattering is not a problem. An alternative approach is fluorescence resonance energy transfer (FRET) (29, 30).

The basic idea is to preferentially excite a donor fluorophore on the protein and monitor the loss of fluorescence as the protein binds to a vesicle containing a molecule with an acceptor fluorophore (or the increase in fluorescence of the acceptor probe). Rapid lateral diffusion of lipids in a vesicle means that only a few % of a derivatized phospholipid need contain the acceptor fluorophore for efficient transfer of energy from bound protein. The key to successfully obtaining a $K_d$ is the donor-acceptor pair selection. Many pairs can transfer energy within a distance of 30-60Å so that the donor and acceptor need not be in direct contact. Another useful use of FRET is to probe the intramolecular interaction between proteins. By attaching donor fluorophore and acceptor fluorophore, the occurrence and intensity of FRET are used to examine the protein-protein interactions. The fluorophore attachment position and fluorophore selection are the key consideration in this method. In my work, I used NBD-W280C as the donor and Rho-W280C as the acceptor to try and detect oligomerization of PI-PLC on membrane surfaces. However, the difficulty of the method is finding an appropriate donor/acceptor pair effective over a short enough distance to specifically monitor complex formation.

Another possible method is to measure ligand binding to proteins by NMR and line-width changes. If the ligand is a small molecule, and exchange between the enzyme-bound and free
states is fast, then a weight-averaged line-width will report on binding. However, this is problematic for SUVs as ‘ligands’. The phospholipids in SUVs have broad linewidths, notably $^{31}\text{P}$ and $^1\text{H}$ resonances, and changes with the addition of a protein can be difficult to relate to a $K_d$. Changes in exchange rates or mixed relaxation mechanisms contributing to the linewidth make this particularly difficult for $^{31}\text{P}$ studies, although our group has used this nucleus to assess how proteins alter phospholipid aggregate properties. In my work, I have used either the filtration/centrifugation or intrinsic fluorescence assay to measure protein binding.

While bulk binding assays are useful, what is really needed to understand PI-PLC is a technique that directly assesses which segments of the protein interact directly with the membrane and which undergo significant conformational changes. Since the protein is cycling on and off the membrane it is very difficult to trap the protein in a membrane-bound conformation. All the PI-PLC crystallographic studies to date have protein without lipids bound, although the soluble proteins myo-inositol in the case of cPI-PLC and I(1,4,5)P$_3$ in the case of PI-PLC$\delta$1 have inositol moieties in the active site. One method that can help assess which regions of the protein interact with membranes is H/D exchange coupled with analysis of deuterium incorporation by mass spectroscopy (HXMS). This method requires small amounts (<1 mg) of protein and can even be used with impure proteins, since exchange in a given protein is detected by mass of specific peptides derived from the target protein sequence. Amide protons within proteins can exchange with deuterium (D$_2$O) in the environment. If the amide proton is involved in a hydrogen bond or buried deep inside a protein structure, the exchange rate will be greatly reduced. However, if an amide proton is exposed to the solvent or in a very flexible structure where any hydrogen bonds are easily broken and reformed, the deuterium exchange will be very fast. Short time snapshots of deuterium exchange can provide information on
discrete intra- or inter-molecular interactions, while longer time points can suggest whether transient conformational changes or changes upon binding, release, and rebinding.

The general procedure of HXMS is shown in Figure 1.9 (31). The target protein is incubated with D$_2$O buffer for different times from 10 up to 3000 seconds. The reaction is quenched by decreasing the system temperature to 0°C and lowering the pH value to very acidic to prevent any back exchange. The desired protein is later digested by pepsin, an efficient protease at very low pH values. The resultant peptides are injected into an HPLC-MS system to analyze the peptides m/z values. A shifting of m/z to higher value indicates deuterium incorporation.
Figure 1.9 Scheme for hydrogen exchange mass spectrometry experiments. (31)
4. Basic questions addressed in this work

Before I entered the laboratory, mutagenesis studies of the *B. thuringiensis* PI-PLC had pointed to two very specific features as critical for binding to PC vesicles. Trp47 and Trp242 in the bacterial enzyme were necessary for tight binding to PC vesicles and the increase in $K_d$ when these residues were removed was consistent with both side chains partitioning into the membrane (15). Since the interaction with PC surfaces also was shown to enhance catalysis toward water-soluble substrates, it was theorized that PC binding via these tryptophan residues also had an allosteric effect separate from just binding and proximity of the bound enzyme to higher local concentrations of substrate. This was used to suggest that tryptophan insertion induces a conformational change that is propagated to the active site. It was postulated (14) that a hydrophobic ridge in the barrel rim is the portion of enzyme that penetrates into the membrane during catalysis. The W47A/W242A protein was crystallized by Carl Zambonelli, a postdoctoral associate in the laboratory. X-ray diffraction data was obtained prior to my entry into the laboratory. However, it was not well refined. From the rough crystal shooting information, this mutant had two monomers in each asymmetric unit and formed a dimer.

Another interesting feature in the *B. cereus* PI-PLC structures available when I started was a concentration of clustered tyrosine residues at various regions on the protein surface including Tyr200, Tyr204, Tyr246, Tyr247, Tyr248, Tyr251, Tyr275. It has been observed that a tyrosine/tryptophan belt occurs in membrane proteins in the region of the phospholipid glycerol backbone and headgroup (21). Rather than a tyrosine belt, in *B. thuringiensis* these residues occur indiscrete clusters, some of which could play a role in enzyme reorientation and association on lipids surfaces. One of these tyrosine clusters is located in what turns out to be the dimer interface in W47A/W242A. Limited mutagenesis suggested removal of three or four of
these residues reduced binding of the protein to PC surfaces. These results provided a hint that bPI-PLC may exist as dimer form on membrane surfaces, although previous studies showed that wild-type PI-PLC exists predominately as a monomer in solution (12).

My Ph.D. work was geared to answer several questions. (i) In crystal structures of mutant proteins that are interfacially impaired, are there any obvious differences from wild type that could explain the reduced activity? (ii) Is there evidence for PI-PLC dimerization and its relationship to activity? (iii) What segments of the protein are in contact with the membrane?

My ultimate goal was to examine how *B. thuringiensis* PI-PLC changes upon binding to a membrane and to assess if there was any change in oligomerization for the bound protein. To answer these questions, we constructed several PI-PLC mutant proteins where some or all of the Tyr residues on the dimer interface were removed. These were characterized for specific activities and membrane binding. With the help from Dr. Chenghua Shao in the Boston University School of Medicine, I refined and analyzed the W47A/W242A crystal structure as well as structures of two tyrosine cluster mutants – Y247S/Y251S in the presence or absence of myo-inositol and Y246S/Y247S/Y248S/Y251S to assess any structural changes that might explain the loss of activity. To further explore if any dimerization happens on vesicles, FRET was also used to probe the interaction between one donor NBD-PLC and acceptor Rho-PLC. Lastly, HXMS was used to monitor which parts of the enzyme are in contact with the membrane or take part in intermolecular interactions.
References


Chapter II: Materials & Methods
**Chemicals**

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), L-ß-phosphatidylinositol (PI) from bovine liver, and the short chain lipids dihexanoyl-PC (diC6PC) and diheptanoyl-PC (diC7PC) were purchased from Avanti Polar Lipids, Inc., and used without further purification. Dihexanoylphosphatidic acid (diC6PA) was prepared from diC6PC by phospholipase D (partially purified *Streptomyces* sp. PLD obtained from Sigma) cleavage. D2O was purchased from Sigma. cIP was generated enzymatically from crude PI using recombinant PI-PLC as described previously (1). All other chemicals were reagent grade. Reagents for specific experiments (e.g., introduction of fluorophores on PI-PLC) and their sources are listed in the experimental section for those protocols.

**PI-PLC Mutations, Overexpression, and Purification**

**Preparation of PI-PLC:** Plasmid preparations were performed using a Qiagen plasmid miniprep kit (purchased from Qiagen, Inc.). The plasmid was transformed into appropriate competent cells (BL21-Codonplus (DE3)-RIL) by the heat-shock method (42°C for 30 seconds). One colony was inoculated into a 5 ml culture tube, which was incubated overnight at 37°C with constant shaking in the presence of 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. A 5 ml aliquot of the overnight culture was added to 2 L of LB media. The newly inoculated media was then incubated at 37°C in the presence of 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. When the O.D.600 was between 0.6 to 1.0, IPTG was added to a final concentration of 1.0 mM, and the culture was incubated overnight at 16°C. The cells were then harvested by centrifugation at 5000 rpm for around 15 min and frozen overnight. The cell pellets were dissolved in 20 mM Tris HCl,
pH 8.9, and lysed by sonication on ice for 5 minutes. Cell debris was pelleted using centrifugation (30 min at 15,000 rpm). The supernatant containing the enzyme was ready for subsequent purification steps.

**Purification of PI-PLC:** Cell extract (dialyzed against 20 mM Tris HCl, pH 8.9) was passed through a 0.22 µm filter to remove any particulate material and applied into a Q-sepharose fast flow column (15 mm X 12 cm) equilibrated with 20 mM Tris HCl, pH 8.9. The protein was eluted using a NaCl gradient ranging from 0 to 0.6 M in 20 mM Tris HCl, pH 8.0 at rate of 2 ml/min. The protein was further purified using a phenyl-sepharose column (10 mm X 10 cm) equilibrated with 0.6 M NaCl in 20 mM Tris, pH 8.0 and eluted with a decreasing NaCl gradient ranging from 0.6 to 0 M at rate of 1 ml/min. Enzyme purity, as checked by SDS-PAGE, was >90%. The fractions containing PI-PLC were collected and dialyzed against Tris buffer to remove high concentrations of salt. Millipore Centriplus 10 filters were used to concentrate the protein and often to exchange the buffer. Recombinant wildtype (WT) protein concentrations were determined by absorbance at 280 nm using an extinction coefficient based on the sequence ($\varepsilon_{280} = 64,150 \text{ M}^{-1} \text{ cm}^{-1}$) or Lowry assay (2). The Lowry assay was specifically used to estimate the concentrations of Trp and Tyr mutants.

**Construction of PI-PLC mutants:** A series of PI-PLC mutants were constructed using QuikChange methodology (3) with a site-directed mutagenesis kit from Stratagene. The QuikChange site directed mutagenesis was performed in several steps as instructed by the manufacturer. (i) The supercoiled double stranded DNA vector (5-50 ng) was incubated in the presence of a dNTP mix, two synthetic oligonucleotide primers (at least 125 ng of each) that contain the desired mutagenesis, and 2.5 units of PfuTurbo DNA polymerase. (ii) Using PCR, the oligonucleotide primers were extended by temperature cycling with PfuTurbo DNA
polymerase and cycling parameters listed in the manual. This led to generation of a mutated plasmid that contained staggered nicks. (iii) The product was then treated with 10 units of Dpn I endonuclease (target sequence: 5’-GmATC-3’) for 1 hour at 37°C. This endonuclease is specific for methylated and hemimethylated DNA and was used to digest the parental DNA template. (iv) The nicked mutated vector DNA was then transformed into XL1-Blue Supercompetent cells. Colonies were inoculated into 5 ml LB media and incubated overnight at 37°C. The mutated plasmids were isolated using the Qiagen plasmid miniprep kit.

The plasmids with the W242C and W47A/W242A mutations were constructed by former students in the laboratory. The mutant genes were overexpressed as for WT PI-PLC and the resultant protein was purified using similar methods to those used for WT protein. Due to the reduced hydrophobic binding affinity of W47A/W242A mutant, the gradient eluting buffer for the phenyl-sepharose column was changed to 1.0 to 0.6 M to enhance purity. For the W242C dimer, after purification, the protein was concentrated to over 5 mg/ml and left in fridge for at least 2 days to insure disulfide bound formation to generate a covalent dimer. Another cysteine mutant (W280C) of PI-PLC was also prepared (using QuikChange™ mutagenesis) to introduce extrinsic fluorophores into the protein at a position near but not at the putative dimer interface. All mutated genes were sequenced to confirm that the desired mutation was introduced. All mutant protein purities were checked by SDS-PAGE, and were over 90%. The yields of these mutants were similar and over 10 mg purified enzyme per 2 liters cell culture.

Mutant proteins with modifications in a surface tyrosine strip were also prepared. Because all four Tyr residues contribute to stability of the W47A/W242A dimer, single mutations converting each Tyr to Ser were not prepared. Instead, a minimum of two Tyr were replaced. The primers were designed for use with the Exsite mutagenesis kit (Stratagene) to
produce the double mutants Y246S/Y248S and Y247S/Y251S. The plasmid containing the double mutant Y247S/Y251S went through another round of Exsite mutagenesis to introduce mutations replacing Tyr^{246} and Tyr^{248} (to form Y246S/Y247S/Y248S/Y251S); the gene for Y246S/Y248S was further mutated to introduce Y247S (generating Y246S/Y247S/Y248S).

**Preparation of NBD-Labeled and Rho-Labeled PI-PLC**

Purified W280C PI-PLC (1 mM) was first incubated at 25 °C with 3 mM dithiothreitol for 10 min for full reduction of any intermolecular disulfide bonds that had formed. After dialysis / filtration to remove excess reducing agent, the protein was incubated overnight at 4°C in the dark with 10 mM fluorescent dye, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) or tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA), in 50 mM HEPES HCl, pH 6.5. The labeling reaction was terminated by dialyzing the protein mixture against 4 L of 50 mM HEPES, pH 6.5; this was repeated 4 times to remove free dye. Prior to fluorescence experiments, the dialyzed protein solution was centrifuged at 5000 rpm for 30 min to remove any particulate material. Fluorescently labeled protein solutions, 2.5- 3.3 mg/ml, were stored in the dark at 4°C and used within 1 month.

**Preparation of small unilamellar vesicles**

Small unilamellar vesicles (SUVs) of phospholipids were prepared by sonication. Briefly, lipids dissolved in CHCl₃ were dried under a stream of nitrogen, and the resultant film lyophilized overnight. The dry lipids (either pure POPC, pure DOPMe, pure DOPG, or mixture of POPC/DOPMe, POPC/DOPG) were dissolved in 50 mM HEPES, pH 6.5, to a stock concentration of 20 mM. The aqueous lipid dispersions were sonicated for 10-30 min with a Branson sonifier cell disrupter until the turbidity had cleared.
Circular Dichroism

Wild type and mutant PI-PLC secondary structure and thermal stability as monitored by the thermal denaturation transition \( T_m \) were measured using an AVIV 202 CD spectrophotometer equipped with a thermoelectric sample temperature controller as described previously \((4, 5)\). The temperature dependence of the ellipticity at 222 nm was monitored from 25 °C to 95 °C (with a heating rate of 1°C/min) for a solution of 0.04 mg/ml protein in 1.0 cm cell. Although the transition is irreversible, we can still compare protein stability of a wide range of mutant proteins. Comparison of secondary structure for wild type and mutant PI-PLC in this solution used wavelength scans from 290 down to 195 nm with protein (0.3–0.4 mg/ml) in a 0.1-cm cell at 25 °C. Secondary structure content was estimated with CDNN \((6, 7)\).

Fluorescence Spectroscopy

Intrinsic fluorescence measurements of PI-PLC as a function of added diC7PC were initially carried out on a Shimadzu RF5000U spectrofluorometer, then more recently using a Fluorolog R-3 spectrofluorometer. Measurements were carried out at 25 °C with \(-2\) μM protein in 50 mM HEPES, pH 7.5, with 1 mM EDTA using an excitation wavelength of 290 nm and 5-nm excitation and emission slit widths. At this concentration of protein there was no detectable light scattering for any of the protein samples with PC (or diC₆PA) micelles added. Changes in the fluorescence intensity were usually expressed as \((I - I_0)/I_0\), where \(I_0\) is the intensity of protein alone, and \(I\) is the intensity in the presence of an additive.

Close proximity of PLC molecules on the surface of SUVs was monitored by fluorescence resonance energy transfer experiments. A solution of 0.010 or 0.020 mg/ml NBD-PLC (donor) in 50 mM HEPES, pH 6.5, with 2 or 4 mM total phospholipid in SUVs, was prepared. Various amounts of Rho-PLC (acceptor) were added, from 0.005 to 0.10 mg/ml final
concentration. After the addition of the Rho-PLC molecules, the mixtures were incubated for 2 min, then fluorescence spectra were obtained at 25°C with the excitation monochromator set at 450 nm and a 2 mm slit width. Although the excitation maximum for NBD is 465 nm, a lower wavelength was chosen to minimize the excitation of tetramethylrhodamine (8). Fluorescence emission was measured at 530 nm with a slit width of 5 mm. The percent of energy transfer (% FRET) was determined by calculating the decrease in the quantum yield of the donor (NBD-PLC) due to the addition of an acceptor (Rho-PLC). The % FRET was obtained experimentally from the ratio of the fluorescence intensities of the donor in the presence (FDA) and in the absence (FD) of the acceptor at the emission wavelength of the donor (530 nm). The extent of FRET is given by

\[ \% \text{FRET} = (1 - \frac{F_{DA}}{F_D}) \times 100. \]

A correction to emission at 530 nm for the contribution of the acceptor was made by subtracting the signal produced by the acceptor labeled protein alone mixed with vesicles. The dependence of the %FRET on added Rho-PLC was analyzed with the expression:

\[ \% \text{FRET} = \text{Eff}_{\text{max}} \frac{[\text{Rho-PLC}]^n}{(\text{Rho-PLC}_{0.5})^n + [\text{Rho-PLC}]^n} \],

where \( \text{Eff}_{\text{max}} \) is the extrapolated maximum FRET (in percent), \( \text{Rho-PLC}_{0.5} \) is the concentration of the acceptor labeled PLC for 50% of \( \text{Eff}_{\text{max}} \) and \( n \) is a coefficient used to account for any sigmoidal character in the curves.

