I. Understanding Membrane Interactions of Bacterial Exoproteins; II. Identification and Characterization of a Novel Mammalian cis-Aconitate Decarboxylase

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Boston College

The Graduate School of Arts and Sciences

Department of Chemistry

I. UNDERSTANDING MEMBRANE INTERACTIONS OF BACTERIAL EXOPROTEINS; II. IDENTIFICATION AND CHARACTERIZATION OF A NOVEL MAMMALIAN *CIS*-ACONITATE DECARBOXYLASE

A Dissertation

by

JIONGJIA CHENG

Submitted in partial fulfillment of the requirements

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I. Understanding Membrane Interactions of Bacterial Exoproteins; II. Identification and Characterization of a Novel Mammalian

cis-Aconitate Decarboxylase

Jiongjia Cheng

Under the direction of Dr. Mary F. Roberts

ABSTRACT

Secreted phosphatidylinositol-specific phospholipase Cs (PI-PLCs) are often virulence factors in pathogenic bacteria. Understanding how these enzymes interact with target membranes may provide novel methods to control bacterial infections. In this work, two typical PI-PLC enzymes, from *Bacillus thuringiensis* (*Bt*) and *Staphylococcus aureus* (*Sa*), were studied and their membrane binding properties were examined and correlated with enzymatic activity.

*Bt*PI-PLC is kinetically activated by allosteric binding of a phosphatidylcholine (PC) molecule. MD simulations of the protein in solution suggested correlated loop and helix motions around the active site could regulate *Bt*PI-PLC activity. Vesicle binding and enzymatic studies of variants of two proline residues, Pro245 and Pro254, that were associated with these motions showed that loss of the correlated motions between the two halves of PI-PLC were more critical for enzymatic activity than for vesicle binding. Furthermore, loss of enzyme activity could be rescued to a large extent with PC present

in a vesicle. This suggests that binding to PC changes the enzyme conformation to keep the active site accessible.

SaPI-PLC shows 41.3% sequence similarity with *Bt*PI-PLC but has very different ways its activity is regulated. While it is kinetically activated by PC it does not in fact bind to that phospholipid. Enzymatic and membrane interaction assays showed that SaPI-PLC has evolved a complex, apparently unique way to control its access to PI or GPI-anchored substrate. (i) An intramolecular cation- π latch facilitates soluble product release under acidic conditions without dissociation from the membrane. (ii) There is a cationic pocket on the surface of enzyme that likely modulates the location of the protein. (iii) Dimerization of protein is enhanced in membranes containing phosphatidylcholine (PC), which acts not by specifically binding to the protein, but by reducing anionic lipid interactions with the cationic pocket that stabilizes monomeric protein. SaPI-PLC activity is modulated by competition between binding of soluble anions or anionic lipids to the cationic sensor and transient dimerization on the membrane depleted in anionic phospholipids. This protein also served as a way to test the hypothesis that a cation- π box provides for PC recognition site. This structural motif was engineered into SaPI-PLC by forming N254Y/H258Y. This variant selectively binds PC-enriched vesicles and the enzyme binding behavior mimics that of *Bt*PI-PLC.

Itaconic acid (ITA) is a metabolite synthesized in macrophages and related cell lines by a *cis*-aconitate decarboxylase (cADC). cADC activity is dramatically increased upon macrophage stimulation. In this work, the cell line RAW264.7 was used to show that cADC activity upon stimulation requires *de novo* protein synthesis. MS analyses of partially purified RAW264.7 protein extracts from stimulated cells show a large increase for immunoresponsive gene 1 protein (IRG1) and siRNA knockdown of the IRG1 reduces cADC activity upon stimulation. Suspected active site residues of IRG1 were identified by mutagenesis studies of the recombinant protein based on a homology structure model of fungal cADC. The cloning and overexpression of this enzyme should help clarify the cofactor-independent decarboxylation mechanism of this mammalian enzyme as well as open up future studies into the specific role of ITA in the mammalian immune system and cancers.

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Abbreviations

A. terreus	Aspergillus terreus
ActD	actinomycin D
AF488-Cys	Alexa Fluor 488 C5 maleimide
AF488-N-term	Alexa Fluor 488 carboxylic acid, succinimidyl ester
APS	ammonium persulfate
B. cereus	Bacillus cereus
B. thuringiensis	Bacillus thuringiensis
BSA	bovine serum albumin
BtPI-PLC	Bacillus thuringiensis phosphatidylinositol-specific
	phospholipase C
C2	protein kinase C conserved region 2
cADC	cis-aconitate decarboxylase
CBD	chitin binding domain
CD	circular dichroism
cDNA	complementary deoxyribonucleic acid
cIP	inositol 1,2-cyclic phosphate
СМС	critical micelle concentration
CNS	central nervous system
СоА	coenzyme A
CSA	chemical shift anisotropy

D_2O	deuterium oxide
DAG	diacylglycerol
DC	detergent compatible
diC ₆ PC	dihexanoyl-phosphatidylcholine
diC ₇ PC	diheptanoyl-phosphatidylcholine
diC ₈ PE	dioctanoyl-phosphatidylethanolamine
diC ₈ PG	dioctanoyl-phosphatidyglycerol
diC ₈ PS	dioctanoyl-phosphatidylseine
DLS	dynamic light scattering
DMEM	Dulbecco's minimal essential medium
DMPC	1,2-dimyristoyl-phosphocholine
DMSO	dimethyl sulfoxide
DOPA	1,2-dioleoyl-phosphatidic acid
DOPG	1,2-dioleoyl-phosphatidyglycerol
DOPMe	1,2-dioleoyl-phosphatidylmethanol
DOPS	1,2-dioleoyl-phosphatidylserine
dsRNA	double-stranded RNA
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor

FBS	fetal bovine serum
fc-P-NMR	high resolution field cycling ³¹ P NMR
FCS	fluorescence correlation spectroscopy
f _{max}	apparent maximum fraction bound
FRET	fluorescence resonance energy transfer
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	hydrophobic interaction chromatography
His-tag	poly histidine tag
I-1-P	inositol 1-phosphate
IDS	iminodisuccinate
IFN-γ	interferon γ
IMPDH	inosine monophosphate dehydrogenase
IPTG	isopropyl β -D-1-thiogalactopyranoside
IRG1	immunoresponsive gene 1
ITA	itaconic acid
K _d	apparent dissociation constant
K _m	Michaelis constant
L. monocytogenes	Listeria monocytogenes
LAB	lactic acid bacteria
LP	lipopeptide or lipoprotein

LPS	lipopolysaccharide
MBP	maltose-binding protein
MD	molecular dynamics
MES	2-(N-morpholino) ethane-sulfonic acid
MS	mass spectrometry
MSA	multiple sequence alignment
MTSL	1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethio-
	sulfonate
MyD88	myeloid differentiation factor-88
NAD^+	β -nicotinamide adenine dinucleotide hydrate
NF-κB	nuclear factor κ -light-chain-enhancer of activated B cells
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
NOS	nitric oxide
O.D.	optical density
ORF	open reading frame
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCA	principal component analyses
PCR	polymerase chain reaction
PE	phosphatidylethanolamine

PG	phosphatidylglycerol
РН	pleckstrin homology
pI	isoelectric point
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase
PIA	phosphatidylinositol analog
PI-PLC	phosphatidylinositol-specific phospholipase C
PIP _n	phosphoinositide
РКС	protein kinase C
PLA	phospholipase A
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PLP	pyridoxal 5'-phosphate
РМе	phosphatidylmethanol
PMSF	phenylmethylsulfonyl fluoride
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
PRs	progesterone receptors
PS	phosphatidylserine
QFF	Q-Sepharose Fast Flow
r.t.	room temperature
RMSF	root mean square fluctuation

R.S.A.	relative specific activity
S. aureus	Staphylococcus aureus
S.D.	standard deviations
SaPI-PLC	Staphylococcus aureus phosphatidylinositol-specific
	phospholipase C
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
siRNA	small interfering ribonucleic acid
SUV	small unilamellar vesicle
TCA	tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamide
TIM	triosephosphate isomerase
TLRs	Toll-like receptors
T _m	melting temperature
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
TPP	thiamine pyrophosphate
TX-100	Triton X-100
V _{max}	maximal rate in Michaelis–Menten kinetics
WT	wild type
X _{PC}	mole fraction of PC

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Chapter 1

Introduction

1.1 Bacterial Phosphatidylinositol-specific Phospholipase C (PI-PLC)

1.1.1 Phospholipids

The bilayer matrix of cellular membranes, formed by amphipathic lipids, enables the regulation of cell segregation, cell division, intracellular membrane trafficking, protein aggregation and dissociation. Specific lipids also play important roles in signaling pathways [1]. There are three major classes of amphipathic lipids: phospholipids, glycolipids, and steroids. The lipid composition of different membranes varies throughout the cell, but phospholipids are usually the most abundant class, comprising 20-80% of the membrane lipid mass [1,2]. In eukaryotic membranes, the major phospholipids are the glycerophospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidic acid (PA) (Fig. 1-1) [2]. Phospholipids are composed of a hydrophilic headgroup and hydrophobic acyl chains. The different polar head groups linked by the glycerophosphate moiety generate phospholipids that are either zwitterionic or anionic (Fig. 1-1, bottom table).

Phospholipids can form a variety of aggregates in aqueous solution (Fig. 1-2) because of their amphipathic character [3]. At the air-solution interface, lipids form monolayers with the polar headgroups in the aqueous solution and the acyl chains in the more hydrophobic air. The structures of phospholipid aggregates differ based on different acyl chain lengths and lipid concentrations [4]. For all lipids there is a threshold concentration (termed the critical micelle concentration or CMC) below which they exist as monomers in the aqueous phase. If the acyl chains are moderately long, the molecules aggregate and form bilayer vesicles. Sizes of vesicles range from 20 nm to 5 μ m in diameter. When the lipids are hydrated the large vesicles encapsulate many other vesicles. Unilamellar vesicles, simpler models for cell membranes, require energy to form from than multilamellar vesicles. Small unilamellar vesicles (SUVs) are an easily prepared model system that are highly curved with 20–50 nm diameters; these are easily prepared by sonication [5]. Many exogenous factors, for example metal ions (Ca²⁺) or lipid binding or cleavage proteins (phospholipase), can trigger vesicle fusion [6]. If acyl chain lengths are short (e.g., dihexanoyl-phosphatidylcholine, diC₆PC) or the polar head groups are very large (e.g., ganglioside GM1) the lipid molecules aggregate to form micelles. Micelles do not have an encapsulated aqueous compartment. Both the micelle and vesicle systems are widely used as models to study protein-membrane interactions and other cell processes that have membrane bound constituents.

1.1.2 Enzymatic Mechanism of PI-PLCs

Phospholipases catalyze the hydrolysis of the four ester linkages of phospholipids to generate either two different lipids or a new lipid and a polar molecule. These enzymes are classified into four families (PLA, PLB, PLC and PLD) depending on the ester bond whose cleavage they catalyze (Fig. 1-3A). PLA₁ and PLA₂ specifically hydrolyze the fatty acyl ester bond at the sn-1 and sn-2 position of the glycerol moiety; PLB enzymes possess the ability to sequentially remove both of the sn-1 and sn-2 acyl chains. PLC enzymes catalyze the cleavage of glycerophosphate ester bond and PLD cleaves terminal phosphodiester bond.



Figure 1-1. General phospholipid structure with head group list in bottom table.





Phosphatidylinositol (PI) is a relatively minor phospholipid component located in the cytosolic side of eukaryotic plasma membranes [2]. PI molecules phosphorylated on C3, C4 and C5 of the inositol ring, the phosphoinositides (PIP_n), are important in signaling pathways with several of these specific second messengers [7,8]. Phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes are a large family of phospholipases that specifically catalyze the hydrolysis of polar head group of phosphoinositide substrates. The substrates for bacterial PI-PLC enzymes are PI and glycosylphosphatidylinositol (GPI), while most mammalian enzymes prefer PIP_n over PI. The scheme for bacterial PI-PLC enzymatic mechanism is shown in Fig. 1-3B. These enzymes use a general acid and base mechanism. Bacterial PI-PLC cleaves its substrate via an intramolecular phosphotransferase reaction on the PI moiety to form a cyclic inositol phosphate molecule (inositol 1,2-cyclic phosphate, cIP) and diacylglycerol (DAG) [9]. The second step carried out by the enzyme is a phosphodiesterase reaction that further hydrolyzes the water-soluble intermediate cIP to inositol-1-phosphate (I-1-P) [10,11].

Bacterial PI-PLC is a virulence factor produced and secreted by many Gram-positive bacteria [12], including the pathogens *Bacillus cereus, Bacillus thuringiensis, Bacillus anthracis, Listeria monocytogenes, Listeria ivanovii, Staphylococcus aureus, Clostridium novyi, Rhodococcus equii,* and also non-pathogenic species *Listeria seeligeri, Streptomyces antibioticus, Cytophaga sp.*, and some strains of lactic acid bacteria (LAB) such as *Lactobacillus rhamnosus* [12-18]. Along with the cleavage of nonphosphorylated PI, most of the PI-PLCs secreted by extracellular pathogens (e.g. *Bacillus* and *Staphylococcus* strains) can also cleave GPI anchors [12] to release proteins that are

tethered to the exterior surface of mammalian cell membrane by these anchors. This process causes cellular damage and one of the products, DAG, can translocate across the bilayer [19] to interfere with intracellular signaling processes [20].

B. thuringiensis is an insect pathogen, and earlier work showed that an avirulent mutant lacking both the broad range and PI-specific PLC activities was much less potent in killing cells [21]. This indicates the PI-PLC enzyme can have significant roles in regulating the virulence of pathogens. *S. aureus* is known to cause a broad range of infections in humans and is commonly found in skin infections and abscesses. A *S. aureus* infection can lead to toxemia and lethal bacteremia [20,22]. Secreted *S. aureus* PI-PLC may aid in colonization and replication of the microorganism by generating DAG from GPI-anchored proteins. The production of this PI-PLC was shown to be upregulated upon infection in community-associated methicillin-resistance of this bacterium [23]. This kind of PI-PLC usually exhibits significant activity toward PI in an acidic environment [24].

1.1.3 Mammalian and Bacterial PI-PLC Structures

At present, there are six families of mammalian PI-PLC enzymes with thirteen distinct isoforms: PLC- $\beta_{(1-4)}$, PLC- $\gamma_{(1-2)}$, PLC- $\delta_{(1,3,4)}$, PLC- ϵ , PLC- ζ , and PLC- η (Fig. 1-4) [25]. These PLC isoforms share four EF-hand motifs (usually associated with specific Ca²⁺ binding), the X and Y catalytic domains, a C-terminal C2 domain for calcium-dependent membrane targeting and a N-terminal PH domain (which is not present in PLC- ζ) to localize the enzymes to their substrate in the membrane and also to interact with other signaling components [26,27].

<u>Figure 1-3.</u> The enzymatic mechanism of phospholipases. (A) Cleavage sites for phospholipases are indicated. (B) The mechanism of the two sequentially reactions catalyzed by bacterial PI-PLC is shown.





Figure 1-4. Domain organization of PI-PLC isoforms.

In contrast to their multi-domain eukaryotic relatives, the bacterial PI-PLCs contain only the catalytic domain (homologous to the X-Y domains of mammalian PLC enzymes) that is responsible for both catalysis and membrane binding. Although PI-PLC catalytic domains are members of the triosephosphate isomerase (TIM) barrel (α/β)₈ superfamily [28,29] they lack a number of hydrogen-bonding interactions between βstrands, resulting in a relatively open β-barrel [28,29] (Fig. 1-5B). The conserved active site is located at the C-terminal end of the β-strands in the barrel [28,29], but most of the residues that interact with membranes and control lipid binding in the bacterial enzymes are located in the less conserved and more mobile surface helices and loops.

The crystal structures of *B. thuringiensis* PI-PLC (Y247S/251S) and *B. cereus* PI-PLC are almost identical and highly similar to the catalytic domain of PLC- δ 1 [28,30]. Sequence similarity among the different bacterial PI-PLC enzymes is not exceptionally high. For example, *S. aureus* PI-PLC shows 41.3% identity with *B. thuringiensis* PI-PLC (Fig. 1-5A) in sequence. However, most of the secondary structure superimposes well with the *Bacillus* enzymes. Two key structural features of the bacterial enzymes are short helix B (containing Trp47 in *Bacillus* PI-PLC and Trp45 in *S. aureus* PI-PLC) and a mobile rim loop (containing Trp242 in *Bacillus* PI-PLC and Phe249 in *S. aureus* PI-PLC), both of which have been implicated in membrane binding [31-34]. In *S. aureus* PI-PLC, helix B is one residue longer than in the *B. cereus* structures and has two lysine residues to create a more positively charged feature. This can form an extended positively charged region when combined with the other positively charged residues in the barrel rim. The disposition of the key hydrophobic residue in the mobile rim loop can adopt

different orientations. For example, Phe249 in *S. aureus* PI-PLC is pointed outward and toward the membrane interface compared with the homologous side chain (Trp242) in *Bacillus* enzymes. In the latter the tryptophan residues are pointed away from the membrane interface. This stresses the flexibility of the rim loop [35].

1.1.4 Interfacial Activation of Bacterial PI-PLCs

The kinetic property 'interfacial activation' is observed for many peripheral enzymes, including phospholipases. 'Interfacial activation' is the sharp increase in enzyme (in this case phospholipase) activity when the substrate is presented in an interface as opposed to a monomer molecule [36]. For the catalytic process at interfaces, the enzyme in the solution initially binds to the interface consisted by aggregated substrate. The interfacial-associated enzyme likely undergoes some conformational changes that allow substrate to bind into the active site. Once the substrate is cleaved, the enzyme releases the products to regenerate free enzyme [37]. The overall rate of the catalytic reaction is thus affected not only by the kinetics of interfacial catalysis but also by the kinetics of binding between enzyme and interface. The concentration of substrate dispersed at an interface is characterized by both the bulk concentration and two-dimensional local concentration, with the latter often more important for the overall kinetic rate.

Several of the bacterial PI-PLC enzymes exhibit a specific interfacial activation by the zwitterionic phospholipid PC. The presence of this non-substrate phospholipid leads to PI cleavage with an enhanced k_{cat} and reduced apparent K_m toward aggregated PI compared to monomeric PI [10, 38-41]. Since PI at a surface is a better substrate, the kinetics of bacterial PI-PLC enzymes is usually explored in micelle or vesicle systems.
Figure 1-5. The comparison of sequences and structures of two typical bacterial PI-PLCs. (A) Sequence alignment of *B. thuringiensis* PI-PLC (*Bt*) and *S. aureus* PI-PLC (*Sa*). Active site residues – the two histindines are highlighted in red and other key residues are highlighted in yellow for conserved ones and in cyan for less conserved ones. (B) Structure of bacterial PI-PLC enzymes: (left) *B. thuringiensis* PI-PLC (PDB 1PTD) and (right) *S. aureus* PI-PLC (PDB 3V18). The *S. aureus* structure was obtained at pH 7.4. There is a structural change at lower pH values where an intramolecular Phe249 π /His258 cation latch is formed.

	10	20	30	40	50	60	70	80
Bt	ASSVNELENWS	KWMQPIPDNIPL	ARISIPGT <mark>H</mark> C	SGTFKLQNPI	KQV <mark>W</mark> GMTQEY	DFRYQMDHGAI	RIFDIRGRLTI	DDNTIVL
Sa	SDSLSKSPE	:::	:.::::: TEINIPGS <mark>H</mark> C	SGSFTLKDPV	:.: <mark>:</mark> . ::. ksv <mark>w</mark> aktQdk	DYLTQMKSGVI	 RFFDIRGRAS	ADNMISV
	10	20	30	40	50	60	70	
	90	100	110	120	130	140	15	50
Bt	H <mark>H</mark> GPLYLYVTLI	HEFINEAKQFLK	DNPSETIIMS	LKKEYEDMKG	AEGSFSSTFE	KNYFVDP	IFLKTEGN	IKLGDAR
Sa	::::::::::::::::::::::::::::::::::::::	.::: .:. GKFLDDAKYYLS	:.:::.:: AYPNETIVMS	.::	: :. VTKTFEEIFR	. :: EYYYNNPQYQI	: NLFYTGSNANI	: PTLKETK
	80 90	0 100	110	120	130	140	150	
D+	160 CKINII KDXCC	170	180	190	200	210	220	230
ЫС	GKIVLLKKISG.	•••	• •• ••	· · · ·	••••	IDERVRSIRD.	·	• •
Sa	GKIVLFNRMGG	 TYIKSGYGADTS	GIQWADNATF	'ETKINNGSLN	LKVQDEYKDY	YDKKVEAVKNI	LLAKAKTDSNI	KDN-VYV
	160 170) 180	190	200	210	220	230	
	240	250	260	270	280	290		
Bt	NFTSLSSGGTA	NSP <mark>YYY</mark> AS <mark>Y</mark> IN	PEIANDIKQK	NPTRVGWVIQ	DYINEKWSPL	LYQEVIRAN	NKSLIKE	
Sa	II IIII.I NFLSVASGGSA	.:: <mark>:</mark> :::.:: NST <mark>YNY</mark> AS <mark>H</mark> IN	PEIAKTIKAN	GKARTGWLIV	<pre>:: . : DYAGYTWPGY 290</pre>	DDIVSEIIDS	:: NK	A
		200	270	200	230	000		



Adding more nonsubstrate lipids dilutes the interfacial substrate concentration. If the total concentration of substrate is constant but its surface concentration decreases, enzyme specific activity often decreases. This phenomenon is called "surface dilution inhibition" [42,43]. The bacterial PI-PLCs also exhibit "scooting mode catalysis", where enzyme completes several rounds of substrate turnover at the substrate interface before dissociating from the particle [41]. Scooting mode catalysis becomes more difficult as the surface concentration of the substrate decreases.