**PI-PLC \(^{31}\text{P} \) NMR Kinetic Assays**

The activity of PI-PLC mutants toward PI (typically 4–8 mM) dispersed in either Triton X-100 (1:2 PI/Triton X-100 unless otherwise noted) or in diC7PC (1:4 PI/diC7PC unless otherwise noted) micelles, and toward cIP in the absence or presence of 5 mM diC7PC was measured by \(^{31}\text{P} \) NMR spectroscopy as described previously (1, 9). Assays (in 25 mM HEPES,
pH 7.5) were carried out at either fixed time points (picked so that <10% product was generated) or as a function of time (monitoring cIP intensity over 0.5–1 h) to estimate specific activity for the different mutants. The amount of PI-PLC used depended on whether the phosphotransferase or cyclic phosphodiesterase step was to be followed (1, 9, 10). Typically 0.05–5 µg protein was added for phosphotransferase reactions and 1~5 µg for cIP cleavage.

**PI-PLC Vesicle Binding Assays**

A vesicle binding assay based on centrifugation to separate free and vesicle-bound protein was used to characterize the partitioning of PI-PLC to PC interfaces (5). This assay used 10–15 µg/ml PI-PLC (ET) in 10 mM Tris HCl, pH 7.5. The POPC SUV concentrations ranged from 0 to 2 mM. Filtrates, containing free enzyme, were lyophilized and analyzed by SDS-PAGE. Band intensities were used to calculate the concentration of ET by comparing intensities from vesicle-containing samples to the ET value of the control. The concentration, EB, was then evaluated as ET – EF. Binding was analyzed using a simple binding isotherm and assuming the binding was cooperative: 

\[ \frac{E_B}{E_T} = \frac{L}{E_T}(L + K_d) \]

The apparent Kd derived from this treatment, which is really the concentration of PC needed for half the maximum binding, allows a direct comparison of the extent to which the different mutant PLC proteins partition to PC vesicles.

**X-ray Crystallography**

Crystals of PI-PLC W47A/W242A mutant were grown at 17 °C by vapor diffusion in hanging drop against a reservoir solution containing 15% 2-methyl-2,4-pentanediol, 16% polyethylene glycol 400, and 0.1 M citrate buffer at pH 5.4. Crystals of the PI-PLC Y247S/Y251S mutant protein were grown at 17 °C by vapor diffusion with hanging drop against a reservoir solution containing 9% (w/v) polyethylene glycol 8000, 0.2 M zinc acetate, and 0.1 M
sodium cacodylate, pH 6.5. Later some crystals were soaked for 2 h in the mother liquor with 100 mM myo-inositol added to introduce a soluble substrate analog into the active site. Crystals of the Y246S/Y247S/Y248S/Y251S mutant were grown in 10% (w/v) polyethylene glycol 8000, 0.1 M MnCl₂, and 50 mM MOPS, pH 7.0.

Crystallographic data were collected at the Brookhaven National Laboratory Synchrotron X8C beam line by Dr. James Head, Boston University School of Medicine. Crystals were directly cooled to 80-100 K by vapor nitrogen stream prior to collection. Data were indexed, integrated, and scaled using the DENZO and SCALEPACK software packages (11).

Phases for mutant structures were provided by molecular replacement using the program EPMR (12) and previous structures of wild type PI-PLC from B. cereus (Protein Data Bank entry 1PTD) as a starting model (13). For Y247S/Y251S, the B. thuringiensis mutant W47A/W242A (PDB entry 2OR2) was also used for phasing. Phases of the Y246S/Y247S/Y248S/Y251S mutant and the myo-inositol-bound Y247S/Y251S structures were generated by molecular replacement using the solved Y247S/Y251S structure. Model-building and refinement steps were carried out using O (14) and CNS (15), respectively. Individual B-factors were refined for all atoms. The PDB code for the W47A/W242A structure is 2OR2. Crystallographic figures were all made through Pymol (16).

**Modeling of disulfide-crosslinked dimers**

For two disulfide-linked homodimers, W242C and N243C, models (built in O and generated in CNS, and later energy minimized in CNS) of the covalent dimers were built based on the W47A/W242A dimer structure with the two tryptophan residues reintroduced (17). Prior to the energy minimization, Trp242 or Asn243 was changed to Cys and a disulfide bond between two subunits made manually. Ramachandran plots of the models were checked and most residues
were in favored regions (most favored and additional allowed as 71.8 and 27.3% for the W242C dimer and 70.5 and 27.7% for the N243C dimer; 0.9% generously allowed for each), with 0.4% for W242C and 0.0% for N243C in disfavored regions.

**H/D Exchange and Peptide Mapping by High-Performance Liquid Chromatography (HPLC)–Tandem Mass Spectrometry**

**PI-PLC digestion:** A total of 5 µg of purified wild type or mutant PI-PLC in 100 µL of 50 mM HEPES (pH 7.5) was mixed with 95 µL of 100 mM NaH₂PO₄ (pH 2.4) followed by the addition of 5 µg of porcine pepsin dissolved in 0.05% TFA and H₂O. The PI-PLC protein was digested for 5 min on ice. The digested sample was injected into a micropeptide trap (Michrom Bioresources) connected to a C18 HPLC column (5 cm × 1 mm, Alltech) coupled to a Finnigan LCQ quadrupole ion-trap mass spectrometer (ThermoElectron). Peptic fragments were eluted using a gradient of acetonitrile (Burdick and Jackson) at a flow rate of 50 µL/min for a tandem mass spectrometry experiment to sequence each peptic fragment. Peptic fragments were identified by using the search algorithm SEQUEST (ThermoElectron) and manual inspection.

**H/D Exchange:** A sample containing 5 µg of wild type or mutant PI-PLC in 2.5 µL of 50 mM HEPES (pH 7.5) (in some cases with SUVs containing 40 mM total phospholipid) was diluted 10-fold with 50 mM HEPES (pD 7.5) dissolved in D₂O at 25 °C (Cambridge Isotope Laboratories) to label the sample. The deuteration reaction was quenched at different time points by adding 170 µL of 100 mM NaH₂PO₄ (pH 2.4) and 5 µg of porcine pepsin dissolved in 0.05% TFA and H₂O for pepsin digestion.

**Isotope Analysis by HPLC–Electrospray Ionization Mass Spectrometry (ESIMS):** The digested sample was immediately injected into a micropeptide trap connected to a C18 HPLC
column coupled to a Finnigan LCQ quadrupole ion-trap mass spectrometer. Peptic peptides were eluted in 12 min using a gradient of 15–45% acetonitrile at a flow rate of 50 μL/min. The micropeptide trap and C18 HPLC column were immersed in ice to minimize back exchange.

The peptide sequences were first searched by software Bioworks and later identified manually. If possible, all singly, doubly and triply charged peaks for each peptide peak were checked to confirm the identification. The centroid mass of each peptic fragment was determined using the software package MagTran. To correct for the back-exchange reaction of hydrogen atoms during pepsin digestion and HPLC−MS, a ‘fully’ deuterated sample was prepared by incubating 5 μg of WT or mutant PI-PLC in 6 M guanidine DCl, 50 mM HEPES (pD 7.5) for 4 hours at 25 °C. The deuterium exchange ratio of each peptic fragment, corrected for the back exchange, was calculated using the following equation:

\[ R = \frac{m-m_{0\%}}{m_{100\%}-m_{0\%}} \]

where \( m \) is the mass of deuterated peptic fragment, \( m_{0\%} \) and \( m_{100\%} \) are the mass of the unlabeled and fully deuterated peptic fragments.

HPLC gradients for exchange and MS-MS are listed in the following Table. These gradients are input into HPLC as commands and are used for column desalting, peptide separation and eluting steps. Input Func (5) means ‘start’ and (97) means ‘stop’. The values are the volume percentages of acetonitrile in eluting buffer. In Table 2-1(A), it takes 5 minutes to equilibrate the system with 2% acetonitrile. From minute 5 to minute 17, eluting buffer gradient change from 15% to 45% to generate a nice separation of peptides. From minute 17 to minute 25, the gradient of acetonitrile goes from 45% to 98% to totally wash out uneluted peptides. From minute 25 to minute 45, the system goes through 3 cycles to clean all impurities that
remain in the columns, peptide traps or tubes. From minute 45 to minute 65, the gradient
decreases and the system is equilibrated with 2% acetonitrile. The pump is stopped at minute 66.

Table 2-1(B) is the gradient used for MS/MS of all WT PI-PLC and the mutants. Instead
of 25 minutes in HXMS, 60 minutes was used to separate and analyze the peptide, followed by
10 minutes elution and 2 system washing cycles.
Table 2-1(A) The gradients used for HXMS:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Func</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>(5) B.CONC</td>
<td>2</td>
</tr>
<tr>
<td>4.99</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>98</td>
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<tr>
<td>29</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>33</td>
<td></td>
<td>98</td>
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<td></td>
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</tr>
<tr>
<td>41</td>
<td></td>
<td>98</td>
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<td>45</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>65</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>66</td>
<td>(97)STOP</td>
<td></td>
</tr>
</tbody>
</table>
(B) The gradient used for MS/MS:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Func</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>(5) B.CONC</td>
<td>2</td>
</tr>
<tr>
<td>4.99</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>5.00</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>25.00</td>
<td></td>
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<td>20</td>
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<td>101.00</td>
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<td>98</td>
</tr>
<tr>
<td>105.00</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>106.00</td>
<td>(97)STOP</td>
<td></td>
</tr>
</tbody>
</table>
References


Chapter III: Crystal structure of an interfacially impaired PI-PLC
Trp47 and Trp242 are known to be important for PI-PLC to bind to PC vesicles (1). We postulated that the poor binding of W47A/W242A was because those two side chains were no longer available to insert into target membranes and allow proper orientation of substrate at the active site. To test this, what we needed was a crystal structure of this interfacially impaired mutant protein. When I joined the laboratory, crystallographic data of the protein had been obtained and a preliminary model but little refinement had been done. My task was to learn the basics of protein structure and to help analyze the structure of this PI-PLC protein in conjunction with Dr. Chenghua Shao in the Department of Physiology at Boston University School of Medicine. The crystal was refined to 1.8Å resolution.

The W47A/W242A mutant is an interfacially challenged enzyme, and it has been proposed that one or both tryptophan side chains serve as membrane interfacial anchors (1). The crystal structure supports this hypothesis. Relative to the crystal structure of the closely related (97% identity) wild-type PI-PLC from Bacillus cereus, significant conformational differences occur at the membrane-binding interfacial region rather than the active site. The Trp to Ala mutations not only remove the membrane-partitioning aromatic side chains but also perturb the conformations of the so-called helix B and rim loop regions, both of which are implicated in interfacial binding. The crystal structure also reveals a homodimer, the first such observation for a bacterial PI-PLC, with pseudo-2-fold symmetry. The symmetric dimer interface is stabilized by hydrophobic and hydrogen-bonding interactions, contributed primarily by a central swath of aromatic residues arranged in a quasiherringbone pattern. Evidence that interfacially active wild-type PI-PLC enzymes may dimerize in the presence of phosphatidylcholine vesicles is provided by previous experiments of Xin Zhang in the laboratory. She showed protection of fluorophores placed on cyeteines introduced into the PI-PLC surface when the protein was bound to vesicles.
These were serendipitously placed near what is observed as the dimer interface in W47A/W242A. The combined data suggest that wild-type PI-PLC could form similar homodimers, anchored to the interface by the tryptophan and neighboring membrane-partitioning residues.

**Crystal Structure of the W47A/W242A Mutant of PI-PLC**

The *B. thuringiensis* PI-PLC W47A/W242A mutant crystallized in the *P*2₁2₁2₁ space group with two monomers in the asymmetric unit forming a homodimer of the protein (Figure 3.1). The two monomers are structurally very similar to each other, with r.m.s. deviations of 0.291 Å (main chain) and 0.549 Å (overall) (Table 3.1). Each monomer folds as an incomplete (βα)₈-barrel in which the inner layer of the barrel is composed of eight β-strands, surrounded by the six α-helices of the outer layer (the two helices paired with strands IV and V are absent in the bacterial PI-PLC enzymes). In the W47A/W242A crystal structure, the two C-terminal residues of PI-PLC, Lys297 and Glu298, are omitted due to lack of electron density. The subunit interface of the pseudo-2-fold symmetric dimer is composed of the 27 residues (9.1% of the total number of residues) from one monomer and the same 27 residues from another (Table 3.2). These residues are mostly contributed from helix G, helix B, and the rim loop. The dimer interface is ~27 Å in length and 26 Å in width, with an accessible surface area of 884 Å², covering 7.5% of the surface area of each monomer (Table 3.3). The contacts between the two monomers are primarily hydrophobic, with 64.3% contributed by nonpolar interacting atoms. These are stabilized further by 10 intermolecular hydrogen bonds (Tables 3.4). The dimer interface reveals a striking surface complementarity in which the most significant interactions are contributed by a cluster of aromatic residues. The aromatic rings of Tyr246, Tyr247, Tyr248, and Tyr251 from helix G and Trp280 pack into a quasi-herringbone pattern forming a hydrophobic core within the interface.
The rings of Tyr246, Tyr247, and Tyr251 make strong hydrophobic interactions with residues from the other monomer. Tyr248 and Trp280 remain confined to their own respective monomers and provide stabilization for the hydrophobic core boundary.

Table 3.1. PI-PLC Crystallographic data and refinement statistics.

<table>
<thead>
<tr>
<th>Diffraction Data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>1.1000</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.84</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
</tr>
</tbody>
</table>
| Unit cell dimensions (Å) | a=64.4  
|                  | b=70.0   |
|                  | c=154.1  |
| No. of unique reflections | 60615 |
| Completeness a (%) | 98.8 (90.6) |
| Redundancy       | 4.4      |
| Rmerge b (%)     | 4.2 (15.8) |

<table>
<thead>
<tr>
<th>Refinement</th>
<th></th>
</tr>
</thead>
<tbody>
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<td>Resolution range (Å)</td>
<td>50 – 1.84</td>
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<tr>
<td>R&lt;sub&gt;cryst&lt;/sub&gt; c</td>
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</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; d</td>
<td>0.2148</td>
</tr>
<tr>
<td>No. of protein atoms</td>
<td>4806</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>576</td>
</tr>
<tr>
<td>No. of atoms total</td>
<td>5382</td>
</tr>
<tr>
<td>R.m.s.d. e bond angles (°)</td>
<td>1.2971</td>
</tr>
<tr>
<td>R.m.s.d. bond lengths (Å)</td>
<td>0.0049</td>
</tr>
<tr>
<td>Mean &lt;B&gt; overall (Å²)</td>
<td>23.3</td>
</tr>
<tr>
<td>Mean &lt;B&gt; protein (Å²)</td>
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<tr>
<td>Ramachandran regions (% residues)</td>
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</tr>
<tr>
<td>Most favored</td>
<td>85.2</td>
</tr>
<tr>
<td>Additional allowed</td>
<td>14.4</td>
</tr>
<tr>
<td>Generously allowed</td>
<td>0.4 f</td>
</tr>
<tr>
<td>Disallowed</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a Completeness reported for all reflections and for the highest-resolution shell (values in parenthesis).
\[ R_{\text{merge}} \] reported for all reflections and for the highest-resolution shell (values in parenthesis). 

\[ R_{\text{merge}} = \frac{\sum |I_i - \langle I \rangle|}{\sum I_i} \]

where \( I_i \) is the intensity of an individual reflection and \( \langle I \rangle \) is the mean intensity of that reflection.

\[ R_{\text{cryst}} = \frac{\sum |F_p - |F_{\text{calc}}||}{\sum |F_p|} \]

where \( |F_{\text{calc}}| \) and \( |F_p| \) are the calculated and observed structure factors, respectively.

\[ R_{\text{free}} \] as defined in Brünger et al. (2).

\(^{\text{c}}\) r.m.s.d., root mean square deviation.

\(^{\text{d}}\) 0.4\% residues in the generously allowed region correspond to Glu\(^{146}\) from both monomers in the asymmetric unit, as observed previously (3).

Table 3.2. Dimer interface descriptive parameters.

<table>
<thead>
<tr>
<th>Protein Interface Parameter (^{\text{a}})</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interface Accessible Surface Area (Å(^2))</td>
<td>884.36</td>
</tr>
<tr>
<td>% Interface Accessible Surface Area</td>
<td>7.52</td>
</tr>
<tr>
<td>Planarity</td>
<td>2.50</td>
</tr>
<tr>
<td>Length, Breadth (Å)</td>
<td>27.31, 25.78</td>
</tr>
<tr>
<td>No. residues involved in binding (^{\text{b}})</td>
<td>27 (9.1%)</td>
</tr>
<tr>
<td>% Polar Atoms in Interface</td>
<td>35.67</td>
</tr>
<tr>
<td>% Non-Polar Atoms in Interface</td>
<td>64.30</td>
</tr>
<tr>
<td>Hydrogen Bonds</td>
<td>10</td>
</tr>
<tr>
<td>Gap Volume</td>
<td>4531.48</td>
</tr>
<tr>
<td>Gap Volume Index</td>
<td>2.56</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) Calculation was made through the protein-protein interactions server at the biomolecular structure and modeling group of London’s Global University. The calculation on the server, including parameter definitions, was based on Jones and Thornton (4, 5).

\(^{\text{b}}\) Total number and percentage (in parentheses) of residues includes contributions from either monomer.
Table 3.3. Major hydrophobic interactions of aromatic residues at the dimer interface. Intramolecular interactions (within 4 Å) are explicitly named for only one monomer but apply equally to both.

<table>
<thead>
<tr>
<th>Chain</th>
<th>Residue</th>
<th>Chain</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Pro42</td>
<td>B</td>
<td>Tyr247</td>
</tr>
<tr>
<td>A</td>
<td>Pro42</td>
<td>B</td>
<td>Tyr248</td>
</tr>
<tr>
<td>A</td>
<td>Tyr246</td>
<td>A</td>
<td>Trp280</td>
</tr>
<tr>
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<td>Tyr246</td>
<td>B</td>
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<td>B</td>
<td>Tyr251</td>
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<td>Tyr248</td>
<td>B</td>
<td>Pro42</td>
</tr>
<tr>
<td>A</td>
<td>Tyr251</td>
<td>B</td>
<td>Tyr246</td>
</tr>
<tr>
<td>A</td>
<td>Pro254</td>
<td>A</td>
<td>Trp280</td>
</tr>
</tbody>
</table>
Table 3.4. Intermolecular hydrogen bonds formed at the dimer interface.

<table>
<thead>
<tr>
<th>Atom at monomer A</th>
<th>Atom at monomer B</th>
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</thead>
<tbody>
<tr>
<td>Gln45 Oε1</td>
<td>Gly239 N</td>
</tr>
<tr>
<td>Glu52 Oε2</td>
<td>Tyr247 Oη</td>
</tr>
<tr>
<td>Glu52 Oε2</td>
<td>Tyr251 Oη</td>
</tr>
<tr>
<td>Gly239 N</td>
<td>Gln45 Oε1</td>
</tr>
<tr>
<td>Tyr246 Oη</td>
<td>Ser250 Oγ</td>
</tr>
<tr>
<td>Tyr247 Oη</td>
<td>Glu52 Oε2</td>
</tr>
<tr>
<td>Ser250 Oγ</td>
<td>Tyr246 Oη</td>
</tr>
<tr>
<td>Ser250 O</td>
<td>Lys279 Nζ</td>
</tr>
<tr>
<td>Tyr251 Oη</td>
<td>Glu52 Oε2</td>
</tr>
<tr>
<td>Lys279 Nζ</td>
<td>Ser250 O</td>
</tr>
</tbody>
</table>
Figure 3.1. Structure of the PI-PLC W47A/W242A mutant dimer from *B. thuringiensis* shown in divergent (wall-eyed) stereo view. (A) Ribbon diagram of the dimer. N and C termini are labeled; the boxed region indicates the dimer interface (shown in detail in (B)). The active site residue His32 is shown as a stick model, and the orange arrow points to the location of the active site. Residues Ile43-Gly48, which form helix B in the *B. cereus* structure but not in the mutant structure, and the rim loop region (Ser236-Ser244) are indicated by cyan or blue arrows, respectively. (B) Aromatic residues (stick representation; the subunits to which the residues belong are indicated with A or B in parentheses) located within the hydrophobic core of the symmetric dimer interface. Lightly colored helices are the N-terminal parts of helix G from both monomers. Shown in the cyan network is the electron density from the omit map omitting Tyr246, Tyr247, Tyr248, Tyr251, and Trp280 from both subunits and contoured at 1.2 $\sigma$.

The effects of the W47A/W242A mutation on PI-PLC structure and function can be seen by comparison with other PI-PLC crystal structures. The amino acid sequence of *B. thuringiensis* PI-PLC is similar to that of the *B. cereus* enzyme, with 97% sequence identity. The only
significant differences between the crystal structures of the *B. cereus* wild type (Protein Data Bank entry 1PTD) (3) and *B. thuringiensis* W47A/W242A mutant enzymes are localized to the so-called helix B (Ile43-Gly48) and rim loop (Ser236-Ser244) regions (Figure 3.2). These regions, and their Trp residues in particular, have been implicated in binding of the protein to PC bilayers, since replacement of either Trp47 or Trp242 with alanine dramatically reduces the affinity of the protein for PC bilayers by an amount comparable with what would be expected if a membrane-soluble Trp side chain were replaced by the much less hydrophobic methyl group of alanine (1, 6). The "helix B" region of the W47A/W242A mutant adopts an irregular loop conformation rather than the \( \alpha \)-helix observed in wild type PI-PLC. The overall shape of the rim loop is similar in the two enzymes, but in the mutant, the loop shifts positionally closer to the lower center of the (\( \beta \alpha \))-barrel, which changes the relative orientation between the rim loop and helix B. In the wild-type enzyme, the closest C\( \alpha \)-C\( \alpha \) distance between the rim loop and B helix is 5.1 Å from Val46 to Gly239, whereas the distance between Val46 and Gly239 is changed to 13.5 Å in the mutant enzyme. When residues from the helix B and rim loop are omitted from the superposition calculation, the main chain r.m.s. deviation falls from 1.305 to 0.514 Å, confirming that significant structural differences are confined to these two regions. Comparison of the crystal structures of *B. thuringiensis* PI-PLC mutants W47A/W242A and R69D (Protein Data Bank entry 1T6M) (7), which contains a metal ion site introduced into the active site, produces similar results: main-chain r.m.s. deviation values fall from 1.352 Å (all residues) to 0.586 Å with residues at helix B and the rim loop omitted. On the other hand, the main-chain r.m.s. deviation between *B. cereus* wild-type and *B. thuringiensis* R69D mutant PI-PLC structures is 0.653 Å for all residues, indicating that despite the species and sequence differences, they retain highly similar conformations in the absence of the W47A/W242A mutation. Of particular note is the
observation that the W47A/W242A mutation has no observable effect on active site geometry, which in the mutant aligns closely with the active sites of *B. cereus* wild-type PI-PLC, with or without inhibitors. The r.m.s. deviation for the 13 active site residues (as defined in Ref. (3)) is 0.564 Å (all atoms). These comparisons reveal that significant structural effects of the W47A/W242A mutation are limited to the helix B and rim loop regions, leaving the active site geometry unaltered and capable of catalysis.

**Figure 3.2.** Ribbon representations of the helix B region (magenta) and rim loop region (together with the N terminus of helix G) (cyan) of wild-type *B. cereus* PI-PLC (Protein Data Bank entry 1PTD (3)) (A) and *B. thuringiensis* W47A/W242A mutant PI-PLC (B). Side chains at positions 47 and 242 are shown as stick representations in both A and B.

*Relevance of dimer structure to PI-PLC activity?*
Previous kinetic studies have established that the *B. thuringiensis* PI-PLC W47A/W242A mutant is an interfacially impaired enzyme that no longer binds well to activating PC interfaces (1). Removal of these two tryptophan residues, singly or together, dramatically increases the apparent *K*<sub>d</sub> (or *nK*<sub>d</sub> in a Langmuir adsorption model for binding (8)) for partitioning of the enzyme onto PC vesicles, and the magnitude of the change is consistent with the change in free energy for removing a group that partitions into the PC membrane. X-ray crystallography was used to investigate protein structural changes that might be associated with the W47A/W242A mutation and to determine whether such changes might occur in the active site, resulting in a lower *k*<sub>cat</sub>. The crystal structure demonstrates that the *B. thuringiensis* PI-PLC W47A/W242A mutant retains the overall (β<sub>1</sub>α)-barrel structure (Figure 3.1) observed in the crystal structure of *B. cereus* wild-type PI-PLC (3). Crystal structures of wild-type PI-PLC complexes with inositol have established that the active site is located inside the β-barrel, and the lower barrel rim allows the substrate entry from the membrane bilayer. Comparison of the wild-type and W47A/W242A mutant structures indicates no significant structural differences between the active sites. However, the W47A/W242A mutant reveals a major conformational change in two regions that have been implicated in membrane binding (1, 6). The W47A/W242A mutation perturbs the helix B region, which in wild-type PI-PLC forms a short α-helix (residues Ile43-Gly48) that has been proposed to partially insert into membranes (1, 8). In wild-type PI-PLC, this helix orients the side chains of Ile43, Val46, and Trp47 so that they pack together and form a plug that extends outwards toward solvent, thus forming a hydrophobic protrusion from the protein surface. This hydrophobic protrusion occurs near the lower rim entrance, where the protrusion could act to stabilize membrane binding and perhaps encourage the proper orientation of the β<sub>1</sub>-barrel with respect to the interfacial surface. The W47A mutation disrupts the helical secondary structure, which in turn
alters the relative orientation of these side chains. The Ile43 side chain becomes redirected into
the protein core, and the Cα-Cα distance between residues 43 and 46 increases from 5.3 to 8.3 Å.
These data are consistent with the premise that the decreased catalytic activity of the mutant is
due primarily to the loss of its membrane binding capacity. Deficient membrane binding could
alter the enzyme activity in various ways (e.g. reduction of enzyme-ligand contact or failure to
induce an active site environment that promotes catalysis).

An unexpected feature to emerge from the W47A/W242A studies was the first
crystallographic observation of a PI-PLC dimer. Dimers have been observed crystallographically
for three other phospholipases (A, A2, and the C-terminal tail of phospholipase C-β); however,
the first two of these are structurally and functionally distinct from PI-PLC enzymes, and the last
is an isolated domain. The outer membrane phospholipase A of Gram negative bacteria is an
integral 3-barrel membrane protein that is active in vitro as a dimer (9). Dimerization depends on
substrate and Ca2+ binding, and the complete active site requires dimer formation (10). The
anion-assisted dimer of secreted phospholipase A2 (11) forms a catalytically inactive enzyme.
Avian phospholipase C-β, which possesses a fold unique to these enzymes, forms dimers that
mediate interactions with G-proteins (12, 13). A recent report shows that an isoform of
mammalian PI-PLC can also form homodimers (14). Although crystal structures of mammalian
PLC-β have yet to be determined, the report implicated the involvement of the catalytic domain
in the dimerization. Precedent thus exists for homodimerization in other phospholipases, despite
the diversity in structural motifs and functional properties.

The dimer interface of B. thuringiensis W47A/W242A PI-PLC exhibits high
complementarity, with a striking pseudo-2-fold symmetry in which aromatic side chains form an
interdigitating core involving the same residues from each monomer. Such symmetry is observed
frequently in macromolecular assemblies. In the W47A/W242A dimer, the monomers are oriented parallel to each other, such that the pseudo-2-fold axis of the dimer interface parallels the axes passing through the two β-barrels. This orientation for the monomers would preserve the interfacial active site accessibility for both. The 2-fold symmetry creates some curvature in the dimer (Figure 3.1A), which may be consistent with the strong preference exhibited for small, highly curved unilamellar vesicles of PC compared with large, more planar bilayer vesicles (δ). It is also possible that the dimer may reorient slightly to adopt a more planar shape on a membrane surface. Such a reorientation could be accommodated with only minor adjustments of the aromatic residues at the dimer interface.

The combined data suggest that PI-PLC from B. cereus and B. thuringiensis share an inherent ability to form dimers. The residues that comprise the dimer interface are conserved in PI-PLC from both species. In the wild-type PI-PLC crystal structure, these side chains are surface-accessible and available to participate in protein-protein interactions. Given the similarities between the enzymes, it is intriguing that the crystallographic dimer has been observed solely for the W47A/W242A mutant. The data herein suggest that the crystallization behavior is critically influenced by the local conformation of the B helix and barrel rim regions, particularly the Trp47 and Trp242 side chains. In the conformation observed in the B. cereus wild-type PI-PLC crystal structure, the hydrophobic protrusion from the B-helix extends outwards in an orientation that interferes with formation of the lower boundary of the dimer interface; the bulky tryptophan side chains prevent the requisite close intermolecular contacts observed in the dimer structure. However, these regions are conformationally mobile, and partitioning of the protrusion into the membrane could relieve the steric hindrance and permit dimerization. Indeed, in the W47A/W242A mutant, such a conformational transition is effected
by the substitution of alanine for the bulky side chains of tryptophans 47 and 242. The double mutation brings the Cα-Cα distance between residues 47 and 242 in the same monomer 2.3 Å closer to each other. The distance between the Ala47/Ala242 pair from one monomer to the other is also close, ∼5 Å (Cα-Cα). These close distances are compatible with dimerization. One possible model for the dimer interface of wild-type PI-PLC, based on the W47A/W242A crystal structure, is shown in Figure 3.3.

**Figure 3.3.** Possible conformation (based on the W47A/W242A dimer structure) adopted by wild-type PI-PLC at the PC membrane interface (indicated by the gray shadow). Subunits A and B are shown in green and yellow, respectively. Tyr246 side chains are included to indicate the orientation of the molecule similar to that shown in Fig. 3.1B.
The crystallographic data could be used to support two very different models: (1) a model in which dimer formation is promoted by membrane binding, or (2) a model where the dimer is the inactive species of the enzyme. The crystallographic data for wild type PI-PLC shows that Trp242 forms part of the barrel rim loop abutting the entrance to the active site. The Trp side chain has been proposed to insert into the bilayer (1), helping to anchor the enzyme to the membrane surface.

We propose a model in which the dimer interface is substantially stabilized when wild-type PI-PLC is bound to membranes. Partitioning of Trp47 and Trp242 into a PC membrane provides the primary driving force for changes in the rim loop that promote dimer formation. The rim loop is highly mobile in both mutant and wild type structures with much higher B factors than the rest of the structure, and its conformation or position can be easily changed. The proposed dimerization is a dynamic process that occurs in several steps. (i) In the absence of a membrane surface, the Trp side chains (Trp242 in its flexible loop as well as the helix B hydrophobic plug) sterically hinder dimer formation. (ii) At the PC membrane surface, the Trp residues partition into the membrane. (iii) This attachment of the Trp "plug" to the PC membrane allows adjacent PI-PLC molecules to approach one another while bound to the membrane interface. (iv) The tyrosine herringbone pattern forms, bringing the two monomers together on the PC interface. Dimer formation should increase the residence time of the protein on a vesicle surface and would lead to enhanced catalysis. There are likely to be other more subtle changes as well, since the enzyme when bound to PC surfaces is much more active toward water-soluble substrates (both diC₄PI and cIP) (8, 15).
References


phosphatidylinositol-specific phospholipase C: specific phospholipid binding anchors the
enzyme to the interface, *Biochemistry* 36, 10089-10097.
Chapter IV: Mutation of the Tyr strip – dimerization or a PC site?
A detailed description of peripheral protein attachment to membranes is often difficult to achieve yet crucial to understanding the functions of these proteins. Few examples exist where a well defined docked structure has been determined. The PI-PLC enzymes from several *Bacillus* species are good structural models for the catalytic domain of the larger and more complex mammalian enzymes that play key roles in PI signal transduction (1-3). The enzyme from *Bacillus thuringiensis* closely resembles the catalytic barrel of PLCδ1 (1). It can be activated toward both PI cleavage to cIP and cIP hydrolysis to I-1-P by non-substrate phosphatidylcholine (PC) (4-6). Studies have shown that tryptophan residues in two discrete surface structural features, short helix B and a longer loop, are needed for the enzyme to bind to activating PC surfaces and for optimal catalytic activity (7, 8). A crystal structure (9) of an interfacially impaired mutant, W47A/W242A, showed that the enzyme could form dimers with a string of tyrosine residues (Tyr246, Tyr247, Tyr248, and Tyr251), forming the hydrophobic core of this dimer interface. The W47A/W242A dimer structure could not be formed by replacing the alanine at positions 47 and 242 with the original tryptophan residues without significant conformational rearrangements of the loop and helix B residues. Fluorescence quenching experiments were consistent with dimer formation of pyrene-labeled Cys mutants placed in the vicinity of the dimer interface (9). However, the hydrophobic probes introduced could drive formation of a dimer that does not occur with wild type protein and is not directly relevant to membrane binding and catalysis.

To further explore the relevance of this surface region of the protein to PI-PLC activity, we have constructed a series of mutants converting the tyrosine residues in the W47A/W242A dimer interface to serines (Y247S/Y251S, Y246S/Y247S/Y248S, and Y246S/Y247S/Y248S/Y251S). As more tyrosines are replaced by serine, there is a decrease in
catalytic activity, both phosphotransferase cleavage of PI to cIP and diacylglycerol (in a variety of assay systems) and cyclic phosphodiesterase hydrolysis of cIP to I-1-P. There is an impairment of protein binding to PC vesicles when two or more tyrosine residues are removed that correlates with reduced specific activity in assays with PI/PC vesicles. Binding of the mutant proteins to diC7-PC micelles was also examined (intrinsic fluorescence and $^{31}$P NMR methods) and provides evidence that this interaction is very different when three or four tyrosines have been replaced by serine. Crystal structures of two of the mutant enzymes, Y247S/Y251S and Y246S/Y247S/Y248S/Y251S, have been solved, and these show that the proteins are very similar to the very closely related Bacillus cereus PI-PLC (10) in that they fail to crystallize as dimers (presumably because helix B and Trp242 prevent close approach of two monomers (9)). Their active sites also superimpose well on that of the B. cereus PI-PLC. The only difference is in the loss of Tyr side chains in the area that forms the crystallographic dimer interface in W47A/W242A. These results can be interpreted as evidence for either (i) surface-induced dimerization of the PI-PLC or (ii) identification of a very specific PC binding site on the protein.