B. thuringiensis PI-PLC, like other PI-PLC enzymes, catalyzes the specific cleavage of PI in two steps. The unique property of B. thuringiensis (and B. cereus) enzyme is that both of these steps are specifically activated by PI-PLC binding to PC interfaces, either in a micelle form or vesicle matrix. Two critical tryptophan residues, Trp47 in the helix B region and Trp242 in a disordered loop (Fig. 1-5B, left), are involved in the PC activation [31,32]. Considering the homodimer crystal structure of a *B. thuringiensis* PI-PLC mutant (W47A/W242A), the wild-type PI-PLC enzyme was suggested to be activated by forming a dimer at PC interface [44]. In the W47A/W242A structure, the major contribution to stabilizing the symmetric dimer interface is a central swath of aromatic residues (the 'Tyr strip' in Fig. 1-5B, left). These side chains are arranged in a quasiherringbone pattern. The W47A/W242A dimer structure was only observed in this interfacially impaired mutant, so that whether or not the protein does indeed form a dimer at membrane surfaces is unclear. Mutagenesis studies of these Tyr residues clearly showed a loss of activity and membrane binding affinity [30]. Since initially there was no information on where, if at all, a discrete PC molecule would bind to the PI-PLC, the proposed model for interfacial activation of *B. thuringiensis* PI-PLC suggests that the enzyme is a monomer in solution with an intact helix B to ensure Trp47 and Trp242 at the correct position for the initial binding. Helix B is then disrupted to assist in the active dimer forming at interface upon binding to the membrane [33]. However, more recent work combining high-resolution field-cycling ³¹P NMR relaxation experiments [34] with mutagenesis studies [30] showed that there was a discrete binding site for substrate and a separate one for a tightly bound PC (activator). The specific PC binding site was suggested to be rich in tyrosines that could form choline cation/Tyr π boxes or sandwiches. The tyrosine strip (Tyr246-247 and Tyr251 in Fig. 1-5B, left) near the membrane interface was proposed to have the PC recognition motif. However, all the evidence for the dimerization model and PC binding pocket were indirect requiring further experiments to validate one or both of these hypotheses.

The *Bacillus* PI-PLC enzymes are activated by strong binding to PC [10,34,38,39]. However, this is not a universal mechanism of interfacial activation for bacterial PI-PLCs. The enzyme from *Listeria monocytogenes* is activated by many noncharged or zwitterionic amphiphiles in a somewhat nonspecific fashion. For that enzyme, the diluting amphiphile prevents cationic enzyme/anionic lipid aggregation and the resulting sequestration of the enzyme in a nonproductive state [39]. This nonspecific activation mechanism also likely reduces penetration of the highly cationic protein into a negatively charged bilayer [10,38,39]. Consistent with this mode of PC activation, *L. monocytogenes* PI-PLC has very low affinity for PC small unilamellar vesicles (SUVs) but very high affinity for anionic phospholipid interfaces. When I started there was limited knowledge about the interfacial activation of PI-PLC from S. aureus.

1.1.5 Lipid Recognition Specificity

Cells are dynamic systems where spatially and temporally localized signaling facilitates responses to the local environment. Variations in lipid composition between different organelles and between organelles and the plasma membrane [1] provide spatial localization of peripheral membrane proteins that recognize specific lipids. For example, a number of proteins specifically recognize rare, anionic phosphoinositides with stereospecific recognition of the phosphoinositide headgroups [45,46]. For pleckstrin homology, PROPPIN β propellers, and other domains, stereospecificity often depends on a network of hydrogen bonds between the phosphoinositide headgroup and conserved basic residues [45-47]. Similarly, proteins specific to the anionic phospholipid phosphatidylserine (PS) may coordinate Ca^{2+} ions for PS binding using conserved loop motifs as observed in annexin V [48] or directly bind PS via C2 domains as in coagulation factor V [49] and lactadherin [50] where polar side chains allow specific recognition of PS. The affinity of the proteins for the membrane may be further modulated by insertion of hydrophobic and aromatic amino acids into the bilayer [31,48,49], and/or multivalent interactions [46] via domain repeats [51] or specificity for more than one type of lipid [10,51].

Although there are numerous examples of specific binding to anionic lipids, less is known about specificity for zwitterionic lipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Specific binding via a trimethylammonium moiety, such as that found in PC headgroups, has been observed in proteins that bind proline betaine and glycine betaine [52,53] or choline [54,55] as well as proteins that bind methylated lysine in histones [56-58] or methylated arginine [59]. In these structures, the methylammonium moiety is the center of a cation- π box with the faces of 2 to 4 aromatic residues located within 4–5 Å of the methylated amine allowing cation- π interactions with the aromatic residues. In search of the trimethylammonium binding motif in Protein Data Bank database, the only known structures containing PC with the choline held in a cation- π box are those for phosphatidylcholine transfer proteins. However, additional interactions with the acyl chains assist in PC binding by the transfer protein [60], and it was unclear whether the cation- π interaction by itself would provide sufficient binding energy to transiently bind a protein to the membrane. Further work was clearly needed to support the hypothesis that the cation- π box is an indeed motif for PC recognition.

1.2 cis-Aconitate Decarboxylase (cADC)

1.2.1 Metabolites and Macrophages

Small molecule metabolites can play important roles in cell growth and survival, amino acid and nucleotide biosynthesis, as well as energy production. In metabolism, the enzyme-catalyzed reactions, which change and transform small molecules in the cells, ensure the conversion of carbon and energy sources into usable metabolites. These endogenous molecules can also act as allosteric or competitive inhibitors in regulating expression and activity of related enzymes [61]. As the substrates and products of numerous metabolic reactions, the metabolites encompass a wide variety of chemical compounds, including organic acids, amino acids and amines, fatty acids and lipids,

sterols, vitamins, mono- and polysaccharides, as well as hormones [61]. The levels of a given metabolite vary depending on the availability and uptake of precursor molecules, enzyme activity, the number of pathways involved, and also fluctuated extracellular environment [62-64].

Macrophages are a type of differentiated monocyte that can be activated by diverse pathogen derived stimuli via numerous receptors. These processes will stimulate transcription of genes that regulate innate and adaptive immune responses. Such genes encode cytokines, chemokines, costimulatory molecules, and enzymes of cytokines and microbial products, leading the induction of an antimicrobial effector system [65,66]. These various endogenous and exogenous stimuli include cytokines secreted by immune cells. Such stimuli include interferon gamma (IFN- γ) and tumor necrosis factor (TNF), pathogen components like lipopolysaccharide (LPS), phagocytosed antigens, etc. [67-69] LPS usually stimulates the macrophage via binding to Toll-like receptors (TLRs), which is the most commonly used reagent in macrophage activation [65,70]. In the immune response, the activated macrophage can produce a large amount of reactive oxygen species or secret cytokines to kill invading microorganisms and maintain the proliferation and development of certain T cells [68,71,72]. The RAW264.7 tumor cell line is a widely used model for macrophages and has been used in studies of metabolism, macrophage activation and lipid synthesis [69,73,74]. This cell line was first isolated from a murine lymphocytic lymphoma induced by the Abelson leukemia virus [75]. It was found to be activated by LPS and produce nitric oxide (NOS) and tumor necrosis factor (TNF) [76,77].

1.2.2 Biosynthesis Pathway of Itaconic Acid (ITA) in Fungi

Itaconic acid (ITA) is a small-molecule effector of cells that has been overlooked in the biochemical community until recently. However, it has been very prominent in the field of polymers. ITA is an unsaturated dicarboxylic acid that is used for industrial synthesis of resins and bioactive compounds. By employing selective enzymatic transformations, ITA can also be used to create useful polyfunctional building blocks and was categorized as one of the "top 12" building block molecules from sugars [78]. ITA was originally discovered as a product of pyrolytic distillation of citric acid by Kinoshita (1932) in an osmophilic strain of green *Aspergillus species* [79]. After many attempts made to improve the economics of ITA manufacture, an optimized industrial process by using *A. terreus* was established. However, the economic and environmental circumstances around ITA production are still under investigation and development. Moreover, investigations into new properties of ITA have opened up possibilities for novel applications in the fields of polymer chemistry, pharmacy, and agriculture [80].

In the industrial production processes to generate ITA, sugars such as glucose and sucrose are the generally used starting materials. For several decades, the metabolic pathway for ITA production was hotly debated and was not fully established. It was not clear whether ITA was produced as an offshoot of the tricarboxylic acid (TCA) cycle, from citramalate or from the condensation of acetyl-CoA [81-85]. Among the several postulated biosynthetic pathways leading to ITA accumulation, the one via aconitase and *cis*-aconitate decarboxylase (cADC) (Fig. 1-6A) is generally supported [81,82]. ITA biosynthesis starts with glycolysis in the cytosol where glucose is metabolized to

pyruvate mainly via the Embden-Meyerhof-Parnas pathway (EMP) pathway. Pyruvate is then either transported to the mitochondria and dehydrogenated into acetyl-CoA, or carboxylated to oxaloacetate in the cytosol. Oxaloacetate is first converted to malate, and then converted back to oxaloacetate after being transported into mitochondria. In mitochondria, citrate synthase converts acetyl-CoA and oxaloacetate to citric acid. Further on in the TCA cycle, citrate is converted into *cis*-aconitate and transported back into cytosol and then decarboxylated to itaconate [81]. The formation of ITA involves the shuttling of intermediate metabolites between different intracellular compartments and utilizes the different enzymatic capabilities of the respective compartments [86]. ITA biosynthesis was analyzed by fractionating cell extracts to distinguish the enzymatic activity of a mitochondrial from a cytosolic enzyme.

1.2.3 cis-Aconitate Decarboxylase (cADC)

That ITA biosynthesis requires *de novo* protein synthesis in fungi confirmed the importance of enzymes such as aconitase and cADC. As an important step for the basic understanding of ITA biosynthesis, Dwiarti et al. purified a 55-kDa protein from the high ITA producing strain *A. terreus* TN484-M1 [87]. From partial sequencing of this protein, the responsible cADC gene was identified and this protein was shown to produce ITA from *cis*-aconitate [88]. Transcriptional approaches also supported this biosynthetic pathway [85]. cADC contains a conserved domain of the MmgE/PrpD family of proteins from bacteria and fungi, which includes several 2-methylcitrate dehydratases of bacteria that are involved in propionate catabolism.

<u>Figure 1-6.</u> Biosynthesis pathways of ITA. (A) Biosynthesis pathway of ITA in *Aspergillus terreus*. Metabolites are given in normal font and enzymes are given in italic. Dashed lines and arrows stand for the simplified steps [81,82,85]. (B) Biosynthesis pathway of ITA in murine macrophage. ITA labeling scheme from $[^{13}C_5]$ glutamine (I) and $[^{13}C_6]$ glucose (II and III) if the cADC pathway is active. PDH: pyruvate dehydrogenase; PC: pyruvate carboxylase.



The MmgE/PrpD family is a diverse class of proteins from a wide range of organisms, including archaea, bacteria and most eukaryotes. Based on the phylogenetic analysis, it can be divided into two major branches, one that includes the PrpDs, most of which share >60% sequence identities and exhibit 2-methylcitrate dehydratase activity, and a second one that share <25% sequence identity to *E. coli* PrpD and are not associated with any known functions. The PrpD proteins are involved in the conversion of 2-methylcitrate to 2-methylisocitrate; they work together with other enzymes in propionate metabolism pathway [89]. In the second group, the crystal structure of one protein, iminodisuccinate (IDS) epimerase; it exhibited a representative MmgE/PrpD fold [90]. It was suggested that this fold could serve as a scaffold for diverse functions, such as enzymatic dehydration and epimerization, as well as immune response and cell adhesion. This group also includes the mammalian immunoresponsive gene 1 (IRG1), which is highly expressed in macrophages activated by LPS [91] and in the uterus activated by progesterone and required for implantation in mouse [92].

Regarding the localization of cADC, there have been some debates as to whether it is present in the mitochondria or in the cytoplasm since *cis*-aconitic acid (*cis*-aconitate) is produced in the TCA cycle while ITA is finally secreted into the culture broth [93]. Studies of the fungal cADC can provide a way to enhance ITA productivity by biotechnological methods, and more importantly, provide an understanding of the biological roles of ITA.

1.2.4 Mechanisms of Decarboxylases

Decarboxylation is a fundamentally important reaction in biological systems. This chemical transformation usually removes a carboxyl group from carboxylic acids and releases carbon dioxide (CO₂). Enzymes that catalyze decarboxylation are called decarboxylases or carboxylyases (EC number 4.1.1). Decarboxylase enzymes play important roles in a wide variety of catabolic and anabolic pathways, including carbohydrate metabolism and synthesis as well as amino acid conversions [94]. Decarboxylases can be divided into two subfamilies, cofactor-dependent enzymes, which utilize different cofactors to catalyze the decarboxylation, and cofactor-independent enzymes. For the latter, orotidine 5'-monophosphate decarboxylase [95,96] and methylmalonyl CoA decarboxylase [97] are enzymes that do not need any cofactors. The cofactors involved in the enzymatic decarboxylation are either an organic cofactor such as biotin, flavin, NAD⁺, pyridoxal 5'-phosphate (PLP), a pyruvoyl group, and thiamin pyrophosphate (TPP), or an inorganic cofactor such as an iron, zinc, manganese and magnesium ions or complexes [98].

The mechanisms for cofactor independent decarboxylases vary but the basic concept is similar. They all establish a well-defined substrate binding pocket, using amino acid residues of the enzymes to catalyze the decarboxylation of the organic acid. As an example, orotidine 5'-monophosphate decarboxylase (OMPDC) catalyzes the critical final step in the pyrimidine biosynthetic pathway. Based on the crystal structure solved with and without substrate [98], a hydrophobic pocket is the key factor. The proposed stepwise mechanism includes a conserved lysine, which protonates the carbon bound to the pyrimidine's leaving carboxylate, and an aspartate, which destabilizes the substrate for decarboxylation [96,99-101]. Unlike the OMPDC's catalysis mechanism, the of 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylation reaction decarboxylase (OHCUD) was postulated to occur directly by using C=N as an electron sink to stabilize the intermediate carbanion. A glutamate in active site destabilizes the ground state of the substrate to facilitate the carboxylate group release; a conserved histidine causes the subsequent deprotonation and protonation to generate a stereospecific product [102,103]. For methylmalonyl CoA decarboxylase (MMCD), catalysis involves a conserved tyrosine that forms a hydrogen bond with the leaving carboxyl group and orients the substrate. The hydrophobic environment around active site also facilitates decarboxylation and the loss of a neutral carbon dioxide molecule. The backbone amide groups can assist in stabilizing the proposed anionic intermediate and transition state by hydrogen bonding with the substrate [96]. A similar mechanism was observed in another enzyme, malonate semialdehyde decarboxylase (MSAD), which also catalyzes the decarboxylation of a β -keto acid [104,105]. Several other mechanisms exist such as that of arylmalonate decarboxylase (AMDase), which generate a thiol ester intermediate formed through a cysteine residue [106-108], and acetoacetate decarboxylase (AADase), which involves the perturbation of a lysine pKa by the highly hydrophobic environment and a second lysine [109]. The cofactor-independent decarboxylase mechanisms proposed are usually based on the crystal structure combined with other experimental observations. Most of these decarboxylation reactions are facilitated by substrate destabilization in the active site and subsequent carbon dioxide release.

1.2.5 Discovery of Itaconic Acid in Other Species

ITA has been found in other fungi, not just *A. terreus*, and these include *Ustilago zeae* [110], *Ustilago maydis* [111], *Candida* sp. [112], and *Rhodotorula* sp. [113]. More recently, ITA has been identified in the metabolomic analyses of mammalian tissue specimens [114-118]. However, there is little knowledge on how ITA is formed in mammalian cells. A previous study in our lab [114] elucidated the synthetic pathway for ITA in macrophage-derived cells. ITA production was attributed to the decarboxylation of *cis*-aconitate by a specific decarboxylase (Fig. 1-6B). This was verified by using isotope tracers method starting from [$^{13}C_5$]glutamine (Fig. 1-6B, Scheme I) or [$^{13}C_6$]glucose (Fig. 1-6B, Scheme II and III). However, up to the beginning of my project, there was no specific gene identified as encoding this enzymatic activity. At the beginning of this year, a group in Luxembourg identified immunoresponsive gene 1 (IRG1) as the gene coding for an enzyme producing ITA through the decarboxylation of *cis*-aconitate [119].

The physiological role of ITA in mammalian cells is still unknown, as well as where the ITA was synthesized. Based on *in vitro* studies, ITA was shown to inhibit key enzymes of TCA cycle (isocitrate lyase [120] and fructose-6-phosphate 2-kinase [121]). Thus, ITA may act as an antibacterial agent and also may play an important role in central carbon metabolism. Despite an overall lack of information on ITA as a mammalian metabolite, there is evidence that ITA can be catabolized by both guinea pig and rat liver mitochondria [122,123]. In rats it was also shown that an itaconate diet leads to a reduced visceral fat accumulation, because of a suppressed glycolytic flux [121].

1.2.6 Immunoresponsive Gene 1 (IRG1)

Lipopolysaccharide (LPS), which is a major glycolipid component of the outer membrane of Gram-negative bacteria, can dramatically enhance the inflammatory potential and performance of macrophages as a potent and pleiotropic stimulus [124,125]. Many genes are highly induced upon the activation of LPS, including TNF-A, IL-1A and B, Irg2, etc. [126-129]. These play crucial roles in the orchestration of various responses to protect the host against infection. The identification of LPS inducible genes has received a lot of interest in recent years and has been used to understand the complexity of macrophage responses. IRG1 cDNA was first identified by screening a cDNA library from an LPS-activated RAW264.7 cell line by Lee et al. in 1995 [91]. This novel cDNA is 2.3 kb having an open reading frame (ORF) of 1938 bases encoding a polypeptide of 647 amino acid residues. IRG1 contains a glycosaminoglycan motif, many potential phosphorylation sites and was mapped to mouse chromosome 14. The IRG1 message appears 1.5 h after LPS stimulation and reaches its maximum at around 6 h. Message production was not dependent on new protein synthesis but was mediated by tyrosine kinase and protein kinase C (PKC) pathways.

Another novel IRG1 gene was [130] then identified that was regulated by the steroid hormone progesterone (P4) in the uterine epithelium. It was analyzed as the mammalian ortholog of the bacterial methylcitrate dehydratase. IRG1 was demonstrated to be a key marker of the implantation window in modulating steroid hormone responsiveness in the uterine luminal epithelium, which is induced through the PKC pathway. Moreover, IRG1 was also identified as a novel target of progesterone receptors (PRs) in the pregnant mouse uterus [92,131]. The identification of IRG1 showing a hormonally regulated pattern of expression in the uterus suggests novel functions of it during implantation [132].

Microglial cells can secrete reactive species upon activation that can cause damage to neurons. Mahe and coworkers identified the presence of IRG1 in the microglial cells first (2001), and then showed its role as a marker of the IFN- γ -activated microglial-derived clone cells [133]. Microglia are the prime and major component of an intrinsic brain immune system in the central nervous system (CNS). However, the mechanism that determines its role is unclear. It was reported that IRG1 was selectively and functionally expressed in killer microglia *in vivo* and could be a potential therapeutic target for gene therapy of some neurodegenerative and neuroinflammatory diseases, such as Alzheimer's disease and AIDS dementia [134].

In macrophage systems, IFN- γ and TNF are essential cytokines for successful clearance of microbial infections. Microbes are often sensed via TLR signaling through the key adaptor molecule myeloid differentiation factor-88 (MyD88), which plays a critical role in inducing immunity against microorganism. Analysis of the pulmonary transcriptome upon infection with *Chlamydia pneumonia*, an intracellular bacterium that causes pneumonia in humans and mice, demonstrated a major impact of MyD88 on inflammatory responses but not on interferon-type responses. IRG1, as one of many MyD88-dependent genes, was involved in immune responses against *C. pneumonia* infection [135]. In contrast, infection by live, virulent *Mycobacterium tuberculosis* (Mtb) activated macrophages largely through TLR2 or TLR4 in a MyD88-independent pathways which further clarified that IRG1 induction by Mtb was mediated via a pathway that does not require TLR2, TLR4, MyD88, etc. [136,137]. Different transcriptional and posttranscriptional regulation of IRG1 expression has been described for macrophages infected with mycobacteria or stimulated with LPS [138]. IRG1 was then found to be highly up-regulated in murine ANA-1 macrophages after stimulation by several proinflammatory cytokines and TLR agonists, as well as in spleen and lung of *Listeria monocytogenes* or *Toxoplasma gondii* infected mice [139]. The subcellular localization of IRG1 in murine macrophages, as a prototypic TNF- and IFN-γ-coregulated gene, was associated with mitochondria, which are known to play crucial roles in the regulation of cellular processes such as apoptosis and innate immune activation [140,141]

In summary, at the time I started working on the project to find the source of mammalian cADC, IRG1 has not been functionally characterized in macrophages and its up-regulation in stimulated macrophages was worthy of further investigation.

1.3 Thesis directions

The first aim of my dissertation is to define membrane interactions of two bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes: *Bacillus thuringiensis* (*Bt*) and *Staphylococcus aureus* (*Sa*) PI-PLCs. Understanding how the bacterial PI-PLCs interact with target membranes may provide specific ways to selectively inhibit them, and hence provide insights into novel methods to control bacterial infections. The two PI-PLC enzymes studied in my work can also be used as models to explore the considerably more complicated enzymatic kinetics and membrane interaction of the mammalian PI-PLC family.

In order to investigate the importance of PI-PLC dynamics in membrane binding and enzymatic activity, we combined MD simulations, *in silico* and *in vitro* mutagenesis along with binding and activity assays, to study the role of loop motions and helix kinking in regulating the activity of *Bt*PI-PLC. Two proline residues, Pro245 and Pro254, were associated with these correlated motions that appear in the simulations to limit the access of substrate. This observation in MD simulations led us to mutate these Pro residues both *in silico* and *in vitro*. The variations in both enzymatic activity and vesicle binding affinity between mutant protein and WT were measured to suggest how the correlated motions affect on the enzyme.

S. aureus PI-PLC shows 41.3% sequence similarity with *Bt*PI-PLC but only has two of the four tyrosine residues in helix G which were initially predicted to contribute to binding to PC-containing membranes. Thus, I cloned the wild-type *Sa*PI-PLC gene into a His-tagged overexpression system from the genomic DNA of *S. aureus* for further expression and purification. Serendipitously, one of my colleagues, Rebecca Goldstein, solved the structure of a homodimer of *Sa*PI-PLC that suggested one could form transient dimers on the membrane surface. To that end I examined enzyme activity as a function of enzyme concentration, pH, and vesicle composition, measured binding of the protein to vesicles by fluorescence correlation spectroscopy (FCS), and tested our hypotheses with a series of mutations. The results show that *Sa*PI-PLC has evolved a complex, apparently unique way to control its access to PI/GPI substrate.