Characterization of Tyr ridge mutants – secondary structure and thermostability

Four tyrosines in W47A/W242A, Tyr246, Tyr247, Tyr248, and Tyr251, form the hydrophobic core of the dimer interface (Figure 4.1). In PI-PLC protein, their replacement with serine residues should dramatically weaken any membrane-induced dimerization, assuming the dimer is similar to W47A/W242A, but maintain any hydrogen-bonding interactions with solvent or phospholipid head groups. If, instead of direct dimer formation, these residues are involved in specific binding of PC molecules (possibly via a Tyr $\pi$-cation interaction with the choline moiety as has been observed in peptide systems (11)), both activity and PC binding might also be affected.
The mutated PI-PLC proteins Y247S/Y251S, Y246S/Y247S/Y248S, and Y246S/Y247S/Y248S/Y251S were examined by CD for changes in secondary structure. Y246S/Y247S/Y248S exhibited a far-UV spectrum equivalent to WT PI-PLC. However, both Y247S/Y251S and Y246S/Y247S/Y248S/Y251S appeared to have less $\alpha$-helix and more $\beta$-sheet (Figure 4.2A) at low concentrations in 10 mM borate buffer. Upon the addition of myo-inositol (a soluble portion of the substrate molecule that binds, although not with terribly high affinity, to the active site) a CD spectrum identical to WT PI-PLC was observed for Y247S/Y251S (Figure 4.2B). The $\alpha$-helix content for the quadruple Tyr mutant, Y246S/Y247S/Y248S/Y251S, dispersed in this solution was not recovered with added myo-inositol. For comparison, myo-inositol had no effect on the secondary structure of rPI-PLC or the triple tyrosine mutant. This suggested that occupation of the active site with the polar part of the substrate can affect the structure of helical regions in the protein and that the double tyrosine mutant likely resembles...
WT PI-PLC under those conditions. The presence of 30% isopropyl alcohol has also been shown to activate PI-PLC \((12)\). This co-solvent has little effect on PI-PLC secondary structure (if measured at 4 °C) \((13)\); however, it did lead to recovery of \(\alpha\)-helix for the quadruple mutant (Figure 4.2B, inset). To the extent that a co-solvent system may mimic an interfacial environment of a membrane, these results indicate that conditions that lead to kinetic activation (e.g. isopropanol) tend to lead to changes in \(Y246S/Y247S/Y248S/Y251S\) secondary structure that approximate WT PI-PLC.

**Figure 4.2.** Change in secondary structure of tyrosine mutants compared with wild type recombinant PI-PLC in the \((A)\) absence or \((B)\) presence of 60 mM \textit{myo}-inositol. Secondary structure motifs are indicated as follows: \(\text{(filled squares)}\) \(\alpha\)-helix, \(\text{(open squares)}\) \(\beta\)-sheet, \(\text{(diagonal lines)}\) \(\beta\)-turn, and \(\text{(gray squares)}\) random coil. Mutant proteins are abbreviated as follows: \(2Y-S\), \(Y247S/Y251S\); \(3Y-S\), \(Y246S/Y247S/Y248S\); \(4Y-S\), \(Y246S/Y247S/Y248S/Y251S\). The \textit{inset} in \(B\) shows the change in secondary structure for \(Y246S/Y247S/Y248S/Y251S\) in the presence of 30% isopropanol \((iPrOH)\).
Specific activities of Tyr mutants

PI-PLC enzymes catalyze the hydrolysis of PI to I-1-P in two steps (cleavage of PI to water-soluble cIP, then hydrolysis of cIP to I-1-P) that can be analyzed separately. Y247S/Y251S exhibited specific activity toward PI solubilized in diC7PC (1:4 PI/diC7PC) (Table 4.1), comparable with wild type enzyme. Changing the detergent matrix to Triton X-100 instead of an activating PC molecule leads to reduced specific activity for WT PI-PLC, typically 30–40% (6). However, Y247S/Y251S phosphotransferase activity with PI/Triton X-100 micelles was noticeably lower than that of WT PI-PLC. In comparison to Y247S/Y251S, the other tyrosine mutants had even lower specific activity toward PI whether solubilized in diC7PC or Triton X-100 (Table 4.1). A similar trend was observed for cleavage of PI/PC (4:1) vesicles; Y247S/Y251S was closest to WT PI-PLC in cleavage ability, whereas the mutants with three or four of the tyrosines removed were much less active.

Table 4.1. Specific activities of surface tyrosine mutants towards PI and cIP in diverse assay systems.

<table>
<thead>
<tr>
<th>PI-PLC</th>
<th>Specific activity (μ mol min⁻¹mg⁻¹)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI/diC7PC (1:4)</td>
</tr>
<tr>
<td>Wild type</td>
<td>560 ± 130</td>
</tr>
<tr>
<td>Y247S/Y251S</td>
<td>670 ± 100</td>
</tr>
<tr>
<td>Y246S/Y247S/Y248S</td>
<td>301 ± 21</td>
</tr>
<tr>
<td>Y246S/Y247S/Y248S/Y251S</td>
<td>112 ± 5</td>
</tr>
</tbody>
</table>

a In the phosphotransferase assays, the PI concentration was 8 mM with either 32 mM diC7PC, 16 mM Triton X-100, or 2 mM POPC (for the SUVs). The cyclic phosphodiesterase assays used 8 mM cIP in the absence and presence of 5 mM diC7PC.
In the absence of an activating interface, the second step of the PI-PLC reaction, hydrolysis of cIP to I-1-P, was greatly reduced for all the tyrosine mutants compared with WT PI-PLC. In the presence of diC₇PC micelles, the specific activity of Y247S/Y251S increased dramatically, within a factor of two of the wild type enzyme-specific activity (Table 4.1). However, as more tyrosine residues were replaced by serine, the mutant PI-PLC enzymes exhibited a much smaller activation by diC₇PC to the point where PC activation was virtually abolished for Y246S/Y247S/Y248S/Y251S. The larger effect on the phosphodiesterase activity compared with the phosphotransferase activity is better seen when the specific activity for each mutant enzyme is normalized to that of WT PI-PLC for that assay system (Figure 4.3).

Hydrolysis of cIP in the presence of diC₇PC is reduced to a much greater extent for Y246S/Y247S/Y248S and Y246S/Y247S/Y248S/Y251S compared with decreases in any of the PI cleavage assays.

A striking kinetic feature of this bacterial PI-PLC is the lack of surface dilution inhibition until the mole fraction PI is reduced below 0.1 at fixed PI (6). Because removal of surface Tyr residues might alter this, we monitored the specific activity of the Tyr to Ser mutant enzymes in experiments where the bulk concentration of PI was held constant at 6 mM and the amount of diC₇PC increased (Figure 4.4). Y247S/Y251S, like WT PI-PLC, has a moderate affinity for PI as 50% inhibition was not observed until a diC₇PC/PI ratio of 10 (mole fraction of 0.091 PI). In contrast, the other two tyrosine mutants started at lower specific activities and showed a continuously decreasing activity (referenced to the value for 6 mM PI/18 mM diC₇PC), as the diC₇PC concentration was increased. Clearly, removal of three or four of the surface tyrosines that make up the core of the dimer interface in W47A/W242A reduces the activities of both steps.
of the reaction. The enhanced surface dilution suggests that removal of these Tyr reduces the affinity of the protein for interfacial substrate.

Figure 4.3. Relative activities of the three Tyr-to-Ser mutant enzymes compared with rPI-PLC. Data for Y247S/Y251S (2YS), Y246S/Y247S/Y248S (3YS), and Y246S/Y247S/Y248S/Y251S (4YS) are shown for PI (8 mM) cleavage in diC₇PC micelles (gray bars), in Triton X-100 (16 mM) micelles (diagonal lines), or mixed with 2 mM PC in SUVs (open bars), and cIP (8 mM) hydrolysis with 5 mM diC₇PC (black bars).
Binding of Tyr mutant proteins to phospholipid SUVs and diC₇PC micelles

The lower activities as tyrosine residues were replaced with serine could reflect a progressively weaker binding of PI-PLC to phospholipid interfaces. Previous work has shown that *B. thuringiensis* PI-PLC binds to PC vesicles with a large hydrophobic component, whereas
it binds to pure anionic phospholipid vesicles through electrostatic interactions (14).

Y247S/Y251S exhibited a 2-fold weaker affinity for PC vesicles than WT PI-PLC (Table 4.2). Y246S/Y247S/Y248S presented an interesting case in that partitioning of the protein onto the vesicles very much depended on the protein concentration. At moderate protein concentrations (0.01–0.1 mg/ml, 0.29–2.9 µM), the protein bound to PC vesicles with an apparent $K_d$ of 0.5 ± 0.2 mM as measured by the centrifugation/filtration assay. When all four tyrosine residues were replaced by serine, partitioning of the enzyme on PC vesicles was considerably weaker (apparent $K_d$ ~1 mM with the centrifugation/filtration assay). Although this binding assay is not very

Table 4.2 Apparent dissociation constants for PI-PLC tyrosine mutants binding to vesicles and short-chain phospholipid micelles.

<table>
<thead>
<tr>
<th>PI-PLC</th>
<th>Apparent $K_d$ (µM)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>POPC SUV</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.075 ± 0.013</td>
</tr>
<tr>
<td>Y247/251S</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Y246/247/248S</td>
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</tr>
<tr>
<td>Y246/247/248/251S</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ Binding fit with a cooperative model (7).

$^b$ The apparent dissociation constant is the concentration of detergent needed for half the total change in fluorescence intensity. The CMC of diC7PC at is 1.5 mM (32); the CMC for diC6PA at pH 8.5 is 14 mM.

$^c$ The errors in measuring the partitioning of these two proteins onto the PC SUVs were particularly large and varied with small variations in protein concentration (0.05–0.2 mg/ml).

accurate for proteins with weak affinities for PC SUVs, it does stress that removal of these Tyr residues on the surface in a region that is below the barrel rim and not near the active site is clearly correlated with the lower activities in assay systems with PI/PC. However, specific
activities progressively decreased for assay systems with diC7PC micelles as well so that interaction of these micelles with the proteins must be evaluated.

The intrinsic fluorescence of PI-PLC is sensitive to surface binding with the intensity increasing when PLC binds to activating PC interfaces (diC7PC or POPC SUVs) and decreasing when molecules bind at the active site \((15, 16)\). This is a particularly useful method for characterizing PI-PLC binding to short-chain phospholipid micelles \((16, 8)\). For WT PI-PLC, the fluorescence intensity increases above the critical micelle concentration of the short-chain PC, and the steepness of the increase reflects the apparent binding constant of the enzyme to micellar phospholipids. Comparison of fluorescence changes for Y247S/Y215S binding to WT PI-PLC protein showed that the double Tyr mutant protein had a weaker interaction with diC7PC micelles than WT PI-PLC, consistent with vesicle partitioning assays \((Figure 4.5)\). Assuming the critical micelle concentration of diC7PC in the presence of the protein is comparable with the 1.5 mM value for pure diC7PC \((17)\), we would estimate an apparent \(K_D\) of 1.1 mM "micellar" PC for Y247S/Y251S compared with WT PI-PLC, for which the \(K_D\) is 0.5 mM micellar diC7PC. With Y246S/Y247S/Y248S and Y246S/Y247S/Y248S/Y251S, the fluorescence intensity did not increase with increasing micellar diC7PC; rather, it decreased \((Figure 4.5)\). In WT PI-PLC, Trp242 has been identified as the major contributor to the increased intrinsic fluorescence upon PC binding \((8)\). Y246S/Y247S/Y248S clearly binds to diC7PC, as there is a change in protein intrinsic fluorescence. The decrease in intensity as this mutant protein binds to diC7PC micelles must reflect a different conformational change upon diC7PC binding. Interestingly, the decreased fluorescence of Y246S/Y247S/Y248S and Y246S/Y247S/Y248S/Y251S was comparable in magnitude to that for diC6PA binding to WT PI-PLC \((Figure 4.5)\). That anionic phospholipid binds competitively with PI at the active site \((15, 16)\). Indeed, if the quadruple Tyr mutant protein...
is titrated with diC₆PA, a change comparable with WT PI-PLC was obtained. These intrinsic fluorescence changes indicate the Y246S/Y247S/Y248S and Y246S/Y247S/Y248S/Y251S proteins bind diC₇PC but not in the same way as rPI-PLC and Y247S/Y251S. The differences correlate with the lower activities of the three and four Tyr-to-Ser enzymes.

![Figure 4.5](image)

**Figure 4.5** Change in intrinsic fluorescence of recombinant PI-PLC and mutants as a function of added short chain phospholipids. DiC₇PC was added to WT PI-PLC (●), Y247S/Y251S (○), Y246S/Y247S/Y248S (□), and Y246S/Y247S/Y248S/Y251S (×); diC₆PA was added to WT PI-PLC (★) and Y246S/Y247S/Y248S/Y251S (+). The fluorescence change is plotted as \((I - I_o)/I_o\) where \(I_o\) is the initial fluorescence of the sample in the absence of the short-chain phospholipid.

**Structures of Y247S/Y251S and Y246S/Y247S/Y248S/Y251S**

To explore if removal of these surface tyrosine residues alters the PI-PLC active site, we determined the crystal structure of two of the tyrosine mutants, Y247S/Y251S (PDB entry 3EA1) and Y246S/Y247S/Y248S/Y251S (PDB entry 3EA3). Crystallographic data and refinement statistics are presented in Table 4.3. The Y247S/Y251S mutant crystallized in the space group of
P2₁2₁2₁ with two monomers in the asymmetric unit. The PI-PLC Y246S/Y247S/Y248S/Y251S mutant protein crystallized in space group P1; its asymmetric unit also contained two monomers. Both tyrosine-to-serine mutant structures are of high quality at 1.75 and 1.78 Å resolution (Table 4), and the individual monomers in both mutant structures closely resemble the PI-PLC structure from *B. cereus* (PDB entry 1PTD) (10). The proteins fold as an incomplete (βα)₈ barrel (TIM-barrel) with an inner layer composed of eight β-strands surrounded by six major α-helices of the outer layer. As was the case with the structure of W47A/W242A (9), the last two residues of PI-PLC, Lys297 and Glu298, did not have observable electron density. The main-chain r.m.s.d. is 0.610 Å between the structures of the *B. cereus* PI-PLC and the Y247S/Y251S mutant and 0.654 Å between the *B. cereus* PI-PLC and the Y246S/Y247S/Y248S/Y251S (Table 4.4). The wild type-like double tyrosine mutant (Y247S/Y251S) and the PC interface-impaired quadruple mutant structures are even more similar to each other, with an r.m.s.d. of only 0.310 Å (Table 4.4). Structural alignments and comparisons indicate the mutations introduced little conformational change in the crystallized proteins (Figure 4.6A).
Table 4.3. Crystallographic data and refinement statistics.

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<td>0.4</td>
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$^a$Completeness, redundancy, and $R_{merge}$ reported for all reflections and for the highest-resolution shell (values in parenthesis).

$^b$ $R_{merge}$ reported for all reflections and for the highest-resolution shell (values in parenthesis). $R_{merge} = \frac{1}{N} \sum \frac{I_i - \langle I \rangle}{I_i}$, where $I_i$ is the intensity of an individual reflection and $\langle I \rangle$ is the mean intensity of that reflection.

$^c$ $R_{cryst} = \frac{\sum \mid F_{calc} \mid - \mid F_{obs} \mid}{\sum \mid F_{calc} \mid}$ where $\mid F_{calc} \mid$ and $\mid F_{obs} \mid$ are the calculated and observed structure factors, respectively.

$^d$ $R_{free}$ was as defined in Brünger (1998).

$^e$ r.m.s.d. is defined as the root mean square deviation.
Table 4.4. Root mean square deviations (Å) of superimposed PI-PLC structures. a

<table>
<thead>
<tr>
<th>PDB entry b</th>
<th>2Y-S</th>
<th>2Y-S(+Ins)</th>
<th>4Y-S</th>
<th>2OR2</th>
<th>2OR2-HB/RL</th>
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<tr>
<td>1PTD</td>
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<td></td>
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<td>0.629</td>
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<td>2Y-S</td>
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<td>4Y-S</td>
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<td></td>
<td></td>
<td>1.410</td>
<td>0.507</td>
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</table>

a Abbreviations: 2Y-S, Y247S/Y251S; 4Y-S, Y246S/Y247S/Y248S/Y251S; 2OR2-HB/RL is a monomer from the W47A/W242A dimer structure omitting helix B and the rim loop region containing Trp242 in the comparison.
b 1PTD is the structure of the PI-PLC from B. cereus (10); 1PTG is the structure of the B. cereus PI-PLC with myo-inositol bound (10); 2OR2 is the structure of the B. thuringiensis W47A/W242A dimer (9).

Figure 4.6. Myo-inositol complex with B. thuringiensis PI-PLC Y247S/Y251S compared with corresponding complex from wild type B. cereus PI-PLC (PDB code 1PTG). The latter structure is shown in magenta in both A and B. (A) backbone superposition with the Y247S/Y251S structure shown in green, myo-inositol (unlabeled) displayed in the active site, and non-mutated tyrosine side chains (labeled in bold) shown as markers to indicate tyrosine strip region. (B) superposition of B. cereus PI-PLC active site residues with the B. thuringiensis PI-PLC Y247S/Y251S-myo-inositol complex shown in Corey-Pauling-Koltun coloring. Hydrogen bonds are shown as green dotted lines.
position of Trp242 in the flexible rim loop was slightly shifted. The active sites of the \textit{B. cereus} PI-PLC and both tyrosine mutants, Y247S/Y251S, with activity similar to wild type protein, and Y246S/Y247S/Y248S/Y251S, with greatly reduced interfacial activity, are superimposable except for His82, which is rotated by about 90° in both mutant protein crystal structures. A crystal of Y247S/Y251S PI-PLC was later soaked with 100 mM \textit{myo}-inositol (PDB entry 3EA2) for comparison to the \textit{B. cereus} PI-PLC structure with \textit{myo}-inositol in the active site (PDB entry 1PTG) \textsuperscript{(10)}. The r.m.s.d. between those two structures is 0.597 Å, and the structural alignment between them reveals no significant conformational changes for active site residues; bound \textit{myo}-inositol is also at the same position for the two protein structures (Figure 4.6B). Therefore, in the crystal and in the absence of an interface, the tyrosine-to-serine mutations of PI-PLC appear to have little influence on the active site geometry either with or without an inositol moiety bound.