There is little known about specific recognition motifs used by peripheral membrane proteins for binding to the PC headgroup. In cytosolic proteins, the cation- π box provides

a suitable receptor for choline recognition and binding through the trimethylammonium moiety. We proved this hypothesis by engineering the cation- π box into *Sa*PI-PLC, which lacks specific PC recognition, with a double mutation protein, N254Y/H258Y. The interaction of this variant with PC-enriched vesicles was monitored by different techniques (FCS, ³¹P NMR, intrinsic fluorescence and also crystallization). Such simple PC recognition motifs could be engineered into a wide variety of secondary structures providing a generally applicable method for specific recognition of PC.

The second aim of this dissertation was to identify and characterize a mammalian *cis*aconitate decarboxylase (cADC). Itaconic acid (ITA) is a metabolite produced by primary macrophages and cell lines derived from macrophages that is dramatically increased upon stimulation [114]. It is synthesized by a cADC activity. Sequence searches with a known fungal cADC show little homology to ORFs in mammalian cells. I used the macrophage cell line RAW264.7, designed a series of assays, including cell fractionation, partial protein purification, MS proteomics study and siRNA transfections to show that cADC activity upon stimulation is from the protein encoded from immunoresponsive gene 1 (IRG1). To identify the active site, a predicted homology model structure was built for fungal cADC. I cloned both IRG1 and fungal cADC into an *E. coli* expression system, purified and characterized the enzyme and compared their properties. The identification of this enzyme can help to clarify the unique decarboxylation mechanism and also open up future studies into the specific role of ITA in the mammalian immune system and cancers. Chapter 2

Materials and Methods

2.1 Chemicals

2.1.1 Cloning and Mutagenesis Reagent

The QuikChange site-directed mutagenesis kit, all the competent cells used in cloning, mutagenesis (XL1-Blue or XL10-gold), and overexpression (BL21 Codonplus) were obtained from Agilent Technologies (Santa Clara, CA). The QIAprep spin miniprep kit and QIAquick PCR purification kit were purchased from Qiagen (Valencia, CA). *Staphylococcus aureus* genomic DNA from strain FPR3757 genomic DNA was purchased from ATCC (Manassas, VA). *Mus musculus* immunoresponsive gene 1 (*Irg1*) as transfection-ready DNA (NM_008392.1) was purchased from OriGene Technologies (Rockville, MD). pET-21a vector was supplied by Novagen (Darmstadt, Germany). Restriction enzymes (EcoRI, BamHI, XhoI, SapI and NheI-HF), Taq DNA polymerase, pMALTEV vector, IMPACT kit, quick ligation kit, calf intestinal alkaline phosphatase and Antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). All the PCR primers were purchased from Eurofins MWG Operon (Huntsville, AL).

2.1.2 Resin for Purification

The Q Sepharose fast flow resin and phenyl sepharose resin were purchased from GE Healthcare (Piscataway, NJ). Micro bio-spin 6 columns were purchased from Bio-Rad Laboratories (Hercules, CA). Ni-NTA agarose resin was purchased from Qiagen (Valencia, CA). Chitin and amylose resin were purchased from New England Biolabs.

2.1.3 Phospholipids

Most of the lipids used in this work were purchased from Avanti Polar Lipids Inc.

(Alabaster, AL), and used without further purification. These include the long chain lipids 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1,2-dioleoyl-phosphatidylserine (DOPS), 1,2-dioleoyl-phosphatidylmethanol (DOPMe), 1,2-dioleoyl-phosphatidic acid (DOPA), 1,2-dioleoyl-phosphatidylglycerol (DOPG), L- α -phosphatidylinositol (PI) from bovine liver, dihexanoyl-phosphatidylcholine (diC₆PC), diheptanoyl-phosphatidylcholine (diC₇PC), dioctanoyl-phosphatidylethanolamine (diC₈PE), dioctanoyl-phosphatidylseine (diC₈PS) and dioctanoyl-phosphatidyglycerol (diC₈PG).

2.1.4 Molecular Probes

The fluorescence labeling reagent Alexa Fluor 488 C5 maleimide (AF488-Cys) and Alexa Fluor 488 carboxylic acid, succinimidyl ester (AF488-N-term) were purchased from Invitrogen (Grand Island, NY). The spin labeling reagent 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethio-sulfonate (MTSL) was obtained from Toronto Research Chemicals Inc (Canada).

2.1.5 Mammalian Cell Culture Reagent

The RAW264.7 cell line was purchased from the ATCC (Manassas, VA). The media for cell growth, Dulbecco's Modification of Eagles Medium (DMEM, with 4.5 g/L glucose and L-glutamine, without sodium pyruvate), was purchased from Cellgro (Manassas, VA). Heat inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). MycoZap CL was purchased from Lonza Cologne AG (Germany). Mouse TLR 1-9 Agonist Kit was purchased from Invivogen (San Diego, CA). ON-TARGETplus SMARTpool, Mouse IRG1 and Control siRNA were purchased from Dharmacon (San Jose, CA). GenMute siRNA Transfection Reagent for RAW264.7 Cell was purchased from SignaGen Laboratories (Rockville, MD).

2.1.6 Other Chemicals

Most of the other chemicals, such as tris(hydroxymethyl)aminomethane (Tris), 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino) ethanesulfonic acid (MES), bovine serum albumin (BSA), dithiothreitol (DTT), deuterium oxide (D_2O) , imidazole, sodium phosphate monobasic, sodium chloride, potassium chloride, magnesium chloride, manganese(II) chloride, calcium chloride, iron(II) chloride, glycine, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamide (TEMED), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), pyridoxal 5'-phosphate (PLP), thiamine pyrophosphate (TPP), β -nicotinamide adenine dinucleotide hydrate (NAD⁺), chloroform, methanol, acetic acid, glycerol, Triton X-100 (TX-100), itaconic acid, *cis*-aconitic acid, L-glutamine, D-glucose, lysozyme, actinomycin D, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, lipopolysaccharides (LPS, 0111:B4) and iodoacetamide were obtained from Sigma-Aldrich (St. Louis, MO). LB broth and LB agar were obtained from Fisher Scientific (San Jose, CA). Kanamycin sulfate, chloramphenicol, ampicillin and isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from American Bioanalytical, Inc. (Natick, MA). The detergent compatible (DC) BCA protein assay kit, molecular weight markers, agarose and 30% acrylamide/bisacrylamide solution were obtained from Bio-Rad Laboratories (Hercules, CA).

2.2 Cloning and Expression of B. thuringiensis PI-PLC and S. aureus PI-PLC

The original plasmid, *B. thuringiensis* PI-PLC (*Bt*PI-PLC) gene (Fig. 2-1) cloned in the pHN1403 vector, was obtained from Dr. Ming-Daw Tsai (Ohio State University).

An affinity tagged overexpression system, formation of a His-tagged protein, was selected for S. aureus PI-PLC (SaPI-PLC) cloning since it results in relatively pure protein. The wide-type SaPI-PLC gene (Fig. 2-2) was obtained directly from S. aureus genomic DNA (strain FPR3757) by PCR. An EcoRI restriction site was used for cloning the 5'end of target gene, with a forward primer 5'-ggGAATTCtcagattcgttgagtaaaagtccag-3' (EcoRI site underlined). A XhoI restriction site was used for cloning the 3'end of target gene with the reverse primer 5'-ggCTCGAGtttattactatctataatttcacttacg-3' (XhoI site underlined). Target genes flanked with EcoRI and XhoI sites were amplified under Taq DNA polymerase by PCR. 1% agarose gel was run to verify the PCR results. The PCR products were further purified by using the QIAquick PCR purification kit following the manufacturer's instructions. The restriction enzymes EcoRI and XhoI were used to cut both the inserts and the empty pET21a vector. During this incubation time (37 ^oC, 3-4 h), alkaline phosphatase was added at 2-2.5 h to avoid self-ligation. After another PCR purification, the quick ligation kit was used to perform the ligation of digested-insert and digested-vector. The ligation mixture was transformed into XL10-gold competent cells for plasmid propagation and preparations. Positive colonies could be identified by restriction digestion (ampicillin resistance). The QIAprep miniprep kit was used for purification of the plasmid DNA. The entire DNA sequence of the inserted target gene was sequenced with T7-promoter and T7-term by Genewiz (South Plainfield, NJ).

Figure 2-1. B. thuringiensis PI-PLC DNA and corresponding amino acid sequences.

1 GCTAGCTCTGTTAAT GAGCTTGAAAATTGG TCAAAATGGATGCAA CCTATACCTGATAAT 1 Δ SSVN ELENW SKWMO PIPD N ATCCCGTTAGCACGA ATTTCAATTCCAGGA ACACACGATAGTGGG ACGTTCAAGTTGCAA 61 21 IPLAR ISIPG THDSG TFKLO 121 AATCCGATTAAGCAA GTGTGGGGGAATGACG CAAGAATATGATTTT CGCTATCAAATGGAC 41 VWGMT QEYDF N Ρ IKQ RYQMD 181 CATGGAGCTCGCATT TTTGATATAAGAGGA CGTTTAACAGATGAT AATACGATAGTTCTT 61 G Α R FDIRG R L T D D Ν т Ι v Ι \mathbf{L} 241 CATCATGGGCCATTA TATCTTTACGTAACA CTGCATGAATTCATA AATGAAGCGAAACAA 81 н Н G ΡL Y L Y νт LHEF Ι Ν E Α Κ 0 301 TTTTTAAAAGATAAC CCGAGTGAAACAATT ATTATGTCTTTAAAA AAAGAGTATGAGGAT 101 F L Κ D N PSE т I IMSL Κ Κ Е Y E 361 ATGAAAGGGGCAGAA GGTTCATTTAGTAGT ACGTTTGAAAAAAAT TATTTTGTTGATCCT G S F S TFEKN 121 М К G A E S Y F v D Р ATCTTTTTAAAAACA GAAGGAAATATAAAA CTTGGAGATGCTCGT GGGAAAATTGTACTA 421 141 I FL КТ EGN I K LGDAR G K TVT CTAAAAAGATATAGT GGTAGTAATGAATCT GGAGGATATAATAAT TTTTATTGGCCAGAT 481 161 LKRYS G S N E S G G Y N N FYWPD 541 AATGAGACGTTTACC ACAACTGTAAACCAA AATGTAAATGTAACA GTACAAGATAAATAT 181 NETFT ΤΤΥΝΟ ΝΥΝΥΤ V O D K Y 601 AAAGTGAATTATGAT GAGAAAGTAAAATCT ATTAAAGATACGATG GATGAAACGATGAAC 201 EKVKS IKD т М D \mathbf{E} Κ v Ν Y D т M N 661 AATAGCGAGGATTTA AATCATCTATATATT AATTTTACAAGCTTG TCTTCTGGTGGTACA 221 N S E D L NHL Y I NFT S T. S S G G т 721 GCATGGAATAGTCCA TATTACTACGCTTCT TATATAAATCCTGAA ATTGCAAACGATATA 241 У У У A S Y I Ν Р Е Ι Α W Ν S Р Α N D Т 781 AAACAAAAGAATCCT ACAAGAGTAGGCTGG GTAATTCAAGACTAC ATAAATGAAAAGTGG V I Q D 261 К QKNP TRV G W Y Ι ΕKW Ν TCACCATTATTGTAT CAAGAAGTTATAAGA GCGAATAAGTCATTA ATAAAAGAATAA 841 SPLLY QEVIR ANKSL 281 I K E

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Figure 2-2. S. aureus PI-PLC DNA and corresponding amino acid sequences.

1 TCAGATTCGTTGAGT AAAAGTCCAGAAAAT TGGATGAGTAAACTT GATGATGGAAAACAT 1 S DSLS KSPEN WMSKL DDGK н 61 TTAACTGAGATTAAT ATACCGGGTTCACAT GATAGTGGCTCATTC ACTTTAAAGGATCCA 21 LTEIN IPGSH DSGSF TLK D Р 121 41 АКТОР K D Y L VKSVW т O M K S G 181 GTCAGGTTTTTTGAT ATTAGAGGTAGAGCA AGTGCTGATAATATG ATTTCAGTTCATCAC 61 V R F F D I R GRA S A D Ν М I S V Н н 241 GGCATGGTTTATTTG CATCATGAATTAGGA AAATTTCTCGATGAT GCTAAATATTACTTG 81 G М VYL HHELG KFL D D AK Y Y L 301 AGTGCTTATCCAAAC GAAACAATTGTGATG TCTATGAAAAAGGAC TACGATAGCGATTCT 101 S А ΥP Ν Е т Ι V M S M Κ Κ D Y D S D S 361 AAAGTTACGAAGACA TTTGAAGAAATTTTT AGAGAATATTATTAT AATAACCCGCAATAT 121 FEEIF К V т к т R E Y Y Y N N Р Q Y 421 CAGAATCTTTTTTAC ACAGGAAGTAATGCG AATCCTACTTTAAAA GAAACGAAAGGTAAA 141 QNLFY TGSNA NPTL Κ ЕТК G K ATTGTCCTATTCAAT AGAATGGGGGGGTACG TACATAAAAAGTGGT TATGGTGCTGACACG 481 161 IVLFN RMGGT YIKSG Y G A D т 541 TCAGGTATTCAATGG GCAGACAATGCGACA TTTGAAACGAAAATT AATAATGGTAGCTTA 181 SGIOW A D N A T FETKI N N G S L 601 AATTTAAAAGTACAA GATGAGTATAAAGAT TACTATGATAAAAAA GTTGAAGCTGTTAAA 201 D E YKD Y Y D K K Ν LKVO VEA V Κ AATTTATTGGCTAAA GCTAAAACGGATAGT AACAAAGACAATGTA TATGTGAATTTCTTG 661 221 Ν LLAK A K т D S N K D Ν v Y V Ν F Τ. 721 AGTGTAGCGTCTGGA GGCAGCGCATTTAAT AGTACTTATAACTAT GCATCACATATAAAT 241 G G S A F N S т Y Ν S VAS Y A S Н Т 781 CCTGAAATTGCAAAA ACGATTAAAGCAAAT GGGAAAGCTAGAACG GGTTGGCTGATTGTT IAK T I K A N 261 Ρ E G K A R Т G W L т GACTATGCAGGATAT ACGTGGCCTGGATAT GATGATATCGTAAGT GAAATTATAGATAGT 841 YAGY TWPGY D D I V S ΕI 281 D Т D S 901 ААТАААТАА 301 N K *

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2.3 Construction of Mutant Proteins

A series of *Bt*PI-PLC mutants and *Sa*PI-PLC mutants were constructed using QuikChange methodology [142] following the QuikChange Manual supplied by Agilent Technologies. Two complementary primers were designed with the mutated nucleotides near the center of the primer; the QuikChange primer design web-based program (http://www.genomics.agilent.com/primerDesignProgram.jsp) was used. All of the primers used are listed in Table 2-1. The mutated plasmid with staggered nicks was generated by PCR using *PfuTurbo* DNA polymerase for primer extension. The amplification product was digested with the Dpn I (a restriction enzyme used to digest the methylated parental DNA template), and then transformed into XL-Blue supercompetent cells for plasmid propagation. All mutant plasmids were confirmed by DNA sequencing.

2.4 Protein Expression and Purification Protocols

2.4.1 Expression of Recombinant PI-PLCs

The sequenced-ready plasmid was transformed into BL21-codonplus (DE3)-RIL competent cells using the heat-shock method. A single colony was inoculated into culture tubes with 5 mL LB media; the tubes were shaken overnight at 37 °C in the presence of appropriate antibiotics (34 μ g/mL chloroamphenicol and 100 μ g/mL ampicillin). The overnight culture was added to a large volume of fresh LB media (1 L or 2 L, with antibiotics added) in the ratio of 1:1000. This newly inoculated media was then incubated in a rotary shaker (200 rpm) at 37 °C. The over-expression of recombinant PI-PLCs was induced by adding IPTG at a final concentration of 0.8 mM (0.4 mM for cysteine mutant

Mutant	Primer
BtPI-PLC	
K38A	5′-cgatagtgggacgttc <u>GC</u> Gttgcaaaatccg-3′
K44A	5′-gcaaaatccgatt <u>GC</u> Gcaagtgtggggaatg-3′
V46K	5′-ccgattaagcaa <u>AA</u> Gtggggaatgacgc-3′
R71A	5′-gatataagagga <u>GC</u> Tttaacagatg-3′
K122A	5′-gagtatgaggatatg <u>GC</u> Aggggcagaaggttc-3′
N168C	5′-gatatagtggtagt <u>TG</u> Tgaatctggagg-3′
Q190_P_N191 ^a	5′-caactgtaaaccaa <u>CCG</u> aatgtaaatgtaacag-3′
V192C	5′-ctgtaaaccaaaat <u>TGC</u> aatgtaacagtacaag-3′
K201A	5′-cagtacaagataaatat <u>GC</u> Agtgaattatgatg-3′
N226C	5′-gcgaggattta <u>TG</u> Tcatctatatattaattttac-3′
P245A	5'-ggtacagcatggaatagt <u>G</u> CAtattactacgcttc-3'
P245G	5'-ggtacagcatggaatagt <u>GG</u> Atattactacgcttc-3'
P245Y	5′-ggtacagcatggaatagt <u>TAT</u> tattactacgcttc-3′
P254A	5'-cgcttcttatataaat <u>G</u> CTgaaattgcaaacg-3'
P254G	5′-cgcttcttatataaat <u>GG</u> Tgaaattgcaaacg-3′
P254Y	5'-cgcttcttatataaat <u>TA</u> Tgaaattgcaaacg-3'
K279A	5′-gactacataaatgaa <u>GC</u> Gtggtcaccattattg-3′
SaPI-PLC	
V44C	5′-ggatccagtaaaatca <u>TG</u> Ttgggcaaagactc-3′
V44W	5'-ggatccagtaaaatca <u>TGG</u> tgggcaaagactc-3'
H86E	5'-cggcatggtttatttg <u>G</u> A <u>G</u> catgaattaggaa-3'
H86Y	5′-cggcatggtttatttg <u>T</u> ATcatgaattagg-3′
Y211A	5'-gatgagtataaagat <u>GC</u> Ctatgataaaaaagttg-3'
D213C	5′-gagtataaagattactat <u>TG</u> Taaaaaagttgaagc-3′
F249I	5′-ggaggcagcgca <u>A</u> TTaatagtacttataac-3′
F249W	5′-ggaggcagcgcaT <u>GG</u> aatagtacttataactatgc-3′
Y253K	5'-gcgcatttaatagtact <u>AAG</u> aactatgcatcac-3'
Y253S	5'-gcgcatttaatagtact <u>AG</u> Taactatgcatcac-3'
Y253W	5'-gcgcatttaatagtactT <u>GG</u> aactatgcatcac-3'
N254Y	5'-gcatttaatagtacttat <u>T</u> ACtatgcatcaca-3'
H258Y	5'-cttataactatgcatca <u>T</u> ATataaatcctga-3'
W287A	5'-ctatgcaggatatacg <u>GC</u> Gcctggatatgatg-3'
Y290A	5′-ggatatacgtggcctgga <u>GC</u> Tgatgatatcgtaag-3′
Y290S	5′-ggatatacgtggcctgga <u>AG</u> Tgatgatatcgtaag-3′
F249W/N254Y/H258Y ^b	5′-ggaggcagcgcaT <u>GG</u> aatagtacttat <u>T</u> ACtatgc-3′
Y253S/Y255S	5'-gcgcatttaatagtactTCTaacTCTgcatcacatataaatcc-3'
N254Y/H258Y	5'-gcatttaatagtacttat <u>T</u> ACtatgcatca <u>T</u> ATataaatcctg-3'
Y253S/N254Y	5'-gcgcatttaatagtactTCTTACTCTgcatcaTATataaatcc-3'
Y255S/H258Y	

Table 2-1. Primers used in site-directed mutagenesis. The desired mutation codons are in capital and substitution bases are underlined (reverse primers are not listed).

^a Q190_P_N191 stands for inserting a proline between Q190 and N191. ^b These two mutants were designed using N254Y/H258Y as the parent plasmid.

proteins) when the optical density (O.D.) at 600 nm reached 0.7-0.9. The culture was incubated for another 20 h at 16 °C. The cells were then harvested by centrifugation at 5000 rpm for 10 min (using a Beckman J2-MC centrifuge with JA-10 rotor) and frozen at -20 °C for storage.

2.4.2 Purification of BtPI-PLC

The cell pellets were suspended in 20 mM Tris-HCl, pH 8.9, and lysed by sonication on ice using a Branson Sonifier 250 Cell Disrupter (Branson Ultrasonic Corp., Danbury, CT). Cell debris was pelleted by centrifugation at 15,000 rpm (using a JA-17 rotor) for 35 min. The supernatant with enzyme was ready for subsequent purification steps. Cell extract (dialyzed against 20 mM Tris-HCl, pH 8.9 overnight) was applied onto a Qsepharose fast flow (QFF) column (15 mm x 15 cm) equilibrated with 20 mM Tris-HCl, pH 8.9. The protein was eluted using an increasing NaCl gradient from 0 to 0.6 M in 20 mM Tris-HCl, pH 8.0 at the flow rate of 2 mL/min. The fractions collected were analyzed by SDS-PAGE to identify those with *Bt*PI-PLC. The protein was further purified using a phenyl-sepharose column (10 mm x 10 cm) equilibrated with 1 M NaCl in 20 mM Tris-HCl, pH 8.0 then eluted with a decreasing NaCl gradient from 1.0 to 0 M at the flow rate of 1 mL/min. The fractions containing PI-PLC were collected and dialyzed against 20 mM Tris-HCl, pH 8.0, and then concentrated by using Vivaspin 20 mL 10 kDa cut-off filters (Vivaproducts, Inc., Littleton, MA). Purity of PI-PLC variants was >90% as monitored by SDS-PAGE. Protein concentrations were measured by both Lowry assays and the absorption at 280 nm. Extinction coefficients at 280 nm, calculated using the ProtParam tool (http://web.expasy.org/protparam [143]), were $\varepsilon_{280} = 65.32 \text{ mM}^{-1} \text{ cm}^{-1}$ for wild type (WT), the 190s loop variants, and most of the Pro variants, or $\varepsilon_{280} = 66.81 \text{ mM}^{-1} \text{ cm}^{-1}$ for the Pro to Tyr variants. Formation of the disulfide bond in the double Cys variant, Asn226Cys/Val192Cys (N226C/V192C), was confirmed using nonreducing SDS-PAGE. For fluorescence correlation spectroscopy (FCS) experiments, a single Cys residue, N168C, was introduced into the protein. For these cysteine-containing proteins, DTT was added during the purification process and in the storage.