The PI-PLC W47A/W242A mutant was crystallized as a dimer, prompting the conjecture that the membrane state of the enzyme might be a dimer. As shown in Figure 4.1 and 4.7A, Tyr246, Tyr247, Tyr248, and Tyr251 from each subunit of the dimer form a quasi-herringbone pattern that stabilizes the dimer interface \textsuperscript{(9)}. Although both Y247S/Y251S and Y246S/Y247S/Y248S/Y251S mutant monomers are structurally similar to a crystallographic monomer of the W47A/W242A PI-PLC dimer, neither tyrosine mutant crystallized as a dimer. For WT PI-PLC, the lack of dimer formation in solution is presumably because helix B is still intact, and the hydrophobic tryptophan "plug" this feature forms in solution prevents close approach of another PI-PLC and subsequent dimerization via the surface tyrosine residues \textsuperscript{(9)}. A closer examination of the putative dimer interface region for the tyrosine mutants shows that the essential interactions cannot be made when four of the crucial tyrosine residues have been replaced by serine (Figure 4.7C). However, Y247S/Y251S still has Tyr246 and Tyr248 as well as
Trp280 to stabilize the hydrophobic core of a membrane-induced dimer interface (Figure 4.7). The remaining interactions may be adequate to allow dimers to form on the membrane surface. Tyr246 in particular may be a key interaction for a surface-induced dimer that resembles that of W47A/W242A.

**Figure 4.7.** Location of tyrosine-serine substitutions in *B. thuringiensis* PI-PLC mutants relative to the crystallographic dimer interface observed in the W47A/W242A mutant (PDB code 2OR2). Backbone ribbon representation with key side chains is shown in stick representation. A, crystallographic dimer (2OR2), with the second subunit shown in gray. Y247S/Y251S (B) and Y246S/Y247S/Y248S/Y251S (C) mutants, shown in Corey-Pauling-Koltun coloration, have been superimposed onto the first subunit of the A dimer structure; the second subunit from A is included to complete the hypothetical dimer interface. This comparison supports the hypothesis that the more active Y247S/Y251S mutant, but not the catalytically impaired Y246S/Y247S/Y248S/Y251S mutant, retains the key tyrosine residues needed for dimer formation.
The high similarity between the structures of the double/quadruple mutant and the wild type PI-PLC indicate that the reduction in enzymatic activities of the triple and quadruple tyrosine mutant proteins and altered binding affinity toward PC vesicles is not likely to be caused by large conformational changes within the single monomer domain. Two possible explanations may explain how replacement of these surface Tyr residues with Ser reduces enzymatic activity; (i) removal of these tyrosine residues disrupts the transient dimer that forms when protein binds to PC-containing membranes, or (ii) these same tyrosine residues are critical parts of a defined PC binding site that aids in anchoring the protein as a monomer to interfaces.

Dimer or PC binding site?

Although the binding of peripheral proteins to membranes often has a strong electrostatic component, tryptophan insertion into the membrane is also frequently observed, particularly for interfacial binding to PC surfaces (18-20). Tryptophan residues are particularly prominent in the region of the bilayer interfaces in membrane proteins as well (21-23). Previous work has suggested that Trp47 and Trp242 of the B. thuringiensis PI-PLC insert into the membrane surface (7, 8) and that this interaction is facilitated by PC molecules present in the interface (14). Trp insertion in turn helps anchor the PI-PLC to vesicles and is ultimately connected to enzymatic activation in that assay system. A variety of biophysical techniques suggested that binding of the protein to the interface changed the enzyme conformation and shifted the enzyme to an "activated" form (4-6, 24). However, no specifics were available on the structure of that activated form.

Interaction of a phospholipid with a specific binding site on the protein has been detected with a wide variety of peripheral membrane proteins. It usually is identified by enhanced
membrane association (e.g. C2 domains and Ca$^{2+}$-mediated interaction with anionic phospholipids, PH domains, and phosphoinositides) or altered kinetics (if the protein is an enzyme). Unless the interaction is of fairly high affinity, localizing the binding site can be difficult and is usually explored with mutagenesis. Along with specific phospholipid binding, protein oligomerization may also provide a way of regulating function. There are integral membrane proteins that are purified as monomers but are believed to function as dimers or oligomers. For example, a tetracycline-divalent cation efflux protein TetL was suggested to exist as an oligomer rather than a monomer in the membrane based on SDS-PAGE and Western blot analysis of membrane samples (25). However, exploring membrane-induced oligomerization of peripheral membrane proteins, particularly where such aggregation might not be necessary for enzymatic activity but rather enhance catalysis, is very difficult. Chemical cross-linking only works if suitable chemical handles are available (24). Association and dissociation of the proteins from the membrane can also make trapping aggregates difficult.

Initial studies (4-6) with the B. thuringiensis PI-PLC suggested that kinetic activation of cIP (and monomeric diC$_4$PI) hydrolysis by PC involved the protein binding to a PC interface at a site distinct from the active site. That interaction then induced a conformational change in the active site that enhanced catalysis of water-soluble substrates. Localization of a distinct binding region on PI-PLC was restricted to the observation that the Trp47 and Trp242 seemed to aid in binding of the protein to interfaces. More recent work, that of a crystal structure of a dimeric protein when the two key surface tryptophan residues were changed to alanine, suggested that the specific kinetic activation of PI-PLC by PC interfaces was the result of interface-induced dimerization of the protein (9). The extrapolation of that dimer to WT PI-PLC posited that when the Trp side chains were present, a hydrophobic Trp plug centered on helix B prevents dimer
formation in solution. Thus, the initial approach of the protein to the lipid interface would be as a monomer. Changes in this helix B feature as well as the rim of the \( \beta \alpha \)-barrel as a result of partitioning of Trp47 into the membrane would displace the Trp plug and allow transient dimers to form on the surface. In the case of PI cleavage, the dimer could enhance catalytic activity by allowing each monomer to alternate substrate binding and catalysis. Although the product diacylglycerol could easily be released back into the bilayer, water-soluble cIP must be released into solution. This would require the rim of the barrel to be at least partially solvent-exposed. For a monomeric protein, release of cIP might destabilize membrane binding of the protein and cause the protein to dissociate from the membrane. Dimerization could effectively increase the affinity of the protein for substrate by increasing the residence time of the protein on the membrane, essentially promoting "scooting" rather than "hopping" of the enzyme (26, 27). However, anchoring of a PI-PLC monomer via a discrete PC binding site could also keep the protein on the surface long enough for processive catalysis. Can these two possibilities be distinguished or at least constrained with the mutagenesis experiments reported in this work?

A dimer interface similar to what we see with W47A/W242A would be held together by four key tyrosine residues, Tyr246, Tyr247, Tyr248, and Tyr251. Because these tyrosine residues are involved in both hydrophobic and hydrophilic binding between the two subunits, replacing some or all of these Tyr residues should impair dimer formation, reduce the affinity of the protein for interfaces, and reduce catalytic activity. The three different tyrosine mutants constructed to test the importance of PI-PLC dimer formation for interfacial activation of PI-PLC by PC surfaces definitely show that this region of the protein is linked to kinetic activation. At least in the crystal, removal of tyrosines does not alter the active site structure. In solution, there may be a small loss of helix in the area of the tyrosine strip, but at least for Y247S/Y251S this can be
retrieved by occupation of the active site. The difference in crystal and solution conformations does highlight one important point; occupation of the active site can affect the secondary structure of the protein, at least around helix G. So if the dimer is formed, it could influence detailed arrangements of groups in the active site, at least enough to increase $k_{cat}$.

Clearly, when the four key tyrosines are replaced by serine, the herringbone intersubunit interactions can no longer occur. Consistent with this, the quadruple mutant has reduced activity (and reduced PC affinity) in all assay systems. The other two tyrosine mutant enzymes would be expected to have different dimerization potentials. The double mutant Y247S/Y251S behaved kinetically almost the same as rPI-PLC. The crystal structure of this mutant suggests that whereas the putative dimer interface might be somewhat destabilized, many intersubunit interactions can still be formed to stabilize the dimer on a PC-containing membrane. Because occupation of the active by a ligand (myo-inositol) was shown to enhance structure in this protein, it is likely that a phospholipid at the active could work in concert with the Tyr residues in stabilizing, at least transiently, a surface-bound dimer.

The alternate explanation is that these Tyr form part of a well defined PC binding site on a monomer that is distinct from the interaction of Trp47 and Trp242 with interfaces. Tyr aromatic rings could form a binding site for the positively charged choline moiety. This region of PI-PLC is rich in surface Tyr, and several of them could stabilize PC binding via $\pi$-cation interactions. In that case one would also expect removal of the Tyr would dramatically reduce the enzyme activity, particularly for PI/PC cleavage and diC$_7$PC activation of cIP.

Both triple and quadruple mutant enzymes have dramatically altered interactions with diC$_7$PC micelles and this provides clues into what happens to the protein when it binds activator lipids. These less active enzymes still bind diC$_7$PC molecules with fairly good affinity, as
assessed by the intrinsic fluorescence experiments. However, the change in intrinsic fluorescence
of the protein upon binding to diC₇PC is more in keeping with a ligand binding to the active site.
In field cycling experiments carried out previously (by M.F. Roberts and X. Zhang),
Y246S/Y247S/Y248S, compared with WT PI-PLC, converts the polydisperse rod-shaped diC₇PC
micelles to much smaller structures at a ratio of diC₇PC to protein where the maximum line
broadening is observed. At this total concentration of diC₇PC to PI-PLC, 35:1, there are no larger
micelles detected that coexist. A PI-PLC monomer could bind a relatively small and finite
number of these diC₇PC molecules to form a nearly spherical small mixed micelle. However, the
very elongated dimer, like that observed for W47A/W242A, would certainly form larger
aggregates. It would present an extended rim with a number of hydrophobic contacts.

If by replacing the Tyr with Ser we have removed a specific PC binding site, then there
should be little difference in specific activity for PI cleavage in diC₇PC versus Triton X-100 for
the triple and quadruple Tyr mutant proteins. Yet there is a 2–3-fold decrease in the Triton X-100
assay system (even more pronounced than for wt). If transient surface-bound dimers are the
optimally active species, then they must form in PI/Triton X-100 as well as in PC-containing
systems. The high relative activities for the Tyr mutants in both micelle systems compared with
PI/PC SUV or cIP/diC₇PC assay systems would then suggest that an amphiphilic molecule
occupying the active site is also very important for biasing the distribution to dimers.

On balance, then, these experiments seem more consistent with a surface-activated dimer
analogous to W47A/W242A. Could dimers form in the absence of interfaces? Some indication of
this may be provided by examining the second step of the reaction, cIP hydrolysis. In solution in
the absence of interfaces, monomer and a very small fraction of dimers of WT PI-PLC could
exist in equilibrium (indeed, in previous cross-linking experiments a very small amount of dimer
for protein alone in solution could be observed (24), suggesting aggregates might occur).
Removing any of the tyrosine residues that contribute to the dimer interface of W47A/W4242A would be expected to bias the equilibrium even more toward monomer. The reduced cIP hydrolysis rates for all the tyrosine-to-serine mutant enzymes in the absence of diC\textsubscript{7}PC are consistent with this possibility. If a diC\textsubscript{7}PC interface can still enhance dimerization, then the cIP hydrolysis rate should increase dramatically as observed for Y247S/Y251S. These kinetics are harder to explain with the postulate of a specific PC binding site. It is interesting that all the Tyr mutant cIP hydrolysis activities are similar (0.2 to 0.6 µmol min\textsuperscript{-1} mg\textsuperscript{-1}) and considerably smaller than for WT PI-PLC (2.3 µmol min\textsuperscript{-1} mg\textsuperscript{-1}). If disruption of a PC binding site were the result of the Tyr replacements with Ser, then one would not expect much of difference between WT PI-PLC and these mutant enzymes in the absence of diC\textsubscript{7}PC (and this is what is observed with examining the kinetics of W47A, W242A, and W47A/W242A (8), mutants that reduce binding to PC interfaces but would not alter a monomer-dimer equilibrium involving Tyr246, Tyr247, Tyr248, and Tyr251).

In summary, we have probed the importance of the strip of tyrosine residues that form the core of the dimer interface observed previously in the mutant protein W47A/W242A. The reduction in specific activity in all assay systems with the removal of three or four of these tyrosines can arise from either two effects. (i) The lack of the tyrosine strip disfavors the surface-induced dimer formation that is needed for optimal activity. (ii) Removal of these tyrosines abolishes a specific and critical PC binding site. Because both phosphotransferase and cyclic phosphodiesterase activities are reduced by these mutations (and the physical interaction of the mutant protein with PC interfaces is altered), we cannot absolutely distinguish between these two alternatives (and the definition of the activated form of the enzyme they imply). However, the
evidence, particularly kinetic changes in PI/Triton X-100 and cIP in the absence of interfaces, appears more consistent with dimerization of this PI-PLC as the activated form of the enzyme.
References


Chapter V: FRET As A Probe of Interface-Induced Dimerization of PI-PLC
In the third and fourth chapters of this thesis, I provided evidence that was consistent with a model of *B. thuringiensis* PI-PLC dimerizing on membrane surfaces as a way to activate the enzyme. However, there was no direct evidence for protein dimerization on surfaces. One method to test this is to use fluorescence resonance energy transfer (FRET) and to examine under what conditions it occurs between PI-PLC molecules bound on a vesicle surface. FRET between W280C-NBD and W280C-Rho are used to assess dimer formation on vesicles. Results suggest any dimerization only occurs on surfaces. While up to 50-60% efficient FRET was observed with the proteins (typically a ratio of W280C-NBD to total phospholipid of ~1:3500 or 1:7000) binding to pure POPC vesicles, a mixed bilayer with a substrate analog along with the kinetic activator enhanced the extent of the FRET interaction (to nearly 100%) but typically required more W280C-Rho for maximum efficiency. The FRET at a lower mole PC fraction (X_{PC}=0.25) was also tested and little FRET was seen. These interesting results suggested any dimer of PI-PLC that may exist on mixed phospholipid surfaces is transient. In addition, two covalent dimer models, disulfide linked dimers for W242C and N243C, were built based on the W47A/W242A crystal structure to try and explain why the W242C-dimer was more active than WT while the N243C-dimer was actually significantly inhibited. A key structural difference was that the very active W242C-dimer appears to have a more extended surface loop compared with the much less active N243C dimer. These results are used to refine a model of how PI-PLC is activated by PC for both PI and cIP cleavage.

**FRET as a probe of PI-PLC oligomerization on interfaces**

To test if PI-PLC could oligomerize on vesicle surfaces, we prepared W280C and derivatized the enzyme to introduce an NBD or a rhodamine (Rho) group at that position. The Förster distance ($R_0$), at which the FRET efficiency is 50%, of the NBD-PLC/Rho-PLC pair
(donor-acceptor) is about 56 Å \( (1) \). Trp280 was chosen as the site of probe attachments because it is near the dimer interface in W47A/W242A but is not involved in intramolecular interactions with other residues in this region and not dimerization directly. Previous replacement of Trp280 with an alanine showed little loss of phosphotransferase or cyclic phosphodiesterase activities \( (2) \), suggesting that this might be a good site for attachment of a fluorophore. The fluorescently labeled donor, NBD-PLC, was kept at 0.01 or 0.02 mg/ml (0.29 or 0.57 \( \mu \text{M} \)) and added to 2 or 4 mM total phospholipid (either pure POPC or POPC/DOPMe mixtures characterized by the mole fraction of PC, \( X_{\text{PC}} \)) presented as SUVs. For the PC and PC/PMe mixtures, these phospholipid concentrations were considerably above the apparent \( K_d \) determined recently by FCS \( (3) \). The NBD fluorescence at 530 nm was then monitored upon the addition of the acceptor, Rho-PLC.

When the two proteins were mixed at these concentrations in the absence of vesicles, there was no fluorescence resonance energy transfer (FRET). If the two differently labeled fluorescent subunits approach each other when bound to phospholipid vesicles, FRET should occur from the NBD-group on one molecule to the Rho moiety on another, and the intensity of the NBD fluorescence should decrease in a saturable fashion. Although the \( R_0 \) is large for this pair, as long as there is an excess of vesicles to NBD-PLC we should be able to detect if dimers form. If only monomers exist on the vesicle surfaces, the response would be expected to be fairly low at high NBD-PLC dilution (at most 1 protein per several vesicles) and linear as Rho-PLC is increased and the vesicle bound PLC concentration increases until there is enough Rho-PLC on the vesicle so that its lateral diffusion will allow for efficient FRET between donor and acceptor.

In the presence of 1mM POPC SUVs and 0.02 mg/ml NBD-PLC, added Rho-PLC caused a pronounced decrease in the fluorescence intensity at 530 nm (Figure 5.1). To assess the contribution of Rho-PLC in this range, I titrated the same SUVs without NBD-PLC with the
Rho-PLC. As can be seen, there is very little contribution of the Rho-PLC to the fluorescence intensity at 520 nm.