2.4.3 Purification of SaPI-PLC

The cell lysate with enzyme was prepared by suspending cell pellets in 20 mM Tris-HCl, 10 mM imidazole, pH 8.5, followed by sonication and then centrifugation. The cell extract was incubated at room temperature with Ni-NTA agarose resin (1 L cells/5 mL resin) for around 1 h on a shaker. The solution containing the enzyme bound on the resin was loaded into a column and then washed using 200 mL buffer (20 mM Tris-HCl, 10 mM imidazole, pH 8.5) at the flow rate of 2 mL/min. The UV signal was monitored during the washing and elution steps. The His-tagged protein was eluted using 0.01 to 0.15 M imidazole gradient in the 20 mM Tris-HCl, pH 8.5. SDS-PAGE was used to identify the fractions to be collected for the second purification step using a QFF ionexchange resin. After overnight dialysis in 20 mM Tris-HCl, pH 8.3, the protein solution was applied to the QFF column and then eluted with a NaCl gradient increasing from 0 to around 0.1 M (this varied among the different mutant proteins). The concentration of the PI-PLC enzymes at greater than 95% purity (monitored by SDS-PAGE as shown in Fig. 2-3) was measured by the absorption at 280 nm using the calculated extinction coefficient $(\varepsilon_{280} = 60.28 \text{ mM}^{-1} \text{ cm}^{-1})$. The yield of purified protein was 60 mg/L cell culture.

Figure 2-3. Purification of SaPI-PLC monitored by SDS-PAGE.



2.5 CD Spectroscopy to Monitor Protein Stability (T_m) and Overall Folding

Secondary structure content and thermal stability of the PI-PLC variants were measured using far-UV circular dichroism (CD) on a model No. 202 CD spectrophotometer (AVIV Biomedical, Lakewood, NJ). All samples used for CD experiments were prepared by adding the desired amount of protein (0.05 mg/mL) to 10 mM borate, pH 8.0. In the wavelength scanning experiments at 25 °C, CD spectra were collected from 300 to 180 nm with 1 nm wavelength steps. Secondary structure content was analyzed with the CDNN program [144] using the calculated molar ellipticity in the 190-260 nm range. Thermal stabilities for all proteins were measured by monitoring the ellipticity at 222 nm (primarily α -helix) while increasing the sample temperature 1.0 °C per min from 20 to 90 °C, with 1 min equilibration time.

2.6 Preparation of Phospholipid Aggregates

Enzyme kinetics experiments used PI/PC small unilamellar vesicles (SUVs) prepared by sonication; the composition of the SUVs is noted by X_{PC} , the mole fraction of PC. Aliquots of phospholipids (PI, PG, and PC species) in chloroform were mixed, dried under N₂, and then lyophilized overnight. The lipid film was rehydrated with the desired buffer. Small PI only, PI/PC and PG/PC vesicles were generated by sonicating the dispersed phospholipid solution on ice for 20 s and resting for 20 s, for a total time of 15-20 min (the time needed is dependent on the total phospholipid concentration) using a Branson Sonifier 250. The average radius for PI and PI/PC (X_{PC} = 0.5) vesicles characterized by DynaPro NanoStar Dynamic Light Scattering (DLS) instrument (Wyatt Technology Corp., Santa Barbara, CA) was 130 Å, with 20 and 46% polydispersity, respectively. Similar sizes have also been documented for the PG/PC SUVs [145,146].

2.7 PI-PLC Activity Assays via ³¹P NMR Spectroscopy

Specific activities of the PI-PLC enzymes were measured by ³¹P NMR spectroscopy [10,31,38,43] using a VNMRS 600 (Varian, Cary, NC) spectrometer. For BtPI-PLC, the activity assay was usually carried in 50 mM HEPES, 1 mM EDTA, and 0.1 mg/mL bovine serum albumin (BSA is used to stabilize the protein at the very low concentrations used in these assays), pH 7.5, 28 °C. Most assays of SaPI-PLC were in 50 mM MES, pH 6.5, with EDTA and BSA at 28 °C. However, for assays examining the pH dependence of activity, a mixed buffer system of MES and HEPES (total concentration of 50 mM) with the desired pH value was used. Specific activities in the presence of salt were determined using a NaCl (137 mM)/KCl (2.7 mM) mixture. The amount of enzyme added into different assay systems was adjusted so that <20% cyclic inositol phosphate product (and no inositol 1-phosphate) was generated in 30 min for the continuous assay and 15 min for the fixed time method. The amount of BtPI-PLC used was 0.1-0.25 µg/mL for mixed micelle assays, 0.2-2 µg/mL for PI cleavage when presented in vesicles, and 5-100 µg/mL for cIP hydrolysis. SaPI-PLC concentrations used in assays ranged from 0.2 to 8 µg/mL. Cleavage rates for PI solubilized in detergent micelles and hydrolysis of cIP were measured by continuously monitoring the relative integrated intensity of the cIP or I-1-P resonance (with ¹H decoupling but no nuclear Overhauser effect), respectively, as a function of reaction time (Fig. 2-4, inset). The increase in cIP under these conditions is linear with time indicative of an initial rate being measured. Because we are dealing with a vesicle and some fusion of SUVs as diacylglycerol (DAG) is produced, this is not the

same as a steady-state rate with the substrate in solution, but it is operationally useful for comparing rates under different conditions (pH, concentration of enzyme, salt, etc.). Mixed micelles of PI with Triton X-100 (TX-100) or diC₇PC were prepared by addition of the detergent solution to dry PI which will turned optically clear after mixing. Mixed micelles examined were either PI (8 mM)/TX-100 (16 mM) or PI (8 mM)/diC₇PC (32 mM) for BtPI-PLC and PI (4 mM)/TX-100 (8 mM) or PI (4 mM)/diC₇PC (16 mM) for SaPI-PLC. The water-soluble substrate cIP (20 mM for BtPI-PLC and 5 mM for SaPI-PLC) was used in the absence or presence of 8 mM diC₇PC. For the specific activities of PI-PLCs toward PI in small unilamellar vesicles (SUVs), the enzyme and SUVs were incubated for a fixed length of time at 28 °C, and the reaction was quenched by the addition of a few drops of acetic acid (dropping the pH below 4) followed by TX-100 addition to solubilize the remaining lipids in mixed micelles. The relative integrated intensity of the cIP resonance versus the total phospholipid concentration (initial [PI] or [PI] + [PC]) was used to calculate PI-PLC specific activity (as shown in Fig. 2-4). For the enzymatic assays of cysteine mutant variants, 5 mM DTT was added to avoid formation of any protein dimers.

<u>Figure 2-4.</u> Characterization of PI-PLC enzymatic reaction by ³¹P NMR. All the phosphorus species involved in the assay system are identified with arrows, including phosphatidylinositol (PI), phosphatidylcholine (PC), inositol 1,2-cyclic phosphate (cIP), and inositol-1-phosphate (I-1-P). No I-1-P was generated in PI cleavage by controlling the amount of enzyme added so that less than 20% product was generated. Inset: a series of ³¹P NMR spectra are shown monitoring the increase of cIP as a function of time in the continuous assay; the time increment between spectra was 1.7 min.



2.8 PI-PLC Site-Directed Spin Labeling

SaPI-PLC cysteine mutants D213C and N254Y/H258Y/D213C were specifically spin labeled on the cysteine by 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate (MTSL) (Fig. 2-5, Scheme I). MTSL was prepared as a 10 mg/mL (37.8 mM) stock solution in methanol and stored at -20 °C. The SaPI-PLC stock protein was diluted into 50 mM HEPES buffer (50% D₂O, 137 mM NaCl, 2.7 mM KCl, 1 mM EDTA, pH 7.5) with 5 mM DTT as 5 mg/mL. Salt was added to avoid protein precipitations when the protein was mixed with SUVs. After incubation at room temperature for 30 min for full reduction of any intermolecular disulfide bonds, DTT was then removed by Micro Bio-Spin 6 columns. A 5-fold MTSL over total –SH was then added and the resulting solution incubated at room temperature for 2 h on a shaker. The excess spin label reagent was removed by three rounds of Micro Bio-Spin 6 columns. The labeling percentage was >95%, confirmed by DTNB assay, which is used to quantify the concentration of thiol groups in a sample [147]. This assay was done by mixing the DTNB reagent (2 mM stock) with 20% SDS, 1 M Tris-HCl, pH 8.0 buffer and either working buffer (as background) or protein solution, incubating for 15 min, then measuring the absorbance at 412 nm. The similar values of spin-labeled protein and background indicated that almost all the free –SH was labeled. The prepared protein sample was then mixed with desired amount of SUVs solution for field cycling experiments.

2.9 PI-PLC Labeling with Alexa Fluor 488

Fluorescent labeling of proteins was carried out according to the manufacturer's protocol (Invitrogen); schemes for labeling chemistry are shown in Fig. 2-5 (Scheme II

and III). The dye was prepared as a 25 mM stock solution in anhydrous DMSO and stored at -20 °C. For BtPI-PLC, N168C mutant proteins were specifically labeled at Cys168 with the hydrophilic dye Alexa Fluor 488 C5 maleimide (AF488-Cys). Prior to labeling, the proteins were incubated with 5 mM DTT at room temperature for 30 min. The excess DTT was removed using Micro Bio-Spin 6 columns, which were preequilibrated with buffer (20 mM Tris-HCl, pH 7.0). The dye was added immediately (mole ratio of dye:protein=3:1 to 5:1) and the solution incubated in the dark at room temperature for 2 h or longer. For different proteins, different amounts of dye were added and incubation time needed for optimal labeling efficiency was determined. Some proteins required incubation on ice. Since cysteine oxidation can interfere with the labeling, argon gas may be layered on top of the samples to remove oxygen prior to incubation. The tubes should then be sealed with parafilm. For labeling the N-terminus of SaPI-PLC with Alexa Fluor 488 carboxylic acid, succinimidyl ester (AF488-N-term), the dye was added into the protein sample at a slightly higher concentration (7 mg/mL, ~200 μ M, mole ratio of dye:protein = 5:1). The SaPI-PLC protein labeling reaction was carried out in phosphate buffer, pH 7.2, to maximize preferential labeling of the N terminus rather than lysine residues. After removal of unbound dye using three spin columns, the absorption at 280 nm for protein plus dye and at 495 nm for the dye were used to estimate the number of dye molecules incorporated. The equation used for calculating the labeling ratio was:

$$[D]/[P] = (\varepsilon_p / \varepsilon_d) * A_{495} / (A_{280} - 0.1 A_{495})$$
where ε_p was the extinction coefficient of PI-PLC at 280 nm (refer to 2.4), ε_d was the extinction coefficient of AF488 at 495 nm (71,000 cm⁻¹ M⁻¹) and 0.1 A₄₉₅ was the correction. All the BtPI-PLC variants had a labeling ratio of 100±10%, and the SaPI-PLCs typically had 93-100% dye incorporation. The N-terminus labeling efficiency of SaPI-PLC was also measured by Mass Spectroscopy. Electrospray ionization mass spectral data of intact PI-PLC were obtained at the University of Massachusetts Amherst Mass Spectrometry Facility (Amherst, MA). For MS sample preparation, the concentrated protein solution (~0.3 mg total) was transferred into 5 mM ammonium acetate, pH 8.0, using Amicon concentrators and spinning at least eight times to get rid of salts. The sample was then lyophilized for MS. Labeling percentages ranged from 80 to 99% with >70% of the protein containing a single Alexa Fluor 488 moiety and the remainder doubly labeled. At 99% labeling efficiency, 25–30% of the labeled protein has 2 labels, whereas this percentage is lower (20-25%) at 80% labeling. The activity of labeled PI-PLC was tested and showed no significantly difference from that of unlabeled PI-PLC.

<u>Figure 2-5.</u> Labeling schemes for MTSL and Alexa Fluor 488. (I) Site-directed spin labeling of cysteine residues by MTSL; (II) Cysteine labeling with Alexa Fluor 488 C5 maleimide; (III) N-terminus labeling with Alexa Fluor 488 carboxylic acid, succinimidyl ester.



2.10 Binding of PI-PLCs to Vesicles by Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) based binding assays were performed on a lab-built confocal setup based on an IX-70 inverted microscope (Olympus) as shown in Fig. 2-6 [145,146]. Alexa Fluor 488 (AF488) fluorescence was monitored at 22 °C with samples placed in chambered coverglass wells (Lab-Tek, Nunc), containing 10 nM labeled PI-PLCs (*WT, *Mutant where the * denotes AF488 labeling) plus 1 mg/mL BSA in 300 µl PBS, pH 7.4 for BtPI-PLC, and 50 mM MES, pH 6.5 for SaPI-PLC. To prevent protein adhesion to the wells, the chambers were incubated with 10 mg/mL BSA and rinsed with desired buffer prior to use. The anionic substrate analog DOPG was used for all FCS experiments because the PI cleavage product DAG leads to vesicle fusion [148]. SUVs containing DOPG and various mole fractions of POPC, X_{PC}, were titrated into the labeled PI-PLC solutions to assess protein binding. For each X_{PC}, FCS titrations were run in duplicate and repeated a second time with different vesicle and protein preparations in parallel with *WT binding assays for direct comparisons of apparent K_d values. Analysis of the data was based on previous work by Elson, Thompson and others [149-153]. The fitted diffusion coefficient for free, AF488-Cys labeled BtPI-PLC was 54±2 μ m² s⁻¹ and AF488-N-term labeled SaPI-PLC (D_{free}) was 50±2 μ m² s⁻¹; the vesicle diffusion coefficient, Dbound, determined from analysis of vesicles containing fluorescently labeled lipids, was in the range of $12-15 \ \mu m^2 \ s^{-1}$ [145,146].

Figure 2-6. FCS experimental schematic. Individual laser lines from an air-cooled argonkrypton, multi-line laser are separated using a quartz prism (P). For our experiments, the 488 nm laser line is isolated and expanded 5 times by two lenses (L1 and L2), in order to be reflected into the sample using a 500drlp dichroic (D1). In the IX-70 inverted microscope, the 60X water objective (Obj) focuses the laser light into the sample and collects the emission. The emission passes through D1 and any remaining scattered laser light is blocked by an HQ505lp long pass filter (F1). The emission is focused onto a 30 µm pinhole by the tube lens (L3) in the microscope to limit the detection volume and collimated by lens L4. To calculate the cross-correlation, the fluorescence signal is split by a 50-50 beam splitter (BS2) and passes through HQ535/50 bandpass filters (F2 and F3) to be focused on two avalanche photodiodes, APD1 and APD2, by lenses L5 and L6, respectively. The box shows a typical observation volume (1 μ m³) with different species, free AF488 labeled protein and vesicle-bound AF488 labeled protein, diffusing in and out of the volume.



FCS experiments were performed using 488 nm excitation as described previously [145,146]. To account for the possibility that multiple proteins could bind to the same vesicle, autocorrelations (G(τ), obtained in crosscorrelation mode using a 50-50 beamsplitter) for samples containing SUVs were fit to [152,153]:

$$G(\tau) = A_p g_p(\tau) + A_v g_v(\tau) \tag{1}$$

where *p* and *v* denote free protein and SUVs that are fluorescent due to PI-PLC binding, respectively and A_j is the amplitude of species *j*. The correlation function for species *j*, $g_j(\tau)$ is given by [149,151]:

$$g_{j}(\tau) = \left(1 + \frac{\tau}{\tau_{D,j}}\right)^{-1} \left(1 + \frac{\tau}{S^{2}\tau_{D,j}}\right)^{-1/2} \quad (2) \qquad \tau_{D,j} = \frac{\omega_{o}^{2}}{4D_{j}} \quad (3) \qquad S = \frac{z_{o}}{\omega_{o}} \quad (4)$$

where the values of ω_o , the radius of the observation volume, and *S*, which depends on z_o the extent of the observation volume, were determined from fits to the rhodamine 110 calibration data using $D=280 \ \mu\text{m}^2 \text{ s}^{-1}$ at 22 °C [150]. $\tau_{D,j}$ is the diffusion time and D_j is the diffusion coefficient. The fraction of protein bound to the SUVs, *f*, may be determined from A_p and the time-averaged number of proteins in the observation volume in the absence of vesicles, $\langle N_o \rangle$ [152]:

$$f = I - A_p \langle N_o \rangle = I - A_p / A_{p,o} \tag{5}$$

where $A_{p,o}=1/\langle N_o \rangle$ is the amplitude for free PI-PLC prior to titration determined from a single species fit. When calculating *f*, $A_{p,o}$ is corrected for any volume changes from the titration.

The apparent dissociation constant, K_d , representing PI-PLC partitioning onto the vesicle surface, and a cooperativity coefficient, n, were determined using the empirical

equation:

$$f = f_{max} \left[PL \right]^n / (K_d^n + \left[PL \right]^n)$$
(6)

where *f*, is the fraction bound for different total lipid concentrations, [PL], at fixed X_{PC} , and f_{max} is the apparent maximum fraction bound. Representative correlation and binding curves are shown in Fig. 2-7A and B. As previously noted, SUVs are quite heterogeneous in size and this heterogeneity leads to f_{max} values of less than 100% (Table 2-2 [146]). In the *Bt*PI-PLC system with pure POPC SUVs, the binding curves have hyperbolic shapes, so fits were performed with *n* fixed to 1. However, for POPC/DOPG mixtures with X_{PC} <0.4 binding to *Bt*PI-PLC or in the *Sa*PI-PLC system, plots of *f* versus [PL] are noticeably sigmoidal and *n* was greater than 1. This apparent cooperativity in the case of *Bt*PI-PLC likely results from the enzyme's preference either for the subpopulation of vesicles with higher PC content or for PC-rich clusters/regions in the vesicles, both of which are scarce when X_{PC} is low. For single vesicle concentrations at specific X_{PC} compositions, FCS titrations were run in duplicate and were repeated at least one more time with different vesicle and protein preparations.

The fits to Equation 1 assume that the distribution of vesicle sizes is homogenous so that a single diffusion time (diffusion coefficient) can be used for vesicle bound proteins. In fact, the SUV size distributions are quite heterogeneous. Previous work in the lab showed that when FCS data are fit assuming that (i) only one protein binds per vesicle and (ii) there is only one diffusion time for vesicle bound protein, the SUV size heterogeneity has no significant effect on the determined value of the apparent K_d but it does result in underestimation of the fraction bound, *f*, and the maximum fraction bound,

 f_{max} , plateaus at less than 100% binding [146]. The vesicle size distributions from dynamic light scattering (DLS) experiments for pure phosphatidylglycerol (PG) or phosphatidylcholine (PC) vesicles were used to generate simulated datasets in Matlab (Mathworks) [146]. For a given phospholipid concentration and K_d, the fraction bound, *f*, was calculated from Equation 6 with n=1. Datasets were generated using different values of α , the brightness of the vesicle relative to free protein according to:

$$G(\tau) = A_p g_p(\tau) + A_v \sum_j P(D_j) g_{vj}(\tau)$$
⁽⁷⁾

where $P(D_j)$, derived from the DLS data, is the probability that an SUV has diffusion coefficient D_j . A_p and A_v are the amplitudes for the free protein and vesicle bound protein, respectively, and the diffusion only correlation function for species i, $g_i(\tau)$, is given by Equations 2-4. The amplitudes used in Equation 7 were calculated from the values of fand α :

$$A_p = (1-f)A_{po}$$
 (8) $A_v = \alpha(A_{po} - A_p)$ (9)

where A_{po} , the protein amplitude in the absence of vesicles, is set to 1. Equation 7 does not account for the more complicated situation where α is dependent on vesicle size, e.g. that larger vesicles are brighter because they bind more proteins or that PI-PLC prefers smaller vesicles making the small vesicles brighter.

The resulting simulated datasets were fit to Equation 1 using Origin (OriginLab). As a control, data were generated using a single diffusion coefficient for the vesicles, and fits to these data generated randomly distributed residuals and the input values for the

apparent K_d and *f* (data not shown). For the data simulated using a distribution of vesicle sizes and Equation 7, the fitting results were similar to those obtained when one protein was assumed to bind per vesicle [146]: (i) the residuals for the fits to the correlations were structured (ii) the values of *f* were underestimated (iii) the values obtained for the apparent K_d were not significantly different from the input values (Table 2-2). In addition, how much *f* is underestimated depends both on the vesicle size distribution used to simulate the data and on the value of α . As α increases, the values of *f* obtained from the fits decrease (Table 2-2). Therefore, the assumption that the SUV population is homogeneous when it is in fact heterogeneous results in accurate values for the apparent K_d and underestimated values for the fraction of protein bound to vesicles.

For *WT *Bt*PI-PLC, *f* values determined using equations 1 and 5 (Fig. 2-7) were similar to those determined previously [145,146] where it was assumed that on average, one PI-PLC is bound per SUV. The average brightness of the SUVs relative to the protein [152], $\alpha = A_{\sqrt{A_p,o-A_p}}$, is shown in Fig. 2-8 as a function of phospholipid concentration for *WT *Bt*PI-PLC. In low affinity conditions $\alpha \sim 1$ indicating that on average one protein is bound per vesicle. For conditions where *WT *Bt*PI-PLC has high affinity for vesicles, α is dependent on the vesicle concentrations with $\alpha \sim 1$ at low and high vesicle concentrations and $\alpha = 2-3$ for moderate vesicle concentrations indicating that more than one protein binds to each vesicle. For *Sa*PI-PLC, the values of α are around 1 due to the weaker affinity of the enzyme for vesicles, indicating that for *Sa*PI-PLC and these small PG/PC vesicles, on average one protein is bound per SUV during the diffusion time of ~190 ms.

Vesicle Distribution	D _v (μm²/s) from fit	α in simulation	α from fit	K _d (μM) from fit	<i>f_{max}</i> from fit [*]
Distribution of PC SUVs	13.8	1.0	1.0	10	0.94
Distribution of PG SUVs	20.3	1.0	1.0	10	0.92
Distribution of PC SUVs	13.8	0.80	0.79	10	0.95
Distribution of PG SUVs	20.3	0.80	0.79	10	0.93
Distribution of PC SUVs	13.8	1.5	1.6	10	0.91
Distribution of PG SUVs	20.3	1.5	1.6	10	0.87
Distribution of PC SUVs	13.8	2.0	2.1	10	0.88
Distribution of PG SUVs	20.3	2.0	2.2	10	0.83

<u>Table 2-2</u>. Results from fitting the FCS data of *WT *Bt*PI-PLC to Equation 1. The fitting is using $K_d = 10 \mu M$ and size distributions from DLS for pure PC or pure PG SUVs.

^{*}Note the underestimation of $f_{max.}$

Figure 2-7. Representative normalized correlations and binding curves for *WT *Bt*PI-PLC. (A) Normalized correlation curves for 10 nM *WT *Bt*PI-PLC in solution (solid squares), in the presence of 15.5 μ M PC SUVs (open circle), and 3335 μ M PC SUVs (open triangel). The lines through these data are fits to Equation 1 with diffusion coefficient for the SUVs fixed at 15 μ m² s⁻¹. The error bars are the S.D. calculated by subdividing the raw data into 50 sections. (B) Titration curves showing the fraction of *WT *Bt*PI-PLC bound to pure POPC SUVs (solid squares), DOPG/POPC (X_{PC} = 0.25) SUVs (open circles). Error bars represent the S.D. from 3 independent sets of titrations.



Figure 2-8. The dependence of α , the relative brightness of the vesicles, on total phospholipid concentration for WT *Bt*PI-PLC (10 nM). $\alpha = A_v/(A_{p,o}-A_p)$ where the vesicle and protein amplitudes, A_v and A_p , respectively were determined by fits to equation 1 in the main text, and $A_{p,o}$ is the correlation amplitude in the absence of vesicles. The SUV lipid compositions are indicated in the legend.



2.11 Binding of SaPI-PLC to SUVs by Centrifugation-Filtration Assay

Estimation of apparent dissociation constants for *Sa*PI-PLC proteins partitioning onto PG/PC SUVs was also carried out using a centrifugation/filtration assay described previously for this system [154]. Vesicle samples were incubated with 0.2 mg/mL protein in 0.5 mL of 50 mM MES (pH 6.5) or HEPEs (pH 7.5). After incubation for 15 min, the free proteins were separated from vesicle bound proteins by centrifugation through a 100 kDa cut-off filter. Filtrates were analyzed by the DC BCA protein assay (according the instruction manual supplied by Bio-Rad) to calculate the free enzyme concentration, E_{f} , by comparing free protein concentrations from vesicle containing samples to the total enzyme, E_t , for enzyme without vesicles centrifuged through the filter. The fraction bound to vesicles, f_b , can then be calculated by

$$f_b = (E_t - E_f) / E_t$$

For fixed vesicle concentrations, the values of f_b can be used to compare the binding affinity of different mutant protein under certain conditions.

2.12 PI-PLC Line Broadening of the $diC_7PC^{31}P$ Resonance

DiC₇PC was titrated into a solution of 3 mg/mL (0.085 mM) PI-PLCs in the desired buffers containing 1 mM EDTA. Protein-induced line broadening at 242.76 MHz was measured on a Varian VNMRS 600 spectrometer. For the bacterial PI-PLCs with significant PC affinity, the lipid linewidth increases dramatically around the CMC (1.5 mM [10]), as protein/lipid micelle complexes form, and then decreases as more diC₇PC is added to reach a limiting linewidth. Proteins with weakened affinity for PC have very little effect on the diC₇PC 31 P linewidth [30]. Linewidths at a given diC₇PC concentration were measured for duplicate samples.

2.13 Intrinsic Fluorescence of PI-PLC

Intrinsic fluorescence measurements of PI-PLCs (0.2 μ M) were carried out on a Horiba Jobin Yvon FL3-22 Fluorolog Spectrometer (HORIBA Scientific, Edison, NJ). Samples were excited at 282 nm, and changes in the fluorescence intensity at the emission maximum, 337 nm, upon the addition of diC₇PC were expressed as (*I* - *I*₀)/*I*₀, where *I*₀ is the emission intensity of protein alone and *I* is the intensity in the presence of diC₇PC. A small amount of background signal from buffer solution or buffer with different concentrations of diC₇PC was subtracted from the control and sample intensities. The dependence of (*I* - *I*₀)/*I*₀ on diC₇PC concentrations reflects protein binding affinity for that short chain lipid [31].

2.14 High Resolution ³¹P Field-Cycling NMR Spectroscopy

High resolution ³¹P field cycling NMR spin-lattice (R_I) relaxation measurements, using a custom-built high resolution field cycling system on a Varian Unity^{plus} 500 spectrometer (Varian, Cary, NC) [155], were carried out with spin-labeled *Sa*PI-PLC spin-labeled at D213C, placing the spin-label at a comparable position to its most effective position in *Bt*PI-PLC, D205C [34]. The much larger dipole of the unpaired electron can relax ³¹P fast-exchanging into and out of a discrete binding site and back into the bilayer. The field dependence of the increase in R_I caused by the spin label for each lipid (ΔR_1) in a vesicle compared to the control (unlabeled protein) can be fit with the following expression:

$$\Delta R_{1} = R_{P-e}(0)/(1 + \omega^{2}\tau_{P-e}^{2})) + c$$

Here $R_{P-e}(0)$ is the maximum relaxation enhancement for that fraction of ligand bound to the spin-labeled protein, and τ_{P-e} is the correlation time for the bound phospholipid/spinlabeled PI-PLC interaction. A constant residual R_1 at higher fields, c, is likely to reflect a limiting CSA contribution due to the paramagnetic interaction.

The parameters $R_{P-e}(0)$ and τ_{P-e} along with the total spin-labeled PI-PLC concentration, [PI-PLC-SL], and phospholipid concentration, L_o , are related to r_{P-e} , the distance between the phospholipid ³¹P and the nitroxide when the ligand is bound. To a first approximation we assume that if there is a discrete site on the protein for an individual phosphorylated molecule, it is saturated with 3-5 mM of the ligand. We also assume that we are looking at a single PC or PMe binding in a given site for a time approaching 0.5 µs, long enough to suggest a specific complex as opposed to nonspecific lateral diffusion of the lipids around the protein, and that only phospholipid in the outer leaflet of the bilayer is in contact with the protein. For these small vesicles on average about 2/3 of the total of a specific phospholipid is in this monolayer. The average distance of the bound phospholipid on the protein at a specific site is calculated from the following expression:

$$r_{P-e}{}^{6} = [PI-PLC-SL]/\{(2/3)[L]_{o}\} \times (S^{2} \tau_{P-e} / R_{P-e}(0)) (0.3 \ \mu^{2}(h/2\pi)^{2} \gamma_{P}{}^{2} \gamma_{e}{}^{2})$$

 S^2 , the order parameter of the electron spin-³¹P dipolar interaction, is approximated as 1 because of the long r_{P-e} compared to the size of local picosecond motions; μ , γ_P and γ_e are constants defined previously [145,146].

2.15 RAW264.7 Cell Culture and Activation with LPS

The RAW264.7 murine macrophage line (ATCC) was incubated at 37°C in a 5% CO₂ atmosphere at 95% humidity. Cells were maintained below 90% confluence, and the media was changed 2-3 times per week. Cells were sub-cultured at a ratio of 1:4. Adherent cells were passaged by incubating with 0.25% trypsin-EDTA for 2 min and scraping with a cell scraper. The RAW264.7 cell lines was cultured in DMEM supplemented with 25 mM glucose, 4 mM L-glutamine, 10% FBS, and 1 μ L/mL Mycozap CL. LPS stimulation was carried by directly adding the desired amount LPS into the fresh changed medium and incubating for a period of time. The LPS stimulation time and concentration needed for optimal itaconic acid (ITA) production were determined by varying the incubation time (1 to 24 h) and LPS concentrations (0.5 ng/mL to 50 μ g/mL).

2.16 RAW264.7 Cell Stimulation with Toll-like Receptor (TLR) agonists

The murine Toll-like Receptor (TLR) agonists used to stimulate the RAW264.7 cells were included in the Invivogen Mouse TLR 1-9 Agonist Kit (Table 2-3). RAW264.7 cells were grown to confluence in supplemented DMEM by using 60 mm cell culture plates and 3 mL fresh medium was added to each plate an hour prior to stimulation. The cells were stimulated with seven types of TLR agonists at concentrations (Table 2-3) sufficient to match or exceed the minimum working concentrations suggested by the Invivogen (<u>http://www.invivogen.com/mouse-tlr1-9-agonist-kit</u>). The cells unstimulated with PBS, harvested and stored at -80° C for later enzymatic analysis.

Agonist Target Specificity Concentration Pam3CSK4 **TLR1/2** A synthetic lipopeptide (LP) that simulates 10 µg/mL the acylated amino terminus of bacterial LP; a potent activator of the prionflammatory transcription factor NF-KB 10⁹ cells/mL HKLM TLR2 A heat-killed preparation of Listeria monocytogenes Poly(I:C) 500 μg/mL TLR3 Polyinosinic-polycytidylic acid: a synthetic analog of double-stranded RNA (dsRNA) Lipopolysaccharide (LPS) derived from E. 100 µg/mL LPS-EK TLR4 coli K12 FLA TLR5 10 µg/mL Flagellin isolated from Salmonella typhimurium FSL-1 TLR6/2 A synthetic lipoprotein derived from 10 µg/mL Mycoplasma salivarium similar to M. fermentans derived LP 25 μg/mL ssRNA40 TLR7 A U-rich 20-mer phosphorothioate protected single-stranded RNA derived from the HIV-1 long terminal repeat

Table 2-3. The types of murine TLR agonists and concentrations used during cell stimulation.

2.17 Itaconic Acid (ITA) Analysis by ¹H NMR

ITA is produced from *cis*-aconitate by a *cis*-aconitate decarboxylase (cADC). To assay the production of this metabolite by RAW264.7 cells, protein extracts were prepared and then assayed using ¹H NMR. The assay is based on the observation that the chemical shifts of the –CH=CH₂ protons of ITA are quite different from those in *cis*-aconitate (Fig. 2-9). Confluent flasks of RAW264.7 cells were washed with PBS buffer and scraped into ice-cold hypotonic lysis buffer (20 mM HEPES, 3 mM MgCl₂, 1 mM PMSF and 10 μ L/mL protease inhibitor cocktail, pH 7.5) and incubated on ice for 10 minutes in order to obtain a crude lysate. Cells were lysed by Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL) until >90% were visible by Trypan Blue staining. To perform the crude lysate assays, an aliquot of a 200 mM *cis*-aconitic acid solution freshly prepared in lysis buffer and adjusted to neutral pH was added to a final concentration of 10 mM. Lysates were then incubated at 37°C with shaking at 100 rpm for 3 h. For metabolite extraction, a 4fold volume of ice cold methanol was added and samples were incubated on ice for 10 min before centrifugation at 14,000 rpm, 4°C. The supernatant was dried under N₂ gas, lyophilized, and dissolved in D₂O for NMR analysis (Fig. 2-9, Scheme I). For partially purified fractions and enzyme assay, the reaction was quenched by NaOH; after lyophilization the samples were resuspended in D_2O and ¹H NMR spectra acquired at 600 MHz (Fig. 2-9, Scheme II). Data were analyzed using VNMRJ software.

<u>Figure 2-9.</u> Characterization of cADC activity. (I) Characterization scheme for cell extract; (II) Characterization scheme for purified cADCs. The bottom box shows comparison of ¹H NMR spectra of *cis*-aconitic acid (5.61 ppm), itaconic acid (5.82 and 5.34 ppm) and enzymatic reaction with less than 20% product.



2.18 Actinomycin D Asssay

In cell biology, actinomycin D (ActD) is used to inhibit transcription. This compound can bind DNA at the transcription initiation complex and preventing elongation of RNA chain by RNA polymerase. However, ActD also shows toxicity to the mammalian cells. The RAW264.7 cells were monitored hour by hour (cell viability were checked under microscope) after applying different amount of ActD (from 10 ng/mL to 1 μ g/mL) and 10 ng/mL LPS. These cells were harvested to check cADC activity retaining.

2.19 Partial Protein Purification of RAW264.7 Cell Extract

The crude lysate after sonication was further separated by regular centrifuge first (14,000 rpm, 30 min), both the supernatant and pellet was used to do cADC activity characterization. The high-level centrifugation, ultracentrifugation, was used to further separate the supernatant fractions ($100,000 \times g$, 90 min) on the TLA 120.2 rotor of an Optima TLX Ultracentrifuge (Beckman Coulter, Inc., Pasadena, CA).

Several protein purification methods were used to further separate the most active fractions for ITA production from the complex mixture of proteins in the cell lysate. Initially, an ion-exchange column was used to purify supernatant after ultracentrifugation. Upon application of a 0 to 0.6 M NaCl grandient (in 20 mM Tris-HCl, pH 8.0), the protein fractions were characterized for ITA production using the ¹H NMR cADC activity assay. The protein fractions that generated ITA were collected for further purification steps, e.g., a hydrophobic column and gel-filtration. Superdex 75 and 200 gel-filtration columns were used to separate the most active fractions and determine the

molecular weight range of the target enzyme by comparing with standard gel-filtration curve.

2.20 Proteomics Study by Mass Spectrometry (MS)

Attempts to identify the cADC protein were carried out by Mass Spectrometry (MS) by comparing proteins in the same amount of unstimulated and stimulated RAW cells after the same steps of purification (sonication, centrifugation, ion-exchange purification). The protein fractions were collected for in-solution trypsin digestion and MS analysis of peptides. The two solutions were lyophilized first to concentrate the sample (and minimize the volume for MS), then resuspended in 30 µL of 8 M urea in PBS buffer, pH 7.5. 100 mM ammonium bicarbonate was added to make a final volume of 100 μ L, followed by heating the sample at 65 °C for 15 min with 150 mg/mL DTT. The samples then stayed at room temperature for 30 min with 92 mg/mL iodoacetamide. The samples were mixed by vortexing rapidly with adding 120 μ L PBS buffer, after which trypsin was added at a ratio of 1:50 (enzyme: substrate) with 1 mM CaCl₂ to digest the protein samples at 37 °C overnight. Trypsin cleavage was quenched with 5% of formic acid and the samples were centrifuged at 15,000 rpm for 20 min to pellet the undigested and precipitated protein. The supernatant was loaded on the column for MS analysis. The proteomics study of RAW264.7 cells by MS was done by Julianne Martell and Prof. Eranthie Weerapana (Department of Chemistry, Boston College, Chestnut Hill, MA).

2.21 Transfection of siRNA

In order to further confirm the cADC activity of IRG1 protein, the RAW264.7 cells were transfected with siRNA using ON-TARGETplus SMARTpool of Mouse IRG1 genes. The cells were diluted to a density of 200, 000 cells/mL, and around 700,000 cells were plated in each of the 60 mm plates 24 h prior to transfections so that the monolayer cell density reached ~60% confluence at the time of transfection. The IRG siRNA transfection experiment followed the general protocol for transfecting siRNA in RAW264.7 cells provided by SignaGen Lab but with some changes. At 1 h before the transfections, fresh DMEM with FBS and antibiotics was added into each plate. The transfection complex was made by mixing 300 µL GenMute Transfection buffer, siRNA (either Mouse IRG1 siRNA or control siRNA) stock and 12 µL GenMute reagent to get a final concentration of 30 nM siRNA per well (this volume is for 60 mm plates). After incubation for ~15 min at room temperature, which allows the transfection complex to form, the solution was added to the cells dropwise while gently rocking the plate back and forth. The transfection medium was replaced by fresh DMEM ~5 h after transfections. After 16 h incubation, half of the plates were stimulated with 10 ng/mL LPS for 8 h. Changes in the cADC activity of untransfected cells, cells transfected with control siRNA and cells transfected with targeting siRNA of various concentrations were monitored. The DC BCA protein assay was used to quantify the total protein amount in each of the cell lysates to minimize the differences among each transfection cycles.

2.22 Cloning and Overexpression of Immuneresponsive Gene 1 (IRG1) into pTXB1

The mouse IRG1 gene sequence shown in Fig. 2-10, was theoretically calculated to be

unstable by ProtParam (<u>http://web.expasy.org/protparam/</u>) with a computed instability index of 43.19.

A fusion protein tagged overexpression system was used for IRG1 cloning since fusion proteins facilitate protein purification and immobilization by using different kinds of molecular tags that can be used for affinity purification. After trying several overexpression systems, including His-tagged, GST-tagged, MBP-tagged, etc., only the IMPACT system was successful for IRG1 overexpression in *E. coli*. The pTXB1 system, as one vector of IMPACT system, allows the target gene to be cloned immediately adjacent to the intein cleavage site (without any extra amino acids by cloning into the NdeI and SapI sites). A C-terminus self-cleavable intein (~28 kDa) tagged fusion protein, which contains the chitin binding domain (CBD, 6 kDa), is generated and used for affinity purification of the fusion precursor on a chitin column. The native recombinant protein without modifications is then released by thiol induced self-cleavage activity of intein.

For the *IRG1* gene, a NheI restriction site was used for cloning the 5'end of target gene, with the forward primer 5'-gcggGCTAGCatgatgctcaagtctgtcacagag-3' (NheI site underlined). A SapI restriction site was used for cloning the 3'end of target gene with the reverse primer 5'-ggtggtt<u>GCTCTTC</u>cgcaattggtaaacctgggcaacg-3' (SapI site undelined). The C-terminal residue of the target protein has an effect on DTT-induced cleavage with pTXB1. The C-terminal amino acid of IRG1 protein, isoleucine only exhibits 60-85% cleavage after 16 h induction at 23 °C. Therefore, it was deleted to leave asparagine as the C-terminus (Asn exhibits 80-95% cleavage after 16 h induction at 23 °C). Target genes

flanked with NheI and SapI sites were amplified by PCR then inserted into the pTXB1 vector. The entire DNA sequence of the inserted target gene was determined by Genewiz using the T7 promotor and Mxe Intein Reverse II Primer (5'-gattgccatgccggtcaagg-3').

The plasmid pTXB1-IRG1 was transformed into *E. coli* host cells for overexpression. LB media containing 34 μ g/mL chloroamphenicol and 100 μ g/mL ampicillin was inoculated at 37°C with 5 mL freshly grown cultures and inoculated until O.D._{600 nm} reached 0.7-0.8. IPTG was added to the final concentration of 0.2 mM for induction of IRG1 protein expression. The cells continued to grow at 16°C overnight (16 h). Cell extract supernatants from disrupting cells by sonication and centrifuging 15,000 rpm for 35 min exhibited cADC activity. However, only the cell debris showed an obvious overexpressed protein band corresponding to a MW of 82 kDa on SDS-PAGE gel (Fig. 2-12A).