**Figure 5.1.** Fluorescence intensity at 530 nm for the sample, NBD-PLC with Rho-PLC added (●), and a control where no NBD-PLC was present but Rho-PLC was titrated into the SUVs (○).

This concentration of PC was deemed not quite high enough to really look for PLC aggregation on surfaces. So we increased the total phospholipid concentration to 2 or 4 mM and titrated the Rho-PLC into the SUVs containing 0.02 mg/ml NBD-PLC. Typical examples of the data are shown in Figures 5.2 (4 mM PC SUVs) and 5.3 (PC/PMe 2mM:2mM SUVs). PMe has been shown to bind competitively with PI (4) and act as a substrate analogue. Indeed, PI-PLC binds more tightly to a 1:1 mixture of PC/PMe (apparent K<sub>d</sub> ~0.016 mM) than to pure PC SUVs, where K<sub>d</sub> ~ 0.05-0.07 mM (3). Since the Rho-PLC added a negligible amount to the sample fluorescence at 530 nm, we used a different control – vesicles plus NBD-PLC but titrated with unlabeled PLC (W280C). The expectation was that the control would maintain a high intensity
while the sample with added Rho-PLC would exhibit a decrease in intensity indicative of FRET between the two labeled PLC enzymes.

**Figure 5.2.** NBD-PLC (0.02 mg/ml) mixed with 4 mM PC SUVs and then titrated with Rho-PLC. (A) Fluorescence intensity at 530 nm for the sample, NBC-PLC with Rho-PLC added (●), and the control, NBC-PLC with unlabeled PLC added (○). (B) Difference in fluorescence intensity between the sample and control. (C) FRET as a function of added Rho-PLC.
Figure 5.3. NBD-PLC (0.02 mg/ml) mixed with PC/PMe (2mM:2mM) SUVs and titrated with Rho-PLC. (A) Fluorescence intensity at 530 nm for the sample, NBC-PLC with Rho-PLC added (○), and the control, NBC-PLC with unlabeled PLC added (○). (B) Difference in fluorescence intensity between the sample and control. (C) FRET as a function of added Rho-PLC.
FRET between the donor and acceptor PLC enzymes was clearly observed. The added Rho-PLC was kept \( \leq 0.1 \) mg/ml, which would produce a maximum ratio of acceptor to donor of 5. A summary of the average \((A/D)_{0.5}\) and FRET efficiencies is shown in Table 5.1. We originally thought that this ratio and the high total concentration of phospholipids (2 or 4 mM) should minimize nonspecific interactions between the two populations of labeled proteins (6). The maximum FRET was extrapolated as \(~60\%\) whether pure PC or a PC:PMe (1:1) mixture was used. For both cases, the ratio of acceptor to donor for half the maximum FRET was \(~2\). At these concentrations of protein, the ratio of total phospholipids to NBD-PLC varied from 3500 to 7000.

**Table 5.1.** FRET from NBD-PLC (0.02 mg/ml) to Rho-PLC in the presence of PC and PC/PMe SUVs.

<table>
<thead>
<tr>
<th>PC (mM)</th>
<th>PMe (mM)</th>
<th>( \text{Eff}_{\text{max}} ) \text{b} (%)</th>
<th>Rho-PLC(_{0.5}) (mg/ml)</th>
<th>((A/D)_{0.5} ) \text{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>44±5</td>
<td>0.016±0.004</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>55±3</td>
<td>0.069±0.012</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>98±9</td>
<td>0.091±0.022</td>
<td>4.6±1.1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>56±5</td>
<td>0.041±0.008</td>
<td>2.0±0.4</td>
</tr>
</tbody>
</table>

\(^{a}\) FRET efficiency was estimated with up to 0.1 mg/ml Rho-PLC added to 0.02 mg/ml NBD-PLC in the presence of SUVs with the composition of PC and PMe indicated.

\(^{b}\) The parameters extrapolated from the dependence of FRET on added Rho-PLC used the equation \(\%\text{FRET} = \text{Eff}_{\text{max}} \left[\text{Rho-PLC}\right] / \left[\left[\text{Rho-PLC}_{0.5}\right] + \left[\text{Rho-PLC}\right]\right]\).

\(^{c}\) Ratio of acceptor PLC to donor PLC for 50% of the maximum FRET.

the outer monolayer), 2 mM total phospholipid translates to a minimum outer leaflet phospholipid/NBD-PLC ratio of 2300:1, likely about one NBD-PLC per vesicle. We hoped that the PI-PLC was sufficiently dilute so that a small excess of Rho-PLC would not be expected to
exhibit efficient FRET with the NBD-PLC unless a fairly specific complex was formed on the membrane surface. Several factors could contribute to the apparent cooperativity in the FRET profile. If the added Rho-PLC is randomly distributed on vesicles, a small amount added may not interact with a vesicle containing NBD-PLC. Increasing the Rho-PLC enhances the probability that a given vesicle will have both donor and acceptor. If a discrete dimer is formed, the FRET efficiency should be 100%. For these SUVs, the efficiency was mostly below 100%. This likely is more consistent with the FRET caused by lateral diffusion of several acceptors and the donor on a given vesicle. At 0.02 mg/ml NBD-PLC, with twice as much Rho-PLC, there is likely to be enough Rho-PLC on a given SUV that with an R₀ ~50 Å there will be observable FRET just by lateral diffusion of the protein. The way to test that was to increase the ratio of total phospholipid to donor protein. Diffusional proximity (and not a discrete complex) as the basis for the FRET should then require more donor Rho-PLC. With this in mind, the NBD-PLC concentration in these FRET experiments was decreased to 0.01 mg/ml.

With the lower concentration of donor PI-PLC in the pure PC SUV system, very little Rho-PLC was needed for efficient FRET. This suggests that with a PC SUV a PLC complex is likely to occur on vesicle surfaces (and might be related to the lower activity that is observed). However, for the mixed vesicles, 4-8 fold more acceptor was needed, a result more consistent with diffusional FRET.

Another mole fraction of PC was examined, X_{PC}=0.25. Binding of the protein is weaker to these SUVs than to X_{PC}=0.9 SUVs (3). For comparison, 4 mM total phospholipid and 0.01 mg/ml NBD-PLC were used. In this case, less FRET was observed with small amounts of Rho-PLC (data for X_{PC}=0.25 in Figure 5.4) and the amount of Rho-PLC needed for half the maximum
FRET was larger than for other compositions of PC/PG. (Figure 5.6). The \((A/D)_{0.5}\) was 9.6±1.9 for this system with 58±7% FRET as the maximum.

**Table 5.2.** FRET from NBD-PLC (0.01 mg/ml) to Rho-PLC in the presence of PC and PC/PMe SUVs.

<table>
<thead>
<tr>
<th>PC (mM)</th>
<th>PMe (mM)</th>
<th>Eff(_{\text{max}}) (%)</th>
<th>Rho-PLC(_{0.5}) (mg/ml)</th>
<th>((A/D)_{0.5}) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>41</td>
<td>0.014±0.003</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>41</td>
<td>0.015±0.002</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>87</td>
<td>0.081±0.009</td>
<td>8.1±0.9</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>58</td>
<td>0.037±0.007</td>
<td>3.7±0.7</td>
</tr>
</tbody>
</table>

\(^a\) FRET efficiency was estimated with up to 0.1 mg/ml Rho-PLC added to 0.01 mg/ml NBD-PLC in the presence of SUVs with the composition of PC and PMe indicated.

\(^b\) Ratio of acceptor PLC to donor PLC for 50% of the maximum FRET.

In the experimental conditions, the ratio of outer leaflet phospholipid to NBD-PLC is 9200:1, meaning on average every 4th vesicle is likely to have an NBD-PLC. On PC SUVs, FRET appears quite efficient, even at these low donor concentrations. Given the large \(R_0\) for this donor/acceptor pair, we cannot clearly distinguish specific but transient dimer formation from FRET caused by diffusional proximity. However, the results are consistent with PLC dimer formation on PC SUVs. In contrast, having both a substrate analog (PMe) and the PC activator clearly changes the FRET behavior making a discrete complex much less likely. Any dimmers formed with the mixed phospholipid SUVs must be quite transient.

We had previously postulated that protein dimerization on surfaces enhanced catalysis. These results qualify that proposal. PI-PLC specific activity towards PI/PC SUVs drops dramatically above \(X_{PC}=0.5\). Part of this decrease has already been attributed to segregation of PC-rich domains with the enzyme from bulk PI (5). What my FRET study might suggest is that
in this segregated domain PI-PLC complexes occur and may be a contributing factor to the low activity in this regime.

![Graph](image)

**Figure 5.4.** (A) Fluorescence intensity at 530 nm for (●) NBD-PLC (0.01 mg/ml) with 4 mM total PC/PMe (1 mM:3 mM) titrated with Rho-PLC compared to the control titration with unlabeled PLC (○). (B) Efficiency of FRET for this \( X_{PC} = 0.25 \) system.

*Modeling of covalent PI-PLC dimers*

In parallel to my FRET studies assessing dimer formation of PI-PLC, another graduate student in the laboratory, Mingming Pu, constructed covalent PI-PLC dimers by introducing a
cysteine in the protein in the region of the dimer interfaces. She found that the disulfide-linked dimer of W242C was almost twice as active as WT towards PI solubilized in diC7:PC (but comparable to WT with PI/Triton X-100 assay systems), while the N243C-linked dimer was considerably less active that WT in either assay system. The W242C-dimer also showed about a 10-fold drop in apparent K_d towards pure PC SUVs while the N243C-dimer bound more weakly than WT. Based on the structure of W47A/W242A, one would predict that both mutations should produce comparable dimers.

To try and sort this out, I tried to model both the W242C and N243C dimers based on the W47A/W242A structure (6). As shown in Figure 5.7A, the overall structures are quite similar with the greatest change in the disordered loop (residues 236_SSGGTA_242_CNS) where the Cys was introduced. The N243C dimer is very similar to the W47A/W242A dimer with only small backbone changes (r.m.s.d. = 0.93 Å). The distance between the Cys residues in the W242C dimer based on W47A/W242A is significantly longer than when the Cys is placed at residue 243 and a similar dimer generated. Connecting the two W242C Cys residues to form a disulfide requires changes in the loop containing this residue. After energy minimization, the most striking change is that this loop is rotated so that it extends out away from the protein, potentially interacting with the membrane surface (Figure 5.7B). In contrast, the helix B residues in the W242C covalent dimer model remain at almost the same position as in W47A/W242A. There are also slight changes that could occur in the active sites of the covalent dimer models compared to W47A/W242A, but these are the same for the N243C and W242C dimers. Thus, it would appear that the change in orientation of the loop adjacent to the disulfide bond could be important for the tighter binding of the W242C covalent dimer to PC vesicles. The loop containing W242C is composed of small polar and flexible residues, so that differences in
membrane partitioning of the side chains does not seem a likely explanation for the differences in specific activities of W242C versus N243C covalent dimers. It is also possible that the surface-induced oligomer sensed by FRET is not really equivalent to W47A/W242A. However, that double mutant is the only PI-PLC in which the protein crystallized as a dimer, so further explanations really require a structure for the W242C-dimer. Current efforts in the laboratory include attempts to crystallize this covalent dimer.

*Is PI-PLC dimerization critical for activity?*

The observation of FRET between labeled PI-PLC monomers on PC and PC/PMe (1:1) vesicles under fairly dilute conditions clearly shows that the proteins can approach one another when bound to the bilayer. For pure PC vesicles, the (A/D)0.5 characterizing the FRET is more or less the same when the protein concentration or total phospholipid are altered. However, the amount of Rho-PLC needed for half the maximum FRET for PMe/PC SUVs increases either when the concentration of NBD-PLC is decreased. Complexes detected by FRET are not stable but transient and unlikely to exist when the protein is diluted 100-to 1000-fold as in the enzyme kinetics.

These results modify our previously proposed mechanism for the activation of this PI-PLC. As before, this bacterial PI-PLC exists as a monomer in solution, presumably with helix B intact and the loop containing Trp242 exhibiting significant mobility, but in a way that prevents PI-PLC monomers from forming a significant population of dimers in solution. A monomer of PI-PLC can bind to a PC bilayer and this requires both Trp47 and Trp242 (2, 7). However, we suggested that helix B, orienting at the surface because of electrostatic interactions between the anionic substrate and Lys44, unravels when bound to substrate containing vesicles so that the
hydrophobic sidechains in this region can partition into the bilayer. It is possible that the initially bound PI-PLC monomer on pure PC has intact helix B but with Trp47 in the bilayer.

Figure 5.7. (A) The W242C-disulfide dimer model (in magenta) and N243C covalent dimer model (in cyan) compared to *B. thuringiensis* PI-PLC mutant, W47A/W242A (PDB code OR2, in grey). (B) Top view of the disulfide bonds modeled in the W242C and N243C covalent dimers. The disulfide bonds are shown as yellow sticks. (C) Superposition of *B. thuringiensis* PI-PLC W47A/W242A active site residues with those from energy minimized models of the W242C and
N243C covalent dimers.

As proteins diffuse on the surface of the PC vesicle, they may form moderately stable complexes, but these structures and their associated FRET do not correlate with high enzyme activity (3). This orientation on pure PC vesicles (and presumably a similar orientation with diC7PC micelles) is adequate for activating the enzyme for water-soluble cIP hydrolysis. If substrate or substrate analogues are present, helix B unravels, the anionic lipid can occupy the active site, and further anchoring to the surface is supplied by PC presumably binding at a discrete site distinct from the active site. The synergistic effect of both types of phospholipids on PI-PLC binding to vesicles (3) reflects this tighter association with vesicles that in turn enhances processive catalysis of PI cleavage.

How do these different vesicle-anchored PI-PLC conformations relate to water-soluble cIP hydrolysis? PI-PLC catalyzed hydrolysis of cIP, as well as the cleavage of water-soluble diC4PI, is much slower than interfacial PI cleavage (7). Although PC activation increases $k_{cat}$ and reduces $K_m$ for these substrates, there can be no processive catalysis since these molecules have no tendency to partition into vesicles or micelles. Perhaps, the transient PI-PLC dimer on a PC surface is adequate to shift active site residues so that catalysis is, in fact, enhanced. However, high activity for the first step is only achieved with occupation of both active site and PC activator site allowing processive catalysis. This step does not require a PI-PLC dimer.


Chapter VI: H/DXMS of *B. thuringiensis* PI-PLC – assessing membrane-induced conformational changes
Hydrogen exchange coupled with mass spectrometry (MS) is a powerful tool for studying protein dynamics. Protein is incubated in D₂O buffer, and accessible amide protons will exchange with deuterium. Quenching the reaction by lowering exchanging temperature and pH (to pH~2) will largely avoid back exchange. MS is used to monitor the deuterium incorporation by measuring the protein mass increase. Protein conformational changes or binding to lipids are likely to influence the H/D exchange rates of certain peptides. Protein dynamics are often related to protein function, and H/D exchange presents an option to characterize changes in protein motions under different conditions. There are many examples of analyses performed with HXMS (hydrogen exchange mass spectrometry) over the last several years. HXMS experiments were performed to study protein dynamics such as conformational changes upon phosphorylation or introduction of single mutations (1). Protein folding and refolding are widely studied via many analytical methods including H/D exchange coupled with MS using pulse labeling (2-4). Protein binding of ligands or other proteins can influence protein folding, function, and stability, and HXMS has been used to study protein-protein, protein-lipid, protein-ligand and protein-nucleotide associations (5-7).

Determining which areas of a protein interact with the membrane can be difficult. Often, flexible regions of the proteins provide the suspected major contacts with the membranes. Furthermore, it is hard to grow a crystal containing both protein and lipid molecules, particularly for a peripheral membrane protein. NMR is a better way for detecting binding sites in solution, but the requirement of very large amount of protein and solubility at high concentrations have been a problem. FRET is potentially useful, little protein is required, but the labeling step and light scattering of large molecules have been a problematic issue. Resolution is also limited – FRET can monitor binding but proximities are often limited to >10 Å or so. H/D exchange
coupled with MS is another way to examine what sites on a protein bind to (or are occluded by) membranes. The H/D exchange is carried out in solution buffer, only 5 μg protein is needed, the requirement for pure protein is low, fast analysis of each MS profile takes less than 1 hour, which is much less than in X-ray and NMR approaches. In the last 2-4 years this methodology has been applied to peripheral proteins – in particular several phospholipase A2 isozymes – with interesting results (8). In particular, this method can suggest where there are hinge regions near active sites as well as suggesting regions where conformational changes must occur. With this as background, I decided to see if H/D exchange could provide insight into (i) what regions of \textit{B. thuringiensis} PI-PLC were involved in binding to target membranes, and (ii) if evidence for membrane-induced dimerization of this PI-PLC could be obtained.

\textit{Effect of PC/PG SUVs on PI-PLC amide exchange}

\textit{B. thuringiensis} PI-PLC has two discrete binding sites for phospholipids – the active site binding PI (or substrate competitors such as phosphatidylycerol (PG)), and an activator site specific for phosphatidylcholine (PC) (9). The protein binds most tightly to PC-rich small unilamellar vesicles (10). In collaboration with Prof. Patrick Wintrode at Case Western University, I have performed H/D exchange mass spectrometry experiments on wild type PI-PLC in solution or bound to POPC/DOPG (1:4 or 1:9) SUVs. These vesicle compositions were chosen to ensure tight protein binding and maximize the on-vesicle time of the protein.