1	ATGA	TGCT	CAAG	TCT	GTC	ACA	GAG	AGC	TTT	GCI	GGT	ATG	ATT	CAC	GGC	ГTG.	AAA	GTG.	AAC
1	М	M L	Κ	S	V	т	Е	S	F	А	G	М	I	Η	G	L	К	V	Ν
61	CACC	TGAC	AGAT	GGT	ATC	ATT	CGG	AGG	AGC	AAG	AGG	ATG	ATC	CTG	GAT	гст	СТG	GGC	GTT
21	Н	ь т	D	G	I	I	R	R	S	Κ	R	М	I	L	D	S	L	G	V
121	GGCT	TCCT	GGGG	ACA	GGC	ACA	GAA	GTG	TTC	CAT	'AAA	GTC	ACC	CAA	TAT	AGT.	AAA	ATC	TAC
41	G	F L	G	т	G	т	Е	V	F	Н	Κ	V	т	Q	Y	S	Κ	I	Y
181	AGTT	CCAA	CACC	TCC	AGC	АСТ	GTT	TGG	GGT	CGA	CCA	GAC	TTC	AGG	CTC	CCA	CCG	ACA	TAT
61	S	S N	т	S	S	т	V	W	G	R	Ρ	D	F	R	\mathbf{L}	Ρ	Ρ	т	Y
241	GCTG	CTTT	гGTT	'AAT	GGT	GTT	GCT	GTT	CAC	TCC	ATG	GAT	TTT	GAT	GAC	ACA	ГGG	CAC	ССТ
81	A	A F	v	Ν	G	v	А	V	Н	S	М	D	F	D	D	т	W	Н	Р
301	GCCA	CCCA	СССТ	тст	GGG	GCT	GTC	СТА	ССТ	GTC	CTC	ACA	GCT	СТА	TCG	GAA	GCC	СТG	ССТ
101	А	т н	Р	s	G	А	v	\mathbf{L}	Ρ	V	\mathbf{L}	т	А	L	S	Е	А	L	Р
361	CAGA	СТСС	CAAG	TTT	TCT	GGC	СТС	GAC	CTG	СТС	CTG	GCG	TTC	AAC	GTT	GGT.	ATT	GAA	GTA
121	Q	т Р	Κ	F	S	G	\mathbf{L}	D	L	\mathbf{L}	\mathbf{L}	А	F	Ν	V	G	I	Е	v
421	CAGG	GACG	ATTA	ATG	CAC	TTC	тсс	AAG	GAA	GCC	AAA	GAC	ATA	CCA	AAGA	AGA	гтс	CAC	ССТ
141	Q	G R	L	М	Н	F	s	Κ	Е	А	Κ	D	I	Ρ	Κ	R	F	Н	Р
481	CCCT	CTGT	GGTG	GGG	ACT	CTG	GGA	AGT	GCT	GCI	GCT	GCG	TCC	AAG	TTTC	CTG	GGG	CTC.	AGC
161	Р	s v	v	G	т	\mathbf{L}	G	s	А	А	А	А	S	K	F	L	G	L	S
541	TTGA	CAAA	GTGC	CGC	GAG	GCA	TTG	GCT	ATT	GCI	GTT	TCC	CAC	GCA	GGGG	GCA	CCC	АТА	GCG
181	L	т к	С	R	Е	А	L	А	I	А	V	S	Н	А	G	А	Р	I	А
601	AACG	CTGC	CACT	CAG	ACT	AAG	ccc	CTT	CAT	ATT	GGC	AAT	GCA	GCC	AAG	CAT	GGG	ATG	GAA
201	N	А А	т	Q	т	K	Р	L	Н	I	G	N	А	А	K	н	G	М	Е
661	GCCA	CGTT	гстс	GCA	ATG	СТG	GGC	CTC	CAA	GGA	AAC	AAA	CAG	ATC	TTG	GAC	CTG	GGG	тса
221	А	т ғ	L	А	М	\mathbf{L}	G	\mathbf{L}	Q	G	N	K	0	I	\mathbf{L}	D	L	G	s
721	GGGT	TCGG	rgcc	TTC	TAT	GCC.	AAC	TAC	TCC	CCC	GAA	GAC	CTT	CCA	AGC	CTG	GAT	тст	CAC
241	G	F G	А	F	Y	А	N	Y	s	Р	Е	D	L	Ρ	S	L	D	s	Н
781	ATCT	GGCT	GTTG	GAC	CAG	CAG	GAT	GTG	GCC	TTT	'AAG	AGC	TTC	CCG	GCA	CAT	CTG	GCT.	ACC
261	I	wг	\mathbf{L}	D	Q	Q	D	v	А	F	K	S	F	Ρ	А	н	L	А	т
841	CACT	GGGT	GGCA	GAT	GCA	GCT	GCA	GCC	GTG	AGA	AAG	CAC	CTT	GTG	ACAG	CCA	GAA	AGA	GCC
281	Н	w v	А	D	А	А	А	А	v	R	K	Н	L	v	т	Р	Е	R	А
901	CTGT	TCCC	гGCТ	GAC	CAC	АТС	GAG	AGA	ATC	GTG	CTC	AGG	ATC	ССТ	GAC	GTC	CAG	TAC	GTA
301	L	F P	А	D	Н	I	Е	R	I	v	L	R	I	Ρ	D	v	Q	Y	v
961	AACA	GGCC	СТТС	CCG	GAC	тса	GAG	CAT	'GAA	GCC	CGT	CAT	TCT	TTC	CAG	TAT	GTG	GCC	TGT
321	N	R P	F	Р	D	s	Е	Н	Е	А	R	Н	s	F	Q	Y	v	А	С
1021	GCCT	CGCT	GCTC	GAC	GGT	AGC	АТС	ACT	GTC	CCA	TCC	TTC	CAC	AGC	CAG	CAG	GTC.	AAT	AGG
341	А	SL	\mathbf{L}	D	G	s	I	т	v	Р	S	F	Н	s	Q	Q	v	Ν	R
1081	CCTC	AGGT	GAGA	GAG	TTG	CTC.	AAG	AAG	GTG	AAG	CTG	GAG	CAT	ССТ	CCTC	GAC.	AAC	CCG	CCA
361	Р	o v	R	Е	\mathbf{L}	\mathbf{L}	К	K	v	К	\mathbf{L}	Е	Н	Р	Р	D	Ν	Р	Р
1141	AGCT	TCGA	CACG	СТА	TAC	TGT	GAA	АТА	AGC	ATC	ACT	СТА	AAG	GAC	GGG	ACC.	ACT	TTC	ACC
381	S	F D	т	L	Y	С	Е	I	S	I	т	L	К	D	G	т	т	F	т
1201	GAGC	GCTC	ГGAC	ACC	TTC	ТАТ	GGT	CAC	TGG	AGG	AAA	CCA	CTG	AGC	CAG	GAA	GAT	CTG	CGC
401	Е	R S	D	т	F	Y	G	Н	W	R	К	Р	L	S	0	Е	D	L	R
1261	AACA	AGTT	CCGA	GCC	AAT	GCC	тса	AAG	ATG	СТА	TGC	AGG	GAC	ACG	GTG	GAA	AGC	CTT	АТА
421	N	K F	R	A	N	A	S	K	M	L	C	R	D	T	V	E	S	L	I
1321	ACGG	TAGT	AGAA	AAG	CTA	GAA	GAC	СТА	GAA	GAC	TGC	тст	GTG	СТА	ACC	AGA	СТТ	CTG	AAA
441	Т	v v	Е	K	L	Е	D	L	Е	D	C	s	v	L	T	R	L	L	K
1381	GGAC	CCTC	rgtc	CAA	GAT	GAA	GСТ	TCA	AAA	СТА	TCC	AGC	ATG	TCC	TCA	TTC	GAT	CAC	ACA
461	G	PS	v	0	D	Е	A	S	ĸ	L	S	S	M	S	S	F	D	H	т
1441	ACGT	TGCC	CAGG	TTT	ACC	ААТ	ATC	TAA		_	-	-	-		-			-	
481	Т	L P	R	F	T	Ν	I	*											

Figure 2-10. Immunoresponsive Gene 1 DNA and corresponding amino acid sequences.

The first step of purification was elution form a chitin column. After loading supernatant (0.5 mL/min), the chitin column was washed with ~500 mL high-salt column buffer (20 mM Tris-HCl, 1.0 M NaCl, pH 8.5), then ~500 mL medium-salt column buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.5) at flow rate of 2 mL/min and finally quickly washed with 3 column volumes of DTT containing cleavage buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.5, 50 mM DTT). The on-column cleavage of the fusion protein was carried at 4 °C for 16-20 hours with 2 column volumes of DTT containing buffer to ensure a sufficient yield of IRG1 protein (room temperature was avoided because of the predicted low stability of the target protein). The target protein with the intein-tag cleaved was eluted from the chitin column and dialyzed at 4 °C. That solution was then applied to a QFF column. Proteins were eluted with a 0 to 0.5 M NaCl gradient (in 20 mM Tris-HCl, 1 mM DTT, pH 8.0). Fractions from 0.09 to 0.2 M NaCl gradient had the purest protein. Elution at that salt concentration is consistent with where cADC activity eluted in the partial purification from the RAW 264.7 cell extract. Recombinant IRG1 protein was collected and concentrated for storage. The best yield of IRG1 was 2.5 mg/L.

2.23 Cloning and Overexpression of Fungal cis-Aconitate Decarboxylase (cADC)

The *cis*-aconitate decarboxylase (cADC) gene from *Aspergillus terreus* was a generous gift from Dr. A. Li, TNO Microbiology and Systems Biology, The Netherlands. The gene and amino acid sequence are shown in Fig. 2-11. The cADC enzyme was theoretically calculated to be unstable by ProtParam with a computed instability index of 43.89.

Figure 2-11. A. terreus cis-aconitate decarboxylase DNA and corresponding amino acid sequences.

1 ATGACCAAGCAATCT GCGGACAGCAACGCA AAGTCAGGAGTTACG GCCGAAATATGCCAT 1 М Т К О Ѕ A D S N A K S G V T AEICH 61 TGGGCATCCAACCTG GCCACTGACGACATC CCTTCGGACGTATTA GAAAGAGCGAAATAC 21 W A S N L A T D D I PSDVL ERAK Y 121 CTGATTCTCGATGGT ATTGCATGTGCCTGG GTTGGTGCAAGAGTG CCTTGGTCAGAGAAG ILD G I A C A W V G A R V P W 41 L S \mathbf{E} Κ 181 TATGTGCAGGCAACA ATGAGCTTTGAGCCG CCAGGAGCCTGCAGG GTGATTGGATATGGG Q A T M S F E Ρ Ρ G A C 61 Y V R v Т G Y G 241 CAGAAACTGGGGCCT GTTGCAGCAGCCATG ACCAATTCCGCTTTC ATACAGGCCACAGAG 81 Q K L G P VAAAM т N S A F ΙΟΑΤ E 301 CTTGACGACTACCAC AGCGAAGCCCCCCTA CACTCTGCAAGCATC GTCCTCCCTGCGGTC 101 L D D Y H SEAPL H S Α S Ι V L P Α V 361 TTTGCAGCAAGTGAG GTCTTAGCCGAGCAA GGCAAAACAATTTCT GGTATAGATGTCATT 121 F A A S E VLAEQ GKTIS G I D V Т 421 CTAGCCGCCATTGTG GGGTTTGAATCTGGC CCGCGGATCGGCAAA GCAATTTACGGATCG 141 L A A I V GFESG Ρ R I G K A I Y G S 481 GACCTCTTGAACAAC GGCTGGCATTGTGGA GCCGTGTATGGTGCT CCAGCTGGTGCGCTG 161 D L L N N G W H C G А VYGA PAGAL 541 GCCACAGGAAAGCTC CTCGGTCTAACTCCA GACTCCATGGAAGAT GCTCTCGGAATCGCG G L T P 181 A T G K L L DSME A L D GΙ Α 601 TGCACGCAAGCCTGT GGTTTAATGTCGGCG CAATACGGAGGCATG GTCAAGCGCGTGCAA 201 C T Q A C G L M S A Q Y G G M V K R V 0 661 CATGGATTCGCAGCG CGTAATGGTCTTCTT GGGGGGACTGTTGGCC TATGGTGGGTACGAG 221 H G F A A RNGLL GGLLA ΥG G Y Ε 721 GCCATGAAGGGTGTC CTGGAGAGATCTTAT GGCGGTTTCCTCAAA ATGTTCACCAAGGGC LERSY 241 A M K G V GGFLK ΜF т к G 781 AATGGCAGAGAGCCT CCCTACAAAGAGGAG GAAGTGGTGGCCGGT CTCGGTTCATTCTGG 261 N G R E P ΡΥΚΕΕ EVVAG LG S F W 841 CATACCTTTACTATT CGCATCAAGCTCTAT GCCTGCTGCGGACTT GTCCATGGTCCAGTC 281 H TFTI R Ι K L Y A C С G L νн G Ρ V 901 GAAGCTATCGAAAAG CTTCAGAGGAGATAC CCCGAGCTCTTGAAT AGAGCCAACCTCAGC 301 E ΑΙΕ K LQRRY PELLN RANL S 961 AACATTCGCCATGTT TATGTACAGCTTTCA ACAGCCTCGAACAGT CACTGTGGATGGATA IRHV YVQL S 321 N т Α S Ν S H C G W Ι 1021 CCAGAGGAGAGGCCC ATCAGTTCAATCGCA GGGCAGATGAGTGTC GCATACATCCTCGCC 341 P E E R P ISSI Α G Q M S v A Y Α I L 1081 GTCCAGCTGGTCGAC CAGCAATGTCTTCTG GCTCAGTTTTCTGAG TTTGATGACAACTTG 361 V OLVD 0 0 C L L Α O F S E F D D Ν L 1141 GAGAGACCAGAAGTG TGGGATCTGGCCAGG AAGGTTACTCCATCT CATAGCGAAGAGTTT 381 E RPEV WDLAR Κ νт Р S Н S Ε Ε F 1201 GATCAAGACGGCAAC TGTCTCAGTGCGGGT CGCGTGAGGATTGAG TTCAACGATGGCTCT C L S A G 401 D Q D G N RVRIE F G Ν D S 1261 TCTGTTACGGAAACT GTCGAGAAGCCTCTT GGAGTCAAAGAGCCC ATGCCAAACGAACGG 421 S V T E T VEKPL G V K E P M P E Ν R 1321 ATTCTCCACAAATAC CGAACCCTTGCGGGT AGCGTGACGGACGAA TCCCGGGTGAAAGAG 441 I L H K Y RTLAG SVTDE S R V Κ Ε 1381 ATTGAGGATCTTGTC CTCAGCCTGGACAGG CTCACCGACATTACC CCATTGCTGGAGCTG 461 I E D L V LSLDR L ТОІТ PLL \mathbf{E} L 1441 CTTAATTGTCCCGTG AAATCGCCACTGGTA TAA 481 L N C P V Κ S Р L V

Figure 2-12. Purification of (A) IRG1 and (B) A. terreus cADC monitored by SDS-PAGE.



A maltose-binding protein (MBP) fusion was used for the fungal cADC cloning. The pMALTEV system allows the target gene to be cloned adjacent to the MBP tag with a TEV cleavage site. The N-terminal MBP fused protein is generated and purified on amylose resin based on the affinity of MBP for the resin matrix. The native recombinant protein without modifications is released by TEV enzyme induced cleavage of the MBP tag at 4 °C. Further chromatographic steps can lead to very pure protein.

A BamHI restriction site was used for cloning the 5'end of the cADC gene, with the forward primer 5'-ggcgGGATCCatgaccaaacaaagtgctgattctaacg-3' (BamHI site underlined). A XhoI restriction site was used for cloning the 3'end of target gene with the reverse primer 5'-gcggCTCGAGttacaccagcggggatttaaccggac-3' (XhoI site undelined). The target gene flanked with BamHI and XhoI sites was amplified by PCR and then inserted into the pMALTEV vector. The entire DNA sequence of the inserted target gene was sequenced by Genewiz using the MalE Primer (5'-ggtcgtcagactgtcgatgaagcc-3') and T7-term primer.

Cell growth was the same as for IRG1 expression. Cell extract supernatants after cell lysis by sonication and centrifugation at 15,000 rpm for 35 min showed an obviously overexpressed MBP-fused protein band corresponding to a MW of 97 kDa on SDS-PAGE gel (Fig. 2-12B). The lysis buffer used was 20 mM phosphate, pH 6.5, with 1 mM DTT, 1 mM PMSF, 5 μ L/mL protease inhibitor cocktail and 0.3 M NaCl. The protein was immobilized on the amylose beads by mixing crude extract supernatant gently with the resin, equilibrated in the same buffer, at 4 °C for around 3 h. The column formed from the treated resin was washed first with ~500 mL high-salt buffer (20 mM phosphate, 1

mM DTT, 0.5 M NaCl, pH 6.5) then with another ~500 mL of salt-gradient buffer (from 0.5 to 0.1 M NaCl). The MBP-fused protein was eluted with 3-4 mL (for 1 L cells) of the final column buffer (20 mM phosphate, 1 mM DTT, 0.1 M NaCl, pH 6.5) mixed with 10 mM maltose.

2.24 Kinetic Measurements of cADC Activity

For the kinetic assays of purified enzymes, the *cis*-aconitate reactions were quenched by NaOH as shown in Scheme II in Fig. 2-9. To obtain the pH optimum of IRG1 and fungal cADC, buffer at a particular pH was prepared with a MES-HEPES mixed buffer system. The pH range tested for IRG1 was 6.5-8.5, and that for fungal cADC was 5.0-8.0. The amount of enzyme used was adjusted so that <20% ITA production was observed. For determining the K_m , the enzymatic activities were monitored by increasing the concentration of substrate (*cis*-aconitic acid) from 0.1 to 20 mM for IRG1 and 1.0 to 20 mM for fungal cADC. For cofactor and inhibitor screening, the particular additive was mixed directly with the substrate solution in assay buffer at the optimal pH. The amount of cofactor added was based on reported values for those molecules for other decarboxylase. The ratio of potential inhibitor to substrate started 2:1 then decreased to 1:1 if any inhibition was observed.

2.25 Mutagenesis Studies of IRG1 and Fungal cADC

The construction of mutant proteins followed protocols used for the PI-PLC enzymes. All the primers used are shown in Table 2-4.

Mutant	Primer
Fungal cADC	
E100A	5'-cttttatccaagccacaGCActggacgattatcac-3'
Y104A	5'-ccacagaactggacgat <u>GC</u> Tcacagtgaggcg-3'
H111A	5'-gtgaggcgccgctg <u>GC</u> Ctcagcgtccattg-3'
H168A	5'-gctgaacaacggctgg <u>GC</u> Ttgtggtgcagtatacg-3'
K217A	5'-tacggcggtatggtg <u>GC</u> Gcgtgtccaacacgg-3'
K288A	5'-cttttactattcgtatc <u>GC</u> Gctgtacgcatgctg-3'
H396A	5'-cgtaaagtcacaccgagt <u>GC</u> Cagcgaggagttcg-3'
K428A	5'-caccgagactgtcgaa <u>GC</u> Gccgctgggtgtg-3'
IRG1	
D93A	5'-gctgttcactccatgGCTtttgatgacacatgg-3'
H103A	5'-ggcaccctgccacc <u>GC</u> Cccttctggggctg-3'
H159A	5'-gacataccaaagagattc <u>GC</u> Ccctccctctgtg-3'
K207A	5'-cgctgccactcagact <u>GC</u> Gccccttcatattgg-3'
K272A	5'-gcaggatgtggccttt <u>GC</u> Gagcttcccggcac-3'

<u>Table 2-4.</u> Primers used in site-directed mutagenesis with desired mutation codons in capital and substitution bases underlined (reverse primers are not listed).

Chapter 3

Role of Pro245 and Pro254 in Membrane Binding and Enzymatic Activity of *Bacillus* Phosphatidylinositol-specific Phospholipase C

3.1 Introduction

Peripheral membrane proteins interact transiently with bilayers. Often the interactions occur via loops whose conformations can change prior to or during membrane binding. B. thuringiensis PI-PLC (BtPI-PLC) fits into this category with loops above the active site (in the center of the $\alpha\beta$ -barrel) that might be important for membrane binding. Initial NAMD simulations of *Bt*PI-PLC in solution, carried out by Prof. Patrick Wintrode of the Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, were used to investigate the importance of PI-PLC dynamics, focusing on the loops, in membrane binding and enzymatic activity. He started with the X-ray crystal structure of free Bacillus cereus PI-PLC [28]. The MD simulations for this Bacillus PI-PLC alone (no membranes present) showed anti-correlated motions between the first two-thirds and last one-third of the PI-PLC structure, essentially the 2 halves of the β barrel (Fig. 3-1). Such anti-correlated barrel motions were not observed in MD simulations for the intracellular bacterial pathogen L. monocytogenes PI-PLC (data not shown). In addition, Bacillus PI-PLC showed coordinated motion closing of loops over the active site. Multiple sequence alignments (MSA) revealed that a number of residues in these loops are conserved in Bacillus, Staphylococcus and other extracellular bacterial pathogens, but not in Listeria (this analysis was done by Prof. Anne Gershenson, Department of Biochemistry & Molecular Biology, University of Massachusetts, Amherst, Massachusetts). Both the MD simulations and conservation results suggested that proline residues 245 (cap) and 254 (kink) in helix G of *Bacillus* PI-PLC might be important for the correlated motions. Considering this, we constructed a series of mutants for Pro245 and Pro254 in B.

thuringiensis PI-PLC. Both *in silico* and *in vitro* mutagenesis studies were applied to identify the roles of these two proline residues in membrane binding and enzymatic activity. Further tests of the role of dynamics in PI-PLC activity were performed by altering the flexibility of the 190s loop. This loop is far from the active site and membrane binding regions of PI-PLC, but the simulations suggest that it plays a role in PI-PLC clamshell motions. Mutations in the 190s loop aimed at altering loop flexibility were designed to study the influence of loop motions on enzyme activity and membrane association.

3.2 MD Simulations and Correlated Motions

The MD simulations, calculation of the root mean square fluctuations (RMSF) and principal component analyses (PCA) were done by Prof. Wintrode. He was trying to determine whether any of the dynamic motions could be correlated with activity and/or binding to membranes [156]. Larger scale motions observed in the simulations of WT included a clamshell-like opening and closing behavior that involved many components (highlighted in red in Fig. 3-1A), including the 190s loop (residues 188-192), the helix B region (residues 39-47), as well as the loops N-terminal to helix F (residues 201-203) and helix G (residues 238-245). This collective motion in which the loops and helix B open and close over the active site is associated with the kinking of helix G about Pro254. Previous work also identified the helices B and G as important for PI-PLC membrane binding [30,34,146]. In the triosephosphate iosomerase (TIM) barrel superfamily, the loop between strand 7 and helix G is often part of a phosphate-binding motif [157]. Dynamics of this loop are also important in the activity of other TIM barrels. In

triosephosphate iosomerase, the corresponding loop is the active site lid [158] while motions of this same loop, containing the catalytic Cys319, is important for activity and allostery in inosine monophosphate dehydrogenase (IMPDH) [159].

If helix B, F and G regions that close over the active site are important for enzyme activity, there might be significant sequence conservation in these regions. Analysis of the PI-PLC MSA showed that this region is quite divergent in the PI-PLC family, consistent with the diversity of lipid binding specificities. However, clustering the MSA using just the helix G region revealed that the phosphate-binding loop is relatively conserved in secreted PI-PLCs from extracellular bacterial pathogens and soil bacteria (Fig. 3-1D). In these species, the proline residue in helix G associated with kinking, Pro254, is 100% conserved while the proline that caps helix G, Pro245, is less conserved. The conservation in this region as well as its association with membrane binding led us to mutate these proline residues both in silico and in vitro. We used 3 types of mutations: (i) Pro to Gly which may increase flexibility and thus dynamics, (ii) Pro to Ala aimed at either uncapping or unkinking helix G and (iii) Pro to Tyr mutants designed to alter phospholipid binding. An additional Tyr residue near or in helix G, which is already rich in Tyr residues associated with membrane binding [146], should increase the probability of cation- π interactions with choline moieties in the membrane [34,160,161].

<u>Figure 3-1.</u> Structure, dynamics and conservation of *Bacillus* PI-PLC. (A) The ribbon diagram of crystal structure of *B. cereus* PI-PLC (PDB 1PTG) made using UCSF Chimera [162] shows the N-terminal and C-terminal halves of the β barrel in light blue and dark blue, respectively, helices F and G in turquoise. Regions that show large fluctuations in the WT MD simulations are in dark red and the active site is indicated by histidine residues 32 and 82 (blue) and myo-inositol (green). The two Pro residues of interest are shown in pink. RMSFs from the MD simulations in solution for (B) P(cap) variants and (C) P(kink) variants with WT (black), Pro to Ala variants (cyan) and Pro to Tyr variants (red). Regions with the largest fluctuations are highlighted in gray. (D) A selection from the multiple sequence alignment clustered using the helix G region from Jalview [163] with Blosum62-based coloring. Higher conservation is reflected as darker shading. The abbreviations are: *Bt, Bacillus thuringiensis; Ba, Bacillus anthracis; Sa, Staphylococcus aureus; Ea, Erwinia amylovora (fireblight); Ssp, Streptomyces species; Lm, Listeria monocytogenes.*



In the simulations, all of the Pro variants show reduced RMSFs relative to wildtype particularly for the 190s loop (Fig. 3-1B and C). For the P(cap) mutants, the anticorrelation between movements of the N-terminal two-thirds of the protein and Cterminal one-third is lost. In a similar vein, the clamshell-like motion is damped in P(kink)Y and essentially disappears in P(kink)A and the loop motions over the active site appear much less coordinated in both kink variants, particularly in P(kink)Y. These significant changes in the MD simulations suggested that mutating either of these Pro residues would alter *Bacillus* PI-PLC activity and/or lipid binding. Similar clamshell motions in the PCA and chained correlations, are observed in MD simulations of PI-PLC bound to DMPC membranes (data not shown), suggesting that the clamshell motion and associated correlations may be important both in solution and on the membrane.

3.3 Purification, Secondary Structure and Thermostability

The mutant PI-PLCs were designed and purified as described in sections 2.3 and 2.4. Formation of the disulfide bond in the double-cysteine variant, Asn226Cys/Val192Cys (N226C/V192C) was confirmed using non-reducing SDS-PAGE that will show a gel shift when the intermolecular disulfide bond forms (data not shown). Secondary structure content and thermal stability of the PI-PLC variants were measured and all of the PI-PLC variants showed similar CD spectra and the secondary structure content (Fig. 3-2). Thermal stabilities for all PI-PLC variants were similar to WT with melting temperatures (T_m) within 1.5 °C of the T_m for WT (54±1 °C), except for P254G, which is slightly less stable (T_m = 51 °C). Thus, any differences in activity or binding are not due to significant alterations in protein structure.
<u>Figure 3-2.</u> Comparison of CD spectra for WT (\blacksquare) and P(kink)A (\bigcirc). Similar agreement with the WT far UV CD data is observed for all of the PI-PLC mutants.



3.4 Phosphotransferase Activity in Micelles

PI dispersed in diC₇PC micelles is a better PI-PLC phosphotransferase substrate than PI presented in TX-100 micelles [10,43], and the same trend is observed for the Pro variants. The P(cap) variants all show decreased activity toward PI/TX-100 mixed micelles, with P(cap)A retaining the most activity (Fig. 3-3A). When PI is solubilized in diC₇PC micelles, enzymatic activity is enhanced, but the cap variants still exhibit lower activity than WT. The kink variants exhibit a very different profile with the mixed micelles. For PI/TX-100, P(kink)A was significantly more active than WT (990±170 versus 620±150 µmol min⁻¹ mg⁻¹) while P(kink)G and P(kink)Y have lower activity than WT. For PI in diC₇PC micelles, activities of the kink variants are equivalent to WT. Combining the two Ala mutations, P(cap)A/P(kink)A, results in a variant with ~50% of the P(cap)A activity towards PI in both TX-100 and diC₇PC micelles despite the benign effects of the P(kink)A mutation.

3.5 Phosphodiesterase Activity in the Absence and Presence of Micelles

The second step in the PI-PLC reaction, cIP hydrolysis to I-1-P, can be mimicked by incubating soluble cIP with PI-PLC. Although the enzyme, substrate and product in this reaction are all soluble, addition of PC micelles greatly enhances activity (Fig. 3-3B) [43]. For the cap variants, changes in cIP cleavage rates are complex. In the absence of diC₇PC, P(cap)A and P(cap)G displayed ~50% and ~10% the specific activity of WT, respectively. In contrast, the specific activity of P(cap)Y was ~2-fold higher than WT. This higher activity may reflect improved cIP binding, which is quite weak for WT [34]. In many proteins that bind a myo-inositol moiety, including PI-PLC, a tyrosine is seen to

stack with the inositol ring and its removal destabilizes binding [28,29]. Placing a Tyr in the cap position may aid in positioning cIP in the active site. Addition of diC₇PC increased the activity for all of the cap variants, but the activities were still far below WT. For the kink variants, specific activities were at least 50% of WT and the cIP hydrolysis rate with micellar diC₇PC present increased 24 ± 2 times, similar to that observed for WT PI-PLC. The double Pro variant, P(cap)A/P(kink)A, behaved similarly to P(cap)A suggesting that the cap mutation overrides the increase in activity towards cIP observed for the P(kink)A variant.

For both types of micelle-based assays, the cap variants show greater reductions in activity than the kink variants. The cIP hydrolysis results for the P(cap)A/P(kink)A suggest that the cap is key for activity towards the soluble substrate. Cap mutations also significantly reduce the activity enhancement observed in the presence of PC, suggesting that the cap plays an important role in enhancement of PI-PLC enzymatic activity by PC, perhaps related to the barrel breathing motions observed in the WT simulation that disappear in the simulations for the cap variants.

Figure 3-3. Specific activities of PI-PLC variants towards PI solubilized in micelles and the soluble substrate cIP. (A) Specific activity for PI cleavage in mixed micelles containing 8 mM PI in 16 mM TX-100 (\blacksquare) or in 32 mM diC₇PC (\boxtimes); (B) Specific activity for cleavage of 20 mM cIP in the absence (\blacksquare) and presence (\boxtimes) of 8 mM diC₇PC. The error bars are the S.D. of the specific activities.



3.6 Binding and Phosphotransferase Activity for Vesicles

PI-PLC partitioning to vesicles is inextricably linked to activity, and PC content significantly enhances binding. PI-PLC prefers SUVs over the less curved large unilamellar vesicles [145], and WT PI-PLC has an apparent K_d of 4-6 mM for pure PG SUVs in PBS buffer. Addition of PC leads to substantial drops in K_d for X_{PC} =0.1-0.5 and increases in enzymatic activity. The extent of the activity increase is dependent on the PI concentration with respect to K_d. For 8-10 mM PI, concentrations considerably above the apparent K_d, there is a 2-3-fold increase in specific activity with the addition of PC suggesting this non-substrate phospholipid activates the enzyme by more than just anchoring the protein to the vesicle [145,146]. However, while the apparent K_d continues to drop, reaching a minimum of ~1 mM at X_{PC}=0.8, activity is greatly reduced for X_{PC}>0.5. Very tight binding sequesters the enzyme on the vesicle surface, apparently in PC rich regions, leading to reduced activity [145]. How are SUV activity and binding related for the Pro variants?

Despite its reduced activity in the micelle assays, the apparent K_d of P(cap)A for SUVs is virtually identical to that of WT for pure PG SUVs and only 2-3 times higher than WT in the presence of PC (Fig. 3-4A). Specific activities of P(cap)A towards PI/PC SUVs were almost the same as WT across the X_{PC} range examined (Fig. 3-4B). In contrast to the Ala variant, the P(cap)Y variant binds much less tightly to SUVs, with apparent K_d values between 0.5 and 0.9 X_{PC} that are more than an order of magnitude higher than WT (Fig. 3-4A). This loss of binding affinity is associated with reduced activity of P(cap)Y for PI in SUVs, although the concentration of substrate in SUVs was well above the

apparent K_d values. However, what is striking for P(cap)Y is that the specific activity does not drop at X_{PC} =0.8 where WT activity drops ~50%, in part because of sequestration of the enzyme in PC-rich regions [145]. Rather the specific activity of P(cap)Y is constant in the range of X_{PC} =0.2 to 0.8. Lower affinity for SUVs may be an advantage at high X_{PC} allowing the enzyme to access the next substrate lipid [146].

The kink variants behave quite differently (Fig. 3-4). P(kink)A is relatively nonperturbing with binding affinities and activities that are similar to WT. P(kink)Y binding affinities are similar to WT except for X_{PC} =0.5-0.6 where the apparent K_ds are 2-3 times higher. These differences in K_d would not be noteworthy, except for the anomalous activity of P(kink)Y towards both pure PI SUVs, where its activity is only 60% that of WT despite nearly identical apparent K_ds, and its activity towards SUVs with X_{PC}=0.5 (1:1 PI/PC) where it, reproducibly, has 1.9 times WT specific activity. This increase in activity is not readily explained by its slightly lower affinity for SUVs. The lower specific activity of P(kink)Y in the absence of PC for both pure PI SUVs and cIP, as well as the generally reduced activity of P(kink)Y despite lipid binding affinities that are similar to WT suggest that freezing PI-PLC motions are particularly detrimental for activity.

For these FCS binding experiments, a single Cys residue, N168C, was introduced into the proteins. Residue 168 is far from both the PI-PLC membrane binding region and active site, and fluorescent labeling has only minor effects on binding and activity (Table 3-1).

Figure 3-4. Membrane binding and activity towards SUVs. (A) Binding of PI-PLC variants to DOPG/POPC SUVs as a function of the mole fraction PC, X_{PC} . (B) Variation of the specific activity of PI-PLC Pro variants towards PI/POPC SUVs with 8 mM PI and varying X_{PC} : WT (\bullet), P(cap)A (\blacksquare), P(cap)Y (\Box), P(kink)A (\blacktriangle), and P(kink)Y (\triangle). The error bars are the S.D. determined from two independent protein and SUV preparations.



PI-PLC Variants ^a	Specific Activity (µmol min ⁻¹ mg ⁻¹)		PC activation ^d	
	PI ^{b,c}	PI/PC (4:1)		
WT	40±12	460±40	12	
N168C	23±7	430±60	19	
P(cap)A	30±9	410±20	14	
N168C/P(cap)A	45±14	500±40	11	
P(cap)G	5±1	130±30	29	
P(cap)Y	6±2	160±20	28	
N168C/P(cap)Y	9±3	210±20	23	
P(kink)A	48±14	480±50	10	
N168C/P(kink)A	31±9	470±30	15	
P(kink)G	36±11	500±10	15	
P(kink)Y	24±7	440±20	18	
N168C/P(kink)Y	26±8	470±20	18	

<u>Table 3-1.</u> PI-PLC specific activities towards SUVs. The lipid compositions are PI (8 mM) and PI/PC (8 mM/2 mM).

^{*a*} P(cap) = Pro245 and P(kink) = Pro254.

^b The low activities for these PI-PLCs towards pure PI SUVs have much larger errors, typically 30% if different batches of SUVs are used, so the uncertainties represent 30% of the average value for each mutant (obtained in many cases on different SUV preparations).

^c For a given variant, the activity with or without the N168C mutation is within the uncertainty.

^d PC activation is the ratio of the specific activity towards PI/PC SUVs divided by the specific activity towards pure PI SUVs.

3.7 Restricting Dynamics of the 190s Loop

While the simulations suggest that mutating the Pro residues in helix G alters dynamics, thus affecting enzymatic activity and membrane binding as discussed above, crosslinking the loops involved in the clamshell-like motions would provide a more direct test of the relationship between dynamics and activity. Unfortunately, many of the structural elements that contribute to motions are known to be crucial for binding and activity, e.g., helix B and the phosphate-binding loop. We therefore chose to alter the dynamics of the 190s loop on the other side of PI-PLC, a region of the protein not previously associated with enzymatic activity or membrane binding because it is far from both the active site and the lipid binding regions containing helices B, F and G and associated loops (Fig. 3-1A). Nonetheless, obvious, pendulum-like motions of this loop are correlated with the motions in the helix B, F and G regions, and constraining this loop should alter PI-PLC dynamics. Two sets of mutations were designed to alter 190s loop motions: (i) the loop was constrained in the V192C/N226C construct by introducing a disulfide bond between the 190s loop and an adjacent loop, and (ii) the flexibility of the 190s loop was altered by introducing a Pro residue between Gln190 and Asn191 (Q190 P N191). The changes of enzyme structure caused by these mutations would not have large effect on the enzymatic activity since the 190s loop is fay away from the active site. Despite the distance of this loop from the PI-PLC active site and membrane binding regions, both variants display less than 40% of the wild-type activity towards pure PI SUVs (Fig. 3-5), lower activity than all of the helix G Pro variants except for P(cap)Y which has only 15% of the WT activity. The activity deficits of the 190s loop

<u>Figure 3-5.</u> Constraining the 190s loop lowers activity towards pure PI SUVs. Specific activities of the 190s loop variants towards SUVs with increasing PC content: WT (black), V192C/N226C (white), and Q190_P_N191(gray).



variants are rescued at 0.2 X_{PC} , while at 0.8 X_{PC} the disulfide-bonded variant has slightly more activity than WT and Q190_P_N191 displays 70% of the WT activity. The activity trend for the 190s loop variants resemble those of the P(kink) variants suggesting that these two very different sets of mutations may have similar effects on PI-PLC dynamics and activity.

3.8 Discussion

Experimentally measuring changes in dynamics is particularly difficult for peripheral membrane proteins such as PI-PLCs that dynamically associate and dissociate from the interface. For these enzymes, catalysis requires two discrete steps, the initial partitioning of the protein to the bilayer surface followed by docking of a substrate into the active site in the optimal orientation. MD simulations provide a way to predict how mutations may alter the dynamics of the free protein conformation that first interacts with the membrane and the effects of interesting mutations on protein partitioning and/or activity can then be determined experimentally. Naively, one might expect that alterations in the free protein dynamics would compromise binding but have little effect on the activity of the membrane bound enzyme. However, for PI-PLC variants that are predicted to have altered dynamics, activity is more likely to be perturbed than binding. While partitioning to the membrane likely alters the conformation and/or dynamics of the mobile loops and helices that contact the interface, much of the protein is not in contact with the surface and some of the lowest energy modes may still be accessible, as observed for the MD simulations performed in the presence of a membrane. Constraints on the conformation

and dynamics of the bound protein may also be different between SUVs and the even more highly curved micelles.

The relative conservation of the putative PC binding regions in extracellular bacterial pathogens, the likely importance of this region in membrane binding and the correlated loop motions observed in the MD simulations focused our attention on the helix B and G regions. Helix G is a long helix containing several Tyr residues known to be important for interfacial binding [30,34]. Pro mutations in this helix do disrupt the correlated motions observed in the simulations. P(cap) (Pro245), the Pro cap for helix G, is >9 Å from the active site and could directly interact with substrate as it enters the active site in addition to directly affecting membrane binding as well as protein conformation and dynamics. In contrast, P(kink) (Pro254), the Pro in the middle of helix G around which the helix kinks in the MD simulations, is >15 Å distance from the active site, and both this distance and its location in the helix preclude direct interactions with substrates. Since no significant effects on binding are observed for the P(kink) variants, changes in activity are likely due to altered protein conformations or dynamics.

Despite similarities in how the Pro mutations reduce the RMSF of loops in the MD simulations (Fig. 3-1B and C), the P(cap) variants generally have larger effects on PI-PLC activity and binding than do the P(kink) variants. The Pro cap is N terminal to Tyr residues 246-248 that are important for binding to membranes containing PC [34,146]. Mutagenesis of all three of these Tyr residues to Ser results in low binding affinity for and activity towards SUVs containing PC [146]. Interestingly, of the cap variants, P(cap)Y, which might have been expected to aid in π -choline cation interactions with PC,

exhibits the lowest affinity for PC-rich SUVs. The loss in activity of the P(cap) variants is also observed for micelle based assays and activity towards the soluble substrate cIP. Removing Pro245 likely alters the accessible conformations near the N terminus of helix G, reducing the probability that the Tyr residues bind to the membrane. Alternatively, the alterations in dynamics observed in this P(cap) variant MD simulations could also reduce the active site binding affinity for substrate.

While in silico mutagenesis of P(kink) (Pro254), the Pro about which helix G flexes, has large effects on the MD simulations, damping fluctuations throughout the protein, the P(kink) variants show much smaller deficits in activity and binding. In the P(kink)A MD simulations, the extended rather than kinked helix G freezes motions in a manner that leaves the active site open. This dynamical change would favor substrate access to the active site resulting in little change in activity or overall vesicle binding, as observed experimentally. In WT, helix B as well as the helix F and G loops close and open over the active site in a coordinated manner; however, loop motion is uncoordinated in P(kink)Y, and in the first principal component occlusion of the active site appears more likely. This would translate to the observed lower specific activity of P(kink)Y in the absence of PC (where only active site binding occurs). In the presence of PC, the helix G region, which has been implicated in binding PC, would be more constrained, presumably favoring an open active site. This analysis argues that enhancement of activity by PC results because PC binding constrains both the helix G and the helix B regions, which would otherwise intermittently limit access to the active site. This picture is supported by mutations in the 190s loop designed to restrict dynamics. Decreases in activity are largest in the absence of PC, and rescued by low mole percent PC again suggesting that one of the roles of PC is to subtly alter PI-PLC dynamics.

For both the P(kink) and P(cap) variants, changes in activity are not consistently correlated with changes in binding. The P(kink) variants bind to vesicles with apparent K_{ds} that are similar to wild-type protein despite changes in activity, while defects in P(cap)Y activity appear to be larger than would be predicted from the K_{d} increase. However, in all cases, changes in activity appear to be correlated with the changes in enzyme dynamics observed in the free protein simulations. Some studies of protein models suggested that the dynamic motions were often intrinsic to the protein structure, independent of the membrane environment [164]. This finding is supported by results from the protein plus membrane simulations (data not shown). Thus, despite the constraints imposed by interfacial binding, the dynamics and protein modes of free PI-PLC in solution are likely still relevant for the membrane bound protein.

3.9 Conclusions

The combination of MD simulations, *in silico* and *in vitro* mutagenesis along with binding and activity assays provides a role for loop and helix motions in regulating the activity of *Bacillus* PI-PLC. In this model, the coordinated closing of the helix B region and helix G region in the free enzyme limits access to the active site, while PC binding in the region of helix G leads to an open active site. Structural alignments of the *Bacillus* PI-PLC [28] with the well-studied yeast TIM [165,166] as well as parasite IMPDHs [167,168] show that the phosphate-binding loop N-terminal to helix G in PI-PLC aligns with the active site lid in yeast TIM and the dynamic loop containing catalytic Cys319 in

IMPDH from two parasites, *Tritrichomonas foetus* and *Cryptosporidium parvum*. Interestingly, while the helix C terminal to these loops is capped by a Pro residue in yeast TIM, Pro in this position is only slightly conserved in TIM [169,170]. The corresponding helix is capped by Pro in *C. parvum* IMPDH but not *T. foetus* IMPDH. This is reminiscent of extracellular bacterial PI-PLCs where helix G is capped by Pro245 in *Bacillus* PI-PLC but not in PI-PLC from *Staphylococcus aureus* (Fig. 3-1D). Thus, there are many ways to control the motions of this loop and associated allostery in TIM barrels, including a balance of loop rigidity and flexibility in TIM [171], ion binding in parasite IMPDH [159] and the cation- π latch in *S. aureus* PI-PLC (refer to Chapter 4). The Pro cap on helix G may be one more non-conserved way to modulate loop motions and allostery in TIM barrels. Chapter 4

Cloning, Expression and Characterization of

Staphylococcus aureus PI-PLC

4.1 Introduction

Secreted bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes contribute to virulence in pathogenic bacteria. Several of the PI-PLCs are usually activated by the zwitterionic phospholipid phosphatidylcholine (PC) [10,38,39]. Intriguingly, the activation mechanism varies among the PI-PLCs. *Bacillus* PI-PLC enzymes exhibit high affinity for PC interfaces but *Listeria* PI-PLC is activated due to lipid dilution effect and shows very low affinity for PC [34, 39]. *Staphylococcus aureus* PI-PLC (*Sa*PI-PLC), a likely virulence factor whose production was shown to be upregulated in a study of methicillin-resistance *S. aureus* [23], shows 41.3% sequence similarity with *B. thuringiensis* PI-PLC but presents yet another wrinkle in the properties of these exoproteins.

In this study, the wild-type *Sa*PI-PLC gene was cloned into a His-tagged overexpression system from genomic DNA of *S. aureus* (strain FPR3757) for further expression and purification. The enzymatic activities and membrane association of *Sa*PI-PLC as a function of enzyme concentration, pH, and vesicle composition were examined in comparison with *Bt*PI-PLC. Meanwhile, the structure of *Sa*PI-PLC solved by Rebecca Goldstein, one of my colleagues, suggested the enzyme could form transient dimers on the membrane surface. To that end, a series of mutations was designed to test the hypotheses about dimerization of *Sa*PI-PLC. The results showed that *Sa*PI-PLC has evolved a complex, apparently unique way to control its access to PI/GPI substrate.

4.2 Purification, Secondary Structure and Thermostability

The overexpression and purification of recombinant *Sa*PI-PLC are described in detail in sections 2.3 and 2.4 of this thesis. Secondary structure and thermal stability of *Sa*PI-PLC variants were determined using far-UV CD. Compared with recombinant wild type *Sa*PI-PLC (WT), there are no significant changes in structure and thermal stability for the single mutation enzymes (Table 4-1, Fig. 4-1). Although Y253S/Y255S has the same secondary structure content, its thermal denaturation temperature is 5 °C lower than WT.

4.3 Testing the Role of a Cation- π Latched Loop in SaPI-PLC

4.3.1 Two SaPI-PLC Structures at Acidic and Basic pH

Rebecca Goldstein determined two discrete crystal structures of this enzyme at pH 4.6 and pH 7.5 (as shown in Fig. 4-2A), and found that under acidic conditions, a large section of mobile loop at the $\alpha\beta$ -barrel rim in the vicinity of the active site shows ~10 Å shift. This is due to a titratable intramolecular cation- π interaction between His258 and Phe249 since the loop shift does not exist in the mutant protein H258Y. To explore how the two conformations are related to enzymatic activity, we measured PI-PLC activity toward SUVs composed of either pure PI or PI/PC (4:1) at pH 5.5, 6.5, and 7.5 (as shown in Fig. 4-3). Considering the enzyme mechanism also involves histidine residues, we examined the activity of H258Y, which cannot form the cation- π latch as a control.

Protein	Secondary Structure				T (%0)
	α-Helix	β-Sheet	<i>β</i> -Turn	Random Coil	I _m (°C)
			%		
WT	19.4	32.2	16.8	31.7	62.8
F249W	19.8	31.8	16.9	31.6	61.8
Y253S/Y255S	19.4	32.1	16.8	31.6	57.7
N254Y/H258Y	19.0	32.4	16.8	31.7	62.4

Table 4-1. Comparison of secondary structure content of WT and selected mutant SaPI-PLC enzymes.

<u>Figure 4-1.</u> Comparison of CD spectra for WT and selected mutant *Sa*PI-PLC enzymes: WT(\blacksquare), F249W(\Box), Y253S/Y255S(\bigcirc) and N254Y/H258Y(\triangle). Similar agreement with the WT far UV CD data is observed for all of the PI-PLC mutants.



4.3.2 Influence of Cation- π Interaction on PI Cleavage

A comparison of activities toward PI vesicles at pH 5.5, 6.5, and 7.5 shows that the recombinant enzyme has higher activity at pH 5.5 than H258Y (Fig. 4-3A), which indicated that this inward-folded rim loop position at acidic pH was associated with higher enzymatic activity. Specific activities are similar and lower for both enzymes as the pH increases (and presumably the His–cation- π interaction is broken because of the histidine deprotonation). In the structure, this cation- π latch can enable water-soluble product cIP diffuse from the active site and could allow for processive catalysis for the enzyme bound to membranes under acidic conditions.