When a protein is incubated in D₂O buffer, the amide hydrogens that are solvent-exposed and not involved in a hydrogen bond with other protein groups will exchange with deuterium very quickly. The hydrogens that are buried inside the protein structure exhibit slow exchange rates. If the protein undergoes a conformational change involving breaking intramolecular hydrogen bonds of the amides, the hydrogens protected by local structure will now exchange
with deuterium in the environment. If a conformational change sequesters amides by placing them in shielded intramolecular hydrogen bonds or removes them from solvent access, then the amide hydrogens will show a decrease in the rate of deuterium incorporation. The peptides analyzed in this study typically encompass 90-95% of the entire amino acid sequence of wild-type PI-PLC covering most secondary structural regions (Figure 6.1). HXMS experiments were performed to monitor changes in the deuterium exchange rate change between vesicle-associated and free protein in solution. In particular, we wanted to see if there was any evidence for dimer

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**Figure 6.1.** Peptide mapping of wild type PI-PLC. Peptides found in both MS-MS and H/D exchange MS are shown in green. Helices A to H are shown in red. β–sheets I to VIII are shown in blue.
formation, which would be expected to retard amide exchange of the region around the Tyr strip (-246Y-Y-Y-A-S-251Y-). We also wanted to get a sense for how local or specific conformational changes were in the protein.

In the absence of vesicles, amides in most regions of wild type PI-PLC exchange slowly (Figure 6.2), although the exact extent depends on the ‘fully deuterated’ control. This is not surprising given the well-defined (αβ)-barrel structure of the protein. In contrast, surface loops typically exhibit higher rates of exchange. Peptide loop 161-176, the loop between β-sheet V to VI and the loop 276-286 show some exchange with H/D exchange rates of 30% to 50% at 20 s after introduction of D2O. It may not be surprising that the N-terminus is very flexible even though this region forms a well-defined helix A in the crystal structure (11).

Short helix A is adjacent to a loop, and in solution, this peptide may either be unstructured or partially unraveled because of the proximity of the loop. In either case, it is deuterium accessible. In contrast to the N-terminus, the region with residues 38-55, containing helix B, does not exhibit any exchange when the protein is free in solution. This is an extremely interesting observation because it says that the short helix B is a stable secondary structure element for soluble protein. In the crystal structure, high B factors for this helix suggested it might not be very stable (11). It has also been proposed that this helix unwinds when the protein binds to membranes (12). The H/D exchange analysis indicates the helix is stable in solution and it is more likely that the helix as a whole moves or occupies different positions giving rise to the high B factors.

The 240’s loop with Trp242 and the surface Tyr strip in the N-terminal region of helix G are notable for very fast deuterium exchange, indicating this region is not self associated as the homodimer seen in the W47A/W242A crystal structure. Next to the N-terminus, this is the most
There are also some peptides with intermediate degrees of H/D exchange. These include the loop composed of residues 161-176, residues 182-195 (which contains β−sheet V and part of VI) and loop 276-285. Of note is the time-dependent behavior of peptide 182-195 which contains β−sheet V and VI. This sheet-loop-sheet structure exhibits an increasing percentage of H/D exchange from 30% at 20 s to over 80% at 3000 s. This suggests a slow conformational change.

Figure 6.2. Peptide map of wild type B. thuringiensis PI-PLC in the absence of SUVs at 20, 50, 100, 500, 1000, 2000 and 3000 s (from top to bottom in each row) after introduction of D₂O. Different H/D rates are shown from purple (least exchangeable) to red (most exchangeable).
for this segment of the structure. Interestingly, these sheets in the structure are not covered by α-helices as is seen with the rest of the β-barrel in the protein. This suggests there is some ‘breathing’ of the β-sheets that over time do break and reform hydrogen bonds, and given their exposure to solvent will incorporate deuterium over time.

When the protein is incubated with DOPG/POPC (1:9) SUVs (Xpc=0.9) (Figure 6.3), chosen because the PI-PLC has the tightest affinity for this composition (10), there are some very obvious changes – some expected and some quite interesting. Most of the amides in the structure

<table>
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<th>αA</th>
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**Figure 6.3.** Peptide map of wild type *B. thuringiensis* PI-PLC incubated with 4 mM POPC/DOPG (9:1) SUVs after introduction of D$_2$O at 20, 50, 100, 500, 1000, 2000 and 3000 s from top to bottom in each row. Different H/D rates are shown from purple (least exchangeable) to red (most exchangeable).

show no change in exchange rate (these are primarily those that already exhibit slow exchange
for the protein diffusing in solution). In this category is helix B, containing Trp47 that partitions into membranes. This feature shows little change in the exchange rate when the protein binds to these SUVs. In contrast, some peptides (residues 1-10, 161-175, 232-252 and 276-285) exhibit slower H/D exchange (Figure 6.4).

![Diagram of protein with SUVs](image)

**Figure 6.4.** Wild-type PI-PLC showing the overall H/D exchange map at 20 s after dilution with D$_2$O. The orange stick suggests the orientation of the vesicle surface.

The N-terminal peptide (residues 1-10) exhibits dramatically reduced exchange rates. This segment of the protein is not near the rim of the barrel and unlikely to bind to the membrane. Hence, it is more likely there is a conformational change in this region that tightens secondary
structure and slows exchange. Arg163 is part of the hydrogen-bond network in the active site, so the peptide containing it (residues 161-175) might be expected to exchange more slowly. The same change is observed with peptide containing residues 232-251, which shows a dramatically reduced exchange rate upon binding to lipids.

One of the key membrane binding residues, Trp242, is located in the 232-251 region as well as the surface Tyr cluster (Tyr246, Tyr247, Ty248, Tyr251) that formed the dimer interface in the W47A/W242A crystal structure. The 240’s loop should be in contact with lipids and might be expected to reduce solvent access to nearby regions, notably the Tyr cluster. Alternatively, the Tyr cluster may also be self-associated when the protein is bound to the SUV surface. Either way, the H/D exchange rate in this region should decrease. The 276-285 peptide is further removed from the membrane surface and this loop might not be expected to exhibit much change in H/D exchange when the protein is bound to vesicles. Yet its exchange rate slows dramatically. In the W47A/W242A dimer there are hydrophobic intramolecular interactions between Trp280 and Tyr246 and Trp280 and Pro254; Lys279 also forms intermolecular H-bonds withSer250. So slower exchange in this region may indicate dimer formation.

The peptide starting at residue 91 to 110 is notable for its faster deuterium exchange rate in the presence of these SUVs. Interestingly, this part of protein contains helix D and is not near the active site. One possible explanation for its mobility upon binding to lipids could be that there is an orientation change of the helix that causes the short loop at end of the helix to unwind slightly and become more solvent exposed. Another peptide, residues 182-195 (β-sheet V to VI), also shows enhanced exchange. While exchanging over time in the absence of SUVs, it appears more susceptible to exchange when the protein is bound to SUVs. Yet the following peptide, residues 196-205 containing part of β-sheet VI, remains protected. β-sheet V and VI are two
connected strands of the barrel that form a wall around the active site. A summary of these changes in deuterium exchange when the protein incubated with SUVs is shown in Figure 6.5.

![Front view and back view of protein structure](image)

**Figure 6.5.** Changes in H/D exchange in wild type PI-PLC binding to POPC/DOPG (9:1) 20 s after the introduction of D$_2$O. The protein backbone shown in grey indicates a region with little change in H/D exchange after binding of the protein to the vesicles. Peptides exchanging faster (91-110, 182-195 and 196-205) after lipid binding are colored in red. Peptides exchanging more slowly (1-10, 161-175, 232-252 and 276-285) are colored blue.

It would appear that when the protein interacts with phospholipids in the membrane, there is now increased exchange of these sheets. How can we explain this behavior? In an MD simulation of the free wild type PI-PLC carried out by our HXMS collaborator, Partick Wintrode, peptide 182-195 (and at least part of residues 196-205) is in a region whose motion is high (Figure 6.6). Asp198 in β-sheet VI has interactions in the crystal structures with myo-inositol (11), which would suggest that the 196-205 peptide should show a slower exchange rate, particularly if a substrate analog is present. When a substrate binds to the active site and becomes associated with Asp 198, this may cause some conformational change in the connecting peptide 182-195 and affect the H/D exchange rate.
Figure 6.6. NAMD simulation by Patrick Wintrode (unpublished) for wild type PI-PLC (blue) and P254A (red).

PI-PLC binding to 4:1 PC/PG ($X_{PC}=0.8$) SUVs (Figure 6.7) was also examined. The protein still binds tightly to this composition of SUVs, and initially we expected the two exchange profiles to be fairly similar. The peptide map of PI-PLC binding to 4:1 POPC/DOPG looks very similar to that of 9:1 POPC/DOPG. Peptide 91-110 is still nearly all exchanged at the shortest time point.

However, variation of $X_{PC}$ may have some small effects. The most significant change comparing to binding to 9:1 POPC/DOPG is that the N-terminal peptide is no longer protected with SUVs present and undergoes significant H/D exchange. Peptide 182-195 incorporates deuterium over the time course of the exchange experiment, which is the same as that of free PI-PLC in solution. Also of note is that peptide 196-205 has increased exchange compared to binding to 9:1 PC/PG. It would appear that protection of the N-terminus and deprotection of peptide 196-205 only happen with the very PC-rich vesicles. PI-PLC binds slightly more tightly
to 9:1 PC/PG than 4:1 PC/PG (10), but the difference in binding affinity is not that striking. However, kinetics as a function of $X_{PC}$ (10) show that the enzyme activity is extremely low at $X_{PC} = 0.9$ compared to $X_{PC} = 0.8$. The combination of tighter binding at $X_{PC}=0.9$ and lower activity might indicate that the PI or PI analog resides in the active site longer in the more PC-rich system. Peptide 196-205 has Asp 198, that binds inositol. The faster exchange of this peptide with $X_{PC}=0.8$ SUVs might occur because the molecule in the active site diffuses in and out more rapidly, while the PC acts as a more stable anchor.

Figure 6.7. Peptide map of wild type PI-PLC incubated with 4 mM POPC/DOPG (4:1) SUVs after introduction of D$_2$O at 20, 50, 100, 500, 1000, 2000 and 3000 s from top to bottom in each row.
Figure 6.8 presents the detailed exchange data for peptides 23-37, 77-88, 182-195 and 276-285 as function of time. The 23-37 peptide exchanges 20-25% rapidly and another 10% over time and there is little difference when SUVs are added. Peptide 77-88 and peptide 276-285 clearly exhibit significant protection with SUVs added. In both cases there appears to be a slightly greater degree of protection from exchange at the earliest time points when $X_{PC}=0.9$ SUVs are present.

![Graphs showing exchange data](image)

**Figure 6.8.** Deuterium incorporation into *B. thuringiensis* PI-PLC as a function of incubation time with D$_2$O in the absence (●) and presence of (○) POPC/DOPG (9:1) or (×)
Peptide 182-195 shows the most unusual change in deuterium incorporation over time. The free PI-PLC and PI-PLC binding to 4:1 PC/PG have similar time-dependent exchange behavior. On a short time scale, the peptide only has 40-50% exchange; the deuterium level continues rising up to 80% at longer time points. However, with the higher X_{PC} SUVs, exchange is enhanced such that 80% is exchanged by the first time point. This β / turn / β motif exhibits exchange over time consistent with what might be expected for unprotected strands in a β-barrel. One possible explanation for the enhanced exchange with X_{PC}= 0.9 is that under these conditions, there may not always be a ligand in the active site. What may be different from when the protein is free in solution is that when the protein is bound to the PC in the very PC-rich SUVs, the active site is in some way strained. This may most affect the β / turn / β motif without an external helix.

*Is W47A/W242A a dimer in solution?*

The H/D exchange rates were also determined for a mutant protein, W47A/W242A, with very low affinity for vesicles that may exists as a dimer in solution. While wild type PI-PLC is a monomer in crystal structures (11), the W47A/W242A protein forms a dimer in the crystal with four Tyr residues (Tyr246, Tyr247, Tyr248, Tyr251) clustering in the dimer interface (13). Whether the protein exits as a dimer in solution is not clear since normal methods to analyze for protein tertiary structure are problematic for this and many other PI-PLC enzymes. These proteins interact with sugar residues that form gel filtration matrices or that are used in centrifugation experiments and aberrant sizes are obtained. In the H/D exchange experiments of wild type PI-PLC in solution, the peptide encompassing the Tyr strip shows very rapid exchange. If a similar W47A/W242A crystal dimer form exists in solution (and is stable), the exchange rate of this peptide should be reduced. The results could provide insight into the conformational state
of PI-PLC monomers. Because two critical membrane binding residues, Trp47 and Tp242 are mutated, this mutant PI-PLC cannot bind to vesicle interfaces, therefore, the H/D exchange with lipids was not tested.

**Figure 6.9.** Peptide map of *B. thuringiensis* PI-PLC W47A/W242A, without lipids, incubated with D$_2$O for 20, 50, 100, 500, 1000, 2000 and 3000 s (from top to bottom in each row).

The peptide map of W47A/W242A covered over 75% of the whole sequence (less than for wild type protein but adequate for assessing dimer formation). More interestingly, much more of the protein amide backbone was exchanging quickly with deuterium. The exchange patterns for W47A/W242A are more or less like wild type in solution but with several notable changes (Figure 6.9). In wild type PI-PLC, helix B (containing Trp47) shows little deuterium exchange,
suggesting that in solution the short helix may be moving but as a stable secondary structure element. When Trp47 is removed in W47A/W242A, the peptide 38-55 shows dramatically enhanced exchange. Removal of the hydrophobic Trp47 destabilizes the short helix. This is consistent with what we see in the W47A/W242A crystal structure where the peptide that forms helix B is now a loop structure. Peptide 111-129 is not seen in wild type PI-PLC; in the double mutant protein this segment of the protein is highly exchanged. Lys115 in this peptide has some contact with inositol in the wild type protein crystal structure so likely this portion of the protein is quite solvent accessible. Peptide 194-217 (including part of β-sheet VI and helix F) shows slow exchange, comparable to what is seen with wild type protein in solution. Peptide 185-193 (sheet V and VI) shows reduced exchange over time while the peptide immediately preceding it (residues 162-184) has a tendency to exchange a little faster than wildtype.

Of particular interest is the dimer interface region peptide, residues 232-252. These exchange more slowly than wild type after short incubation times. However, by 3000 s the exchange is equivalent to that seen for wild type protein. This is consistent with either an equilibrium of monomer and dimer in solution or a loose dimer structure for the W47A/W242A. The double mutant can not form a very tight dimer structure in solution or this peptide would have been protected. Given the extensive contacts between the two monomers in W47A/W242A, formation of a similar dimer would have the residues in these peptides well protected from D2O exchange. Therefore, the retarded exchange for W47A/W242A likely monitors the monomer/dimer equilibrium in solution. Detailed views of exchangeable regions superimposed on the PI-PLC structure and compared to wild type protein are shown in Figures 6.10 and 6.11.

Several peptides exhibited time dependent H/D exchange. Figure 6.12 shows the time dependence of two of these peptides, 77-88 and 232-252 in W47A/W242A. Interestingly, these
two peptide exhibit similar deuterium exchange rates. They both increase about 30% from 20 to 3000s indicating the conformational change leading to exchange of these two peptides may happen at the same time. Important catalytic residue His82 (the general acid in the mechanism) is in the 77-88 loop, while the 232-252 peptide contains both the 240’s loop for membrane binding and the Tyr strip. So the correlation from deuterium exchange to conformational change over time between 77-88 is 232-252 is reasonable.
Figure 6.1: Evolution of backbone exchange rates that become more during dilution after the increase in deuterium incorporation into peptides excited in blue. Thus, the exchange rate for W47A/W242A in comparison with wild type are colored red; PI-PLC is as a backbone exchange rate after the increase in deuterium incorporation into peptides excited in blue.

Front view

back view

$W47A/W242A$

Time (s)

$F_D$

1

0.8

0.6

0.4

0.2

0
**W242C covalent dimer – how does exchange compare to wild type PI-LC?**

The H/D exchange rates were also determined for a constructed covalent dimer (disulfide-linked W242C), thought to mimic the membrane-induced dimeric structure. This particular mutant protein was made to see if formation of a covalent dimer linked around the putative dimer interface would enhance enzyme activity and vesicle binding. The W242C-dimer does indeed have about a two-fold enhanced activity towards PI in assay systems with PC (either a detergent such as diC7PC or mixed with PC in vesicles (14). This covalent dimer also binds very tightly to PC SUVs, so a comparison of exchange rates could also be made to wild type PI-PLC.

The peptide map of W242C covered over 80% of the whole sequence (Figure 6.13). Unfortunately, the H/D exchange information in the area around the disulfide is missing in the peptide map. Nonetheless, the rest of the peptide could be analyzed. Several peptides in the W242C dimer exchange rapidly in the absence of SUVs. Peptide 9-26 which is a loop connected to the N-terminal helix shows time-dependent H/D exchange with the amount of deuteration increasing from 50% at 20 s to over 80% at 3000 s. This particular peptide is not observed in wild type PI-PLC, but the N-terminal portion of wild type does exchange rapidly. The peptide loop 112-122 connecting β-sheet III and helix E also exchanges rapidly (this peptide was also not observed in wild type enzyme). Although the dimer interface region peptide 230-249 is missing, the peptides adjacent to it, 216-229 and 250-260, are exchanging rapidly – to a much greater extent than for the wild type protein free in solution. This indicates there are no further inter-molecular associations beyond the disulfide-bonded area. The peptides 196-205 (in helix F) and 276-285 (helix G) also exhibit increased exchange when compared to the same regions in
wild type protein. Most of the other peptides exhibit exchange behavior similar to what is observed for wild type protein. In particular, peptide 36-49, containing the important membrane binding residue Trp47, exchanges slowly, suggesting the helix B is well structured in the W242C-dimer. Since in the W47A/W242A dimer helix B is destabilized and several side chains in the area (Pro42 and Asn46) used to stabilize the crystallographic dimer, the covalent W242C dimer must have a significantly different structure.

W242C WITHOUT LIPIDS

W242C WITHOUT LIPIDS

The PI-P1 exchange behavior similar to what is observed for wild type protein. In particular, peptide 36-49, containing the important membrane binding residue Trp47, exchanges slowly, suggesting the helix B is well structured in the W242C-dimer. Since in the W47A/W242A dimer helix B is destabilized and several side chains in the area (Pro42 and Asn46) used to stabilize the crystallographic dimer, the covalent W242C dimer must have a significantly different structure.

W242C WITHOUT LIPIDS
Since the W242C dimer binds to lipids tightly, H/D exchange rates for the W242C dimer binding to 4 mM POPC/DOPG (9:1) were also examined (Figure 6.14). The only region not significantly protected from exchange was peptide 112-122, containing Lys115, an active site residue. This peptide was not observed in wild type protein. Peptide 216-230 was the only region where protection was not as effective as for wild type protein binding to vesicles. This peptide was also the only one that showed increased deuterium incorporation over time. For wild type PI-PLC binding to SUVs, two other regions still exhibited fast exchange – peptides 91-110 and 182-195. Both these regions show dramatically reduced H/D exchange in the covalent dimer.
Figures 6.15 and 6.16 provide views of H/D exchange mapped onto the structure of W47A/W242A, the dimer we thought W242C might mimic. Although the 240’s loop and most of the Tyr strip are not seen, covalent linkage of the two monomers must distort the secondary structures around this point since peptides adjacent to these features show very fast deuterium incorporation in the absence of SUVs. However, once the protein binds to the SUV surface, solvent exposure is decreased more than for any other system.
If we compare free W242C dimer in solution with wild type PI-PLC in solution (Figure 6.17), the overall structure of W242C is more exchangeable than wild type. Despite the missing 240’s loop, short helix B remains protected, but β-sheet VI, helix G and several loops exchange more rapidly than in wild type PI-PLC. In the MD simulation carried out by Dr. Wintrode (unpublished results), long helix G in solution oscillates between a straight and kinked form at Pro254. In the W242C dimer, the two monomers are connected by disulfide bond in the 240’s loop. This may impede the helix motion and introduce sufficient strain that local (and presumably transient) helix unwinding occurs. If this region is more flexible it may also bind better to membranes. Once bound (Figure 6.18), the W242C dimer is more structured than free in solution and more stable than wild type PL-PLC on SUVs surface. This correlates with tighter $K_d$ for W242C-dimer binding to PC SUVs.
Fig. 6.17. H/D exchange comparison of wild type PI-PLC and W242C in solution.

w.t. PI-PLC with 9:1 POPC/DOPG

W242C with 9:1 POPC/DOPG

9:1
Figure 6.19 shows the time-dependence of H/D exchange for selected peptide in the W242C dimer. Deuterium exchange levels change less than 30% from 20 to 3000s with the change occurring mostly within the first 1000 s.
In summary, many of the results from the HXMS experiments are consistent with previous studies on which parts of the protein interact with membranes. In the wild type PI-PLC peptide map, residues 38-55 containing helix B exchange slowly while peptide 232-251 is very accessible. This indicates helix B is a stable secondary structure element in solution; the loop would be expected to exchange rapidly and does. Since Trp47 and Trp242 are important for membrane binding, the peptides containing those residues would be expected to show slow exchange with vesicles present – and this is indeed observed. In the W47A/W242A mutant, peptide 38-55 shows very rapid exchange suggesting the short helix unwinds and becomes a loop as is observed in the crystal structure of this mutant protein. Thus, Trp47 (and its packing with Ile43) is necessary for the stability of this short surface helix even when no membranes are present.

Both mutant proteins, W47A/W242A and the W242C disulfide-linked dimer, have much higher H/D exchange than wild type protein in solution. This is reminiscent of older $^1$H NMR studies by the Tsai group that showed small perturbations of some active site residues had a large effect on overall protein stability([15]). However, the presence of membranes had a dramatic effect on stabilization of the W242C dimer. Even regions that still exchanged in wild type bound to membranes were not accessible in the covalent dimer bound to vesicles. The further reduced H/D exchange for membrane bound W242C dimer correlates with the higher activity and lower $K_d$ of this homodimer.

HXMS was initially started to evaluate the possibility of dimer formation when PI-PLC binds to membranes. However, the reduced deuterium exchange rate of peptide 232-251 in wild type PI-PLC upon binding to lipids is consistent with dimer formation but also with this region involved in PC binding. H/D exchange in this peptide was more useful in assessing dimerization.
of W47A/W242A in solution. The time dependent increase in deuterium indicates there may be an equilibrium of dimer and monomer in solution. Peptide 232-251 is missing in W242C dimer peptide map, but the regions next to it exhibit very fast exchange for the covalent dimer free in solution. Therefore, this dimer cannot be exactly like the W47A/W242A dimer. The reduced exchange around the 232-251 peptide when the the W242C dimer is bound to membranes does suggest that this structure is likely to reflect the activated form of PI-PLC.
References


Chapter VII: Future Directions
Results summary:

The work in this thesis brings insights into bacterial PI-PLC conformational changes upon binding to membranes. The *B. thuringiensis* PI-PLC folds as a single domain and is a monomer in aqueous solution (1). The crystal structure of a double mutant of PI-PLC, W47A/W242A, was solved (2) and showed the protein as a homodimer. The major interactions (80% of the dimer interface area) came from four clustered surface tyrosine residues (which we call the ‘Tyr strip’) from each monomer with the rest (20%) coming from hydrogen bonds. This structure was the first observed dimer structure in the bacterial PI-PLC family and suggested a possibility of PI-PLC dimerization on membrane surfaces as part of the mechanism for interfacial activation.

To further explore the role of the Tyr strip in PI-PLC structure and functions, three mutant proteins, Y247S/Y251S, Y246S/Y247S/Y248S, and Y246S/Y247S/Y248S/Y251S, were constructed. Binding affinities and specific activities as well as surface dilution behavior of these mutants were tested. Crystal structures of Y247S/Y251S in either the presence or absence of myo-inositol and Y246S/Y247S/Y248S/Y251S were also solved. We found that removal of three or four of these tyrosine residues greatly reduced the enzyme binding affinity for PC vesicles. Results from kinetic assays showed decreased activities of the mutants correlating with the reduced affinity for PC SUVs. Crystal structures of the mutants with or without substrate inositol ring showed there were minor structural changes in either overall structure or active sites. So structural changes in the protein were not the reason that led to the loss of PC binding affinity or reduced specific activities. The disruption of dimer formation on vesicles may be a reasonable explanation for the non-efficient functioning of these PI-PLC Tyr mutant proteins.
FRET was used to try and monitor the oligomer state of PI-PLC on membrane. Position 280 in PI-PLC was chosen for fluorophore attachment and W280C made for derivatization with fluorophores. NBD-PLC and Rho-PLC were prepared and used as the donor-acceptor pair. FRET under various lipids ratios, lipids concentrations and NBD-PLC concentrations were tested. If a dimer with a structure like that in the W47A/W242A dimer structure formed, the distance between donor and acceptor should be around 10Å which is much less than the Forster distance of this fluorophore pair (50Å). The results showed FRET occurred between the donor and acceptor, but it required quite a few Rho-PLC molecules and the FRET was rarely 100%, strongly suggesting that a stable dimer was not forming in these experiments. With 1:10 NBD-PLC to Rho-PLC, the maximum FRET is under 60% which indicated the dimer structure is not very stable. Variation of lipid mixture ratios would lower the FRET and require more acceptor molecules, which again suggested that any dimer structure on vesicle surfaces should be transient.

To examine which portions of the PI-PLC are interacting with membrane and to further explore if there is any evidence for PI-PLC dimerization on membrane surface, deuterium exchange coupled by mass spectrometry experiments were done on wild type PI-PLC, W47A/W242A and the W242C covalent dimer. The HXMS experiments provided interesting insights into this bacterial PI-PLC. The stability of short helix B in wild type PI-PLC and its complete and rapid H/D exchange in W47A/W242C confirmed that (i) in solution, as in the W47A/W242A crystal structure, the helix B peptide is no longer helical, and (ii) Trp47 is critical for stabilization of this unusual helix with its solvent exposed hydrophobic side chains. The reduced deuterium exchange for peptide 230-251 in wild type PI-PLC in the presence of vesicles and the time dependent exchange for the same peptide in W47A/W242A are consistent with
transient dimer formation upon vesicle binding (and enhanced dimer formation in solution for W47A/W242A). Furthermore, the reduced deuterium exchange of peptides connecting to Tyr strips in the W242C covalent dimer when bound to vesicles suggested a possible transient dimer structure that differs from the observed W47A/W242A crystal dimer. Of course, the same protection experiments might also be consistent with the Tyr strip providing a binding site for PC molecules. Discrimination between these two possibilities is exceedingly difficult.

**Future directions:**

The emergence of the Tyr strip as a key feature in PC binding suggests there may be other surface areas where Tyr are clustered that may be relevant to membrane binding. There are two other Tyr residues in the region around Tyr246, Tyr247, Tyr248, and Tyr251. Tyr204 and Tyr275 should be changed to serine residues to see if these residues contribute to lipid binding or kinetic behavior. Although these two residues are involved in the dimer formation in the W47A/W242A crystal, they may be important in membrane insertion of wild type PI-PLC.

Other non-active site conformational features of the protein may contribute to interfacial behavior. According to the MD simulation carried out by Prof. Patrick Wintrode, long helix G is flickers between bent and straight forms with the ‘hinge’ for this movement Pro254. Consistent with such dynamics is the HXMS data for the wild type and for W47A/W242A in solution where peptide 250-260 shows little exchange but the previous region, which includes the 240s loop and also the initial part of helix G, is exchanges with D$_2$O very rapidly. In the W242C covalent dimer in solution, much more of helix G (peptide 250-260) is accessible to H/D exchange. The accessibility to D$_2$O is lost once the covalent dimer binds to vesicles. This information might suggest that helix G conformational changes are important for vesicle binding and activity.
Pro254 is at the pivot position, so a vesicle binding and kinetic study for P254A mutant would be a good place to start. Another region highlighted by the HXMS data includes β-sheets V and VI and α-helix D. β-sheets V and VI form part of the β-barrel and are therefore near the active site. They are unique in lacking surrounding/facing helices. The lack of a stabilizing helix on the outside of this segment of the barrel (β) suggests these strands should be more flexible and could be solvent exchangeable under different conditions. These features show enhanced exchange with vesicles present. One hypothesis to test is that occupation of the active site might cause changes in the barrel that strain the sheet hydrogen bonds and thus make these residues more exchangeable. Trying H/D exchange experiments in the presence of high concentrations of soluble molecules of different complexities to try and mimic substrate (myo-inositol, I-1-P, diC₄PG, 1-hexanoyl-PG, etc.) occupying the active might also cause increased exchange and should be tested. Helix D is further away from the active site and why its H/D exchange is enhanced in the presence of SUVs is unclear. Helix D (-TLHEFINEAKQFLKDN-) is not completely an amphiphatic helix as might be expected for one facing the strands of the β-barrel. Mutation of residues with side chains facing away from the barrel interior might be a good place to start. Changes in vesicle binding and enzyme activity might help us form a hypothesis for why this secondary structure feature exhibits enhanced exchange in the presence of membranes. In some HXMS data, the N-terminus became more structured after binding to vesicles. The N-terminus contains a short helix and should be quite flexible even when the protein is bound to a membrane. This is another case where protection in H/D experiments is interesting. Perhaps when the protein is bound to SUVs, the loop region between αA and β1 adopts a more defined and protected structure. Mutations introduced in this area would also be good targets.
Crystal structures of selected mutant proteins could directly provide useful information. The W242C covalent dimer exhibited higher PC binding affinity and increased enzymatic activity in assay systems with PC present. In the energy minimization, the 240’s loop extended further away from the backbone core and became more exposed, consistent with my HXMS data. A crystal structure of this dimer might provide an explanation for the enhanced function. A complementary structure would be that of the covalent N243C dimer, which is inhibited compared to uncrosslinked protein. A comparison of the two might provide further insight into whether (and which) dimers are critical for activation.

HXMS could be used for further analysis the oligomerization state of PI-PLC in either solution or on a membrane. Trp242 and the Tyr strip (Tyr246/Tyr247/Tyr248/Tyr251) are within the same peptide in pepsin digestion. However, a detailed analysis of deuterium exchange is needed in this region to try and separate the surface loop from the Tyr strip in helix G. If we insert a pepsin cleavage site (some aromatic residues) in between W242 and Y246 that do not change the secondary structure in this area, then two separate peptides, one containing the 240’s loop and the other the Tyr strip, should be obtained. This may allow us to separate dimer formation from membrane binding, although that would be difficult if the two are linked. Protein-substrate binding using soluble substrate analogs would also be interesting – does the H/D exchange rate increase for helix D and βV/βVI region when these molecules occupy the active site. For protein-membrane binding, further variation of XPC may provide insight into how the surface composition (and vesicles binding, which was previously measured by FCS) affects H/D exchange rates. We already analyzed PI-PLC mutants binding to XPC = 0.1 and 0.8 PC/PG SUVs. Experiments with XPC= 0.5 and 0.2 would be quite useful, H/D? exchange could even be done with 9:1 PC/PI – during the preincubation with the enzyme, the PI should be
almost completely hydrolyzed to DAG. While some vesicle fusion will occur, this at least should provide some information on what happens when DAG is present in membranes – does this weaken binding or change interactions to some degree. Other interesting mutated proteins could be examined. For example, Y247S/Y251S, which has two residues in the Tyr strip removed. This one might be particularly useful if we can separate the 240s loop from Helix G in pepsin cleavage.
References


Appendix I: Expression and purification of Akt1 PH domain

The plasmid was transformed into appropriate competent cells (BL21-Codonplus (DE3)-RIL) by the heat-shock method (42 °C for 30 seconds). One colony was inoculated into a 5 ml culture tube, which was incubated overnight at 37°C with constant shaking in the presence of 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. A 5 ml aliquot of the overnight culture was added to 2 L of LB media. The newly inoculated media was then incubated at 37°C in the presence of 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. When the O.D.₆₀₀ was between 0.6 to 1.0, IPTG was added to a final concentration of 1.0 mM, and the culture was incubated overnight at 16°C. The cells were then harvested by centrifugation at 5000 rpm for around 15 min and frozen overnight. The cell pellets were dissolved in PBS buffer, pH 7.3, and lysed by sonication on ice for 5 minutes. Cell debris was pelleted using centrifugation (30 min at 15,000 rpm). The resulting lysate was applied to a glutathione Sepharose 4B (Amersham-Pharmacia Biotech) column that had been washed with PBS. After the binding step, the resin was washed several times with PBS containing 1% Triton X-100, then with 20 mM Tris–HCl, pH 8. After transferring the resin into a column, it was further washed with the same buffer with 0.7 μg/mL pepstatin A, and 0.2 μg/mL aprotinin (optional)(According to the Roche website, aprotinin does not inhibit thrombin). The fusion protein was cleaved with thrombin (Roche) from the resin. The enzymatic reaction was carried out at 4 °C overnight. Then the protein was eluted and the resin was washed several times with PBS for at least 4 times. PMSF was added to 0.25 mM to the fractions to terminate the enzymatic cleavage. The collected fragments were passed through a filter to remove remaining resin. The flowthough fraction contained the desired protein, while impurities appeared to be retained on the resin. Before measurements, the protein solutions were dialyzed to exchange buffers.
Appendix II: Expression and purification of PLC δ1 EF hand domain

The EF-hand fragment was expressed as a fusion protein in BL21(DE3) E. coli (Stratagene). The plasmid was transformed into appropriate competent cells (BL21-Codonplus (DE3)-RIL) by the heat-shock method (42 °C for 30 seconds). One colony was inoculated into a 5 ml culture tube, which was incubated overnight at 37°C with constant shaking in the presence of 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. A 5 ml aliquot of the overnight culture was added to 2 L of LB media. The newly inoculated media was then incubated at 37°C in the presence of 34 μg/ml chloroamphenicol and 50 μg/ml ampicillin. When the O.D.600 was between 0.6 to 1.0, IPTG was added to a final concentration of 1.0 mM, and the culture was incubated overnight at 16°C. The cells were then harvested by centrifugation at 5000 rpm for around 15 min and frozen overnight. The cell pellets were dissolved in PBS buffer, pH 7.3, and lysed by sonication on ice for 5 minutes. Cell debris was pelleted using centrifugation (30 min at 15,000 rpm). The resulting lysate was applied to a glutathione Sepharose 4B (Amersham-Pharmacia Biotech) column that had been washed with PBS. After the binding step, the resin was washed several times with PBS buffer, pH 7.3, containing 0.25% Fos-Choline, then with 20 mM Tris–HCl, pH 8. After transferring the resin into a column, it was further washed with the same buffer with 0.7 μg/mL pepstatin A, and 0.2 μg/mL aprotinin (optional)(According to the Roche website, aprotinin does not inhibit thrombin). The fusion protein was cleaved with thrombin (Roche) from the resin. The enzymatic reaction was carried out at 4 °C overnight. Then the protein was eluted and the resin was washed several times with PBS for at least 4 times. The collected fragments were passed through a filter to remove remaining resin. The flowthrough fraction contained the desired protein, while impurities appeared to be retained on the resin. Before measurements, the protein solutions were dialyzed to exchange buffers.