4.3.3 Effect of Other Phospholipids on PI Cleavage

When 20 mol% PC is present in the vesicles, the activities are low at pH 5.5 but increase as the pH is raised (Fig. 4-3B). This indicated that other more subtle conformational changes in the protein occur with PC added and the pKa values for the active site histidines may be shifted. The higher activity of WT *Sa*PI-PLC at pH 6.5 suggests that the presence of that zwitterionic phospholipid PC provides cationic choline moieties to compete with cationic His258 for interactions with the Phe249 π -system. This could extend the loop structure allowing better partitioning of the Phe side chain into membranes which in turn could lead to more efficient PI cleavage and product release.

Consistent with the idea that the choline cation competes with His258, we have examined the effect of different short chain phospholipids on cleavage of PI dispersed in Triton X-100 micelles. The use of the Triton micelle matrix and micelle forming additives should avoid changes in bilayer behavior that may occur with the different long-chain phospholipids (PE in particular). In this qualitative turbidity assay it is clear that both diC_7PC and diC_8PE activate the enzyme above what is observed with the PI dispersed in TX-100, while diC_8PG and diC_8PS are inhibitory (roughly 10-fold less active than with the short-chain PC added), presumably by binding to the active site. The amino group of PE, like the trimethylammonium group of PC, could interact with Phe249 and similarly alter pKa values or lead to a more productive conformation involving that rim loop.

4.4 Influence of Functional Oligomerization on SaPI-PLC Enzymatic Activity

4.4.1 SaPI-PLC Dimer Structure

Rebecca Goldstein serendipitously found that SaPI-PLC crystallized as a complete homodimer in the presence of diC₄PC and Mg(NO₃)₂ at acidic pH. The dimer interface is a hydrophobic box formed by the direct interaction of the side chains belonging to helix B of each monomer (Val41 and Val44, in Fig. 4-4A and B). Besides this valine box, there are several other interactions that aid in the dimerization, for example the ionic bonding between Lys38 and Asp50 and hydrogen bonding between Thr36 and Tyr285. On the other hand, in the monomeric structure, there is an anion-binding pocket adjacent to helix B made up of Asp38, Lys39, His86 and Lys42 (Fig. 4-4C). However, in the dimer, helix B of one monomer occludes the pocket of the other, limiting water-soluble or membrane-bound anion binding. Thus, in the dimer, this unoccupied positively charged region could contribute to electrostatic steering to anionic phospholipids, but the discrete binding pocket is no longer accessible. Figure 4-2. Overlay of the acidic pH (green, PDB 3V16) and basic pH (blue, PDB 3V18) structures of *Sa*PI-PLC. The red circle highlights the mobile rim loop and the large conformational change in it with pH. Key residues forming the cation- π interaction are labeled. myo-Inositol is depicted in red space-filling representation and the chloride ion as a green sphere. The C-terminus, N-terminus and helix B are labeled. A gray bar represents the membrane interface.



Figure 4-3. The effect of conformational changes between acidic and basic structures on enzymatic activity. The pH dependence of the specific activity of *Sa*PI-PLC (\blacksquare) and the H258Y (\Box) mutant toward (A) 4 mM PI and (B) PI/PC (4 mM:1 mM) small unilamellar vesicles in 50 mM MES/HEPES, 1 mM EDTA with 0.1 mg/mL BSA adjusted to the indicated pH.



4.4.2 SaPI-PLC Enzymatic Activity: Testing for a Functional Oligomer

The pH optimum for SaPI-PLC enzymatic activity depends on the SUV composition (Fig. 4-5A). Toward pure PI SUVs, the pH optimum is pH 5.5-6, whereas for vesicles with 0.5 X_{PC} , the optimum is shifted to pH 6.5–7. Thus, most assays were carried out at pH 6.5. Specific activity increases dramatically when PC is present in the bilayer. Similar PC enhanced activity and a shift in the pH profile were observed with PI dispersed in micelles of diC₇PC versus Triton X-100 (Fig. 4-5C). Unlike *Bt*PI-PLC, where specific activity decreases when $X_{PC}=0.5$ [146], no surface dilution inhibition is observed for SaPI-PLC with increasing PC content up to 0.8 X_{PC} , either for fixed total phospholipid (Fig. 4-5B) or fixed PI with increasing amounts of PC. Because the dimer structure with its occluded anion-binding site was obtained in the presence of PC, SaPI-PLC salt sensitivity might be ameliorated by PC. Indeed, the presence of PC rescues the loss of activity observed at moderate salt concentrations (Fig. 4-5B), and higher X_{PC} results in lower salt sensitivity. This is consistent with a transient dimer that has lost the ability to bind soluble anions in the anion-binding pocket. That this is an interfacial phenomenon is shown by cIP kinetics (Fig. 4-5D), which exhibits both only a small activation by diC7PC micelles and only about a 2-fold drop in specific activity with added salt.

A more direct indication of a functional dimer is the observation that, in the absence of salt, *Sa*PI-PLC specific activity is dependent on enzyme concentration, particularly when the substrate is pure PI SUVs (Fig. 4-6A). For comparison, the specific activity of *Bt*PI-PLC, which is unlikely to form the same dimer through helix B, does not depend on enzyme concentration and is only weakly salt dependent (Fig. 4-6B). If PC is present in the bilayer, there is still an increase in *Sa*PI-PLC-specific activity with increasing enzyme concentration, but this reaches an optimal value at a much lower enzyme concentration (Fig. 4-6C). This dependence of PI-PLC specific activity on protein concentration strongly suggests that the optimally active form of the enzyme is a dimer. The presence of PC in the membrane must shift the equilibrium between protein monomer and oligomer to lower protein concentrations.

As a further test of this hypothesis, I mutated Val44, one of the key components of the dimer interface in the crystal structure. This valine was replaced with: (i) a cysteine, a small hydrophobic amino acid but is capable to form hydrogen bonding to destabilize the dimer interface (a large excess of DTT was present to prevent any disulfide formation) or (ii) a tryptophan, a large side chain with a propensity to partition into membranes that might be expected to strengthen the transient dimer interaction. For PI SUVs, the specific activity of V44C was lower than that of the WT enzyme and increased at most 2-fold (compared with 5-fold for WT) over the protein concentration range examined (Fig. 4-6D). In contrast, the specific activity of V44W was higher than wild type and appeared to reach a maximum value at lower enzyme concentrations. These data strongly suggests that enhanced protein dimerization on the surface is part of the PC activation mechanism for *Sa*PI-PLC.

Figure 4-4. S. aureus PI-PLC can form a homodimer. (A) S. aureus PI-PLC dimer structure (PDB 4F2B); (B) a close-up of the dimer interface; (C) close up view of the anion binding site with key residues labeled. (D, E) View of the anion binding pocket as seen looking down from the membrane interface in the homodimer (D), where it is unoccupied, and the *in silico* Y253S (monomer Y253S: PDB 4F2T) dimer (E), generated from the monomer with a sulfate ion bound in the anion site.



Figure 4-5. PC modulates the pH and salt dependence of *Sa*PI-PLC activity as measured by production of cIP from PI using ³¹P NMR. (A) The pH dependence for *Sa*PI-PLC cleavage of PI in pure PI SUVs (4 mM) (\bigcirc) and PI/PC (4 mM/4 mM) (\bigcirc) SUVs. (B) The specific activity at pH 6.5 increases with increasing X_{PC} (\blacksquare) and is not as sensitive to the addition of 140 mM salt (\boxtimes). The total phospholipid concentration was held constant at 4 mM for the indicated X_{PC}. The enzyme concentration was 4 µg/mL for pure PI SUVs and 0.3 µg/mL for PI/PC SUVs. (C) The specific activity for cleavage of 4 mM PI in 8 mM Triton X-100 (\bigcirc) or 16 mM diC₇PC (\bigcirc) micelles. (D) The specific activity toward 5 mM cIP alone or with 5 mM diC₇PC and in the absence (\blacksquare) or presence of 140 mM salt (\boxtimes). Error bars in each plot represent S.D. from multiple assays.



Figure 4-6. SaPI-PLC relative specific activity (R.S.A.), as measured by production of cIP from PI using ³¹P NMR, increases with enzyme concentration suggesting oligomerization of this protein (but not *Bt*PI-PLC) on interfaces. For SaPI-PLC, specific activities are normalized to the highest specific activity obtained for WT enzyme acting on 4 mM PI SUVs in the absence of added salt (9.8 µmol min⁻¹ mg⁻¹ at pH 6.5). For *Bt*PI-PLC activities are normalized to 164 µmol min⁻¹ mg⁻¹ at pH 7.5, the optimal activity toward 8 mM PI SUVs in the absence of salt. R.S.A. as a function of protein concentration is shown for: (A) *Sa*PI-PLC toward PI (4 mM) SUVs in the absence (\bigcirc) and presence (\bigcirc) of 140 mM salt; (B) *Bt*PI-PLC activity toward PI (8 mM)/PC (8 mM) SUVs in the absence of salt; and (D) V44C (\triangle) and V44W (\blacktriangle) compared with the WT *Sa*PI-PLC (\bigcirc) toward 4 mM PI SUVs.



4.5 Effect of PC on SaPI-PLC Vesicle Binding

For *Bt*PI-PLC, increasing X_{PC} up to 0.8 also increases the affinity of the enzyme for SUVs, and tight binding ($K_d < 10 \mu M$) sequesters the enzyme on the vesicles inhibiting enzyme activity [145]. SaPI-PLC behaves quite differently. SaPI-PLC binds well, with mM K_d values, to either PG or PG/PC (1:1) SUVs at acidic pH, but binding at pH 7.5 is much weaker (Fig. 4-7A), indicating that the primary interaction is electrostatic. At pH 6.5, where optimal activity is observed, the enzyme has high affinity for anionic phospholipid-rich bilayers ($X_{PC}=0.5$), with fairly low affinity for PC-rich bilayers (Fig. 4-7B). Indeed, binding to pure PC SUVs could not be measured. The apparent K_d for pure PG vesicles is 0.38±0.14 mM at pH 6.5, and this value corresponds well with the apparent K_m value (0.23±0.03 mM) for cleavage of PI in pure PI SUVs (Fig. 4-7C). For $X_{PC}=0.5$, again the apparent K_d determined by FCS (2.5±0.5 mM) is approximately the same as the kinetic apparent K_m for PI/PC SUVs (1.9±0.5 mM). SaPI-PLC apparent K_d values increase with PC content and the enzyme binds much less tightly at high PC. For these compositions, and in the absence of salt, the K_m reflects the K_d for vesicle binding as measured by FCS.

Added salt reduces the affinity for PG-rich SUVs to such an extent that the apparent K_d for pure PG SUVs could not be measured. To explore this further we examined the fraction of protein bound to a large excess of vesicles (18 mM PG) in the absence and presence of NaCl (Fig. 4-7A, inset). Without salt, all of the protein partitioned onto the vesicles, whereas increasing the ionic strength dramatically reduced protein binding. With ~130 mM NaCl, only ~20% of the protein was bound. Given the high bulk

phospholipid concentration (18 mM), in the presence of salt the apparent K_d for PG vesicles must be very high (>50 mM). This result suggests that *Sa*PI-PLC binding to pure PG vesicles is almost entirely mediated by electrostatic interactions. However, with PC in the vesicle, the much higher residual activity indicates that binding of the enzyme to the bilayer now has a more substantial hydrophobic component.

4.6 Perturbing Hydrophobic Interactions with the Membrane

In the *Bacillus* enzymes, a number of aromatic residues are critical for tight binding to PC-rich membranes, including Trp242, which partitions into the bilayer [28,31], and a strip of four surface Tyr residues in helix G [30]. In SaPI-PLC, Trp242 is replaced by Phe249 and this enzyme has only two of the four tyrsoine residues (Tyr253 and Tyr255) in helix G. It is also unclear if Phe249 interacts with the membrane because it is tucked into the protein and away from the surface in the monomeric acidic SaPI-PLC crystal structure and in the dimer structure. To test whether these residues interact with the membrane in SaPI-PLC, we constructed the following variants: F249W and F249I, Y253S, and Y253S/Y255S. Tyr290, a single Tyr residue that is unlikely to interact with the membrane, was also mutated to serine as a control. The activity of the control, Y290S, is similar to WT (Fig. 4-8). However, both Y253S and the double Tyr mutant enzyme are much less active under all conditions. Increasing PC content has a very modest effect on PI cleavage by Y253S, whereas Y253S/Y255S is virtually insensitive to the presence of PC. In contrast, mutating Phe249 to a tryptophan significantly enhanced enzyme activity for $X_{PC}=0.5$, whereas the activity of F249I is equivalent to that of the WT. The pH dependence was varied for several of these variants, and whereas F249I and Y290S still behave essentially like WT, Y253S exhibits much lower activity regardless of pH (Fig. 4-9). The loss of activity and PC activation for Y253S and Y253S/Y255S suggest that these residues play a role in the conformational changes that activate the protein in the presence of PC.

Because both F249W and Y253S/Y255S have activities that are significantly different from WT, protein binding to PG/PC SUVs was measured by FCS (Fig. 4-7B). The K_d values for F249W are comparable with WT at X_{PC} =0 and 0.2 but significantly smaller at 0.8 X_{PC} , indicative of enhanced interactions with the membrane as might be expected when the larger tryptophan side chain replaces a phenyalanine that inserts into the membrane. However, this higher affinity for PC-rich SUVs does not translate into significantly higher activity. For comparison, Y253S/Y255S has similar affinities to WT up to 0.7 X_{PC} , but much lower activity. For higher PC content, the fraction of mutant protein bound was small and linear up to 50 mM phospholipid, and the apparent K_d could not be estimated. These surface tyrosine residues are important for PC enhancement of activity; however, except at 0.8 X_{PC} , the loss of activity is not a result of reduced binding. Thus, unlike *Bacillus* PI-PLC where K_d values for SUVs vary by orders of magnitude (from μ M to >100 mM), as a function of X_{PC} and/or enzyme mutations, the range of K_d for *Sa*PI-PLC is much more limited and less easily perturbed.

4.7 Occupation of the Anion Binding Pocket versus Dimerization

Compared with WT, Y253S has little activation by PC but similar membrane affinity. This behavior can be explained by the structure of Y253S solved by Rebecca Goldstein. The monomer structure of Y253S is essentially identical to WT, but in an *in silico* Y253S homodimer structure, the anion binding pocket is empty. Tyr253 aids in formation of a lid for the pocket in the opposing monomer of the homodimer structure (Fig. 4-4D). Replacement of the Tyr side chain with a serine leaves an opening in the adjacent monomer, thus increasing anion access to the anion-binding pocket in any Y253S homodimer (Fig. 4-4E). This increased accessibility could facilitate soluble anion or interfacial anionic phospholipid binding to the site, which in turn would destabilize the dimer interface by shifting helix B slightly away from the surface and separating value residues.

To further explore the role of the anion-binding pocket and activity, two other mutant enzymes were generated: Y253W, where the "lid" for the pocket is now considerably larger, likely preventing anion binding and favoring dimerization, and Y253K, where the positive charge might increase and enhance anion binding electrostatically, although this could be balanced by the methylene chain occluding the pocket. As seen in the inset of Fig. 4-8A, Y253W has specific activity much higher than Y253S and comparable with WT. Y253K has lower activity than Y253W at all X_{PC} examined. However, it is not as compromised as Y253S, suggesting that the bulk of the lysine alkyl chain may be blocking access to the anion site.

As a further test of how modulating the anion-binding pocket can affect activity we also prepared H86Y and H86E. The His86 imidazole forms part of the anion-binding pocket. H86Y will remove the side chain positive charge but will keep the lid in place in the dimer. H86E should antagonize anion binding because we are replacing a positive charge with a negative one. As shown in Fig. 4-8B, H86Y exhibits higher activity toward

pure PI SUVs (14.1±1.0 versus 9.8±0.3 μ mol min⁻¹ mg⁻¹ for WT) and activity comparable with WT with PI/PC SUVs. In contrast, H86E has very low activity toward pure PI SUVs (electrostatic repulsion would impede strong binding to SUVs), but is dramatically enhanced with PC present (a 135-fold increase compared with the more typical 10–15fold for H86Y and WT). Consistent with the low activity toward PI SUVs, H86E showed only a small increase in activity with enzyme concentration compared with the other proteins, reflecting its difficulty in binding to the target membrane (Fig. 4-8C). With X_{PC}=0.5 SUVs, the relative loss of activity for H86E in the presence of salt (29% residual activity) was also less than WT (21% residual activity). This behavior also suggests that the anion-binding pocket is intertwined with dimer formation.

These results suggest a very intriguing proposal: *Sa*PI-PLC is optimally active as a dimer, but an appropriate phospholipid interface and/or high protein concentration is needed to at least transiently stabilize this structure. Soluble anions, such as the physiological anions, phosphate (PO_4^{3-}) or chloride (Cl⁻), can compete for the anionbinding pocket, and prevent dimer formation as well as weakening interfacial binding. An anionic phospholipid might interact with this site, but in doing so, it would also destabilize the dimer, leading to lower activity while aiding in binding the protein to the bilayer. The observation that the double mutant Y253S/Y255S behaves similarly to Y253S supports the importance of this single aromatic group in indirectly promoting interfacial dimerization. Figure 4-7. SaPI-PLC binding affinity, measured by FCS, increases with pH and PC content and is similar to the apparent K_m for the enzyme. Variation of the PI-PLC apparent dissociation constant, K_d , for SUVs as a function of: (A) pH for PG (\bullet) and PG/PC (X_{PC} =0.5) (\bigcirc), and (B) mole fraction PC, X_{PC} , at pH 6.5, for WT (\bullet), F249W (\Box), and Y253S/Y255S (\triangle). The inset in (A) shows the fraction of WT protein bound to pure PG (18 mM) SUVs at pH 6.5 as the concentration of salt is increased. (C) Relative specific activity of SaPI-PLC as a function of total phospholipid concentration ([PI]+[PC]) at $X_{PC}=0$ (\bigcirc) and 0.5 (\bullet) at pH 6.5. The amount of enzyme used was 0.1 µg/mL for pure PI SUVs and 0.2 µg/mL for experiments with PI/PC SUVs. For each system the observed activity was normalized to the maximum observed activity, 11.2 µmol min⁻¹ mg⁻¹ for pure PI SUVs and 282 µmol min⁻¹ mg⁻¹ for the PI/PC SUVs. The error bars are the S.D. from independent experiments.



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<u>Figure 4-8.</u> Specific activities of *Sa*PI-PLC variants as a function of X_{PC} at pH 6.5. (A) The specific activities for WT (■), F249W (⊠), F249I (■), Y253S (□), Y290S (≡), and Y253S/Y255S (■). The inset shows the activity of Y253 variants: Y253S (□), Y253K (ℤ), and Y253W (■). (B) The specific activities for H86 variants toward pure PI SUVs and PI/PC (1:1) SUVs in the absence and presence of added salt: H86Y (□) and H86E (ℤ) are compared with WT (■). The kinetic experiments in (A) and (B) kept the PI concentration at 4 mM and increased the PC to yield the indicated X_{PC} . (C) The dependence of PI-PLC specific activity on protein concentration: WT (●), H86Y (○), and H86E (■). Error bars are the S.D. of activities measured in triplicate.


Figure 4-9. pH dependence of *Sa*PI-PLC variants specific activity (μ mol min⁻¹ mg⁻¹) towards PI/PC SUVs (X_{PC} =0.5): WT (\blacksquare), F249I (\Box), Y253S (\blacksquare), and Y290S (\boxtimes). The concentration of PI was fixed at 4 mM with increasing amounts of PC added. PC activation is only reduced for Y253S.



4.8.1 SaPI-PLC Activity, Dimer Formation and the Membrane PC Content

The kinetic and structural data strongly suggest that *Sa*PI-PLC is optimally active as a dimer, and that this dimer is transiently stabilized by the membrane surface. The small dimer interface in the crystal structure suggests that dimers are likely to be transient, making them difficult to observe. *Sa*PI-PLC might aggregate to a small extent in solution, but obtaining accurate sizes for bacterial PI-PLCs in solution is difficult because they have an affinity for carbohydrates. At the protein concentrations required for gel filtration or analytical ultracentrifugation, these PI-PLCs interact with the resin or sucrose leading to aberrant sizes [172]. Furthermore, increased ionic strength, which suppresses interaction of the *B. thuringiensis* enzyme with resins [10], would favor monomeric *Sa*PI-PLC However, the simple membrane-induced dimer model does not explain why *Sa*PI-PLC activity increases with increasing PC content despite its lower affinity for PC-rich membranes. The presence of PC in the vesicle also rescues activity lost by the enzyme in moderate concentrations of salt (Fig. 4-5B), consistent with a switch from electrostatic to hydrophobic interactions.

A better understanding of *Sa*PI-PLC might be gained by comparing it to the wellstudied PI-PLC from *B. thuringiensis*, which has a specific binding site for PC near helices F and G [34]. For *B. thuringiensis* PI-PLC, apparent K_ds range from ~4 mM for anionic SUVs to ~2 μ M at X_{PC}=0.75 with apparent K_ds of 200-30 μ M from 0.2 to 0.5 X_{PC} where enzyme activity is optimal [146]. *Sa*PI-PLC binds more weakly to PC containing SUVs with apparent K_ds of 0.3 to 50 mM for 0 to 0.8 X_{PC}, likely due to its lack of a PC specific binding site near the F and G helices [34].

Why then does SaPI-PLC activity increase with PC content when the affinity for vesicles is decreasing? Incorporation of PC may dilute the surface concentration of the anionic phospholipids in vesicles, and that in turn could enhance SaPI-PLC dimer formation. This mechanism is consistent with the observation that much lower enzyme concentrations are required to increase PI-PLC specific activity in the presence of PC (Fig. 4-6C) even though the apparent K_m increases as PC is increased. Alternatively, there are a number of aromatic residues in loops and N-terminal portions of helices around the barrel rim that could form transient cation- π interactions with the choline headgroup. This could alter the conformation of loops or side chains and facilitate transient protein dimerization. It should also be noted that the cation- π latch is still observed in the dimer structure (obtained at pH 4.6), and its presence is likely to aid in soluble cIP release, at least at acidic pH. However, the altered pH profile in the presence of PC might suggest that the zwitterionic phospholipid leads to alterations of pKa values for the many histidine residues in and around the active site. This could be the result of a conformational change or change in the surface charge of the membrane. Additionally, interactions with PC and transient dimerization could better orient SaPI-PLC relative to the membrane surface, facilitating enzyme activity by increasing substrate access.

4.8.2 The Anion-binding Pocket – A Key to Inhibiting Dimer Formation

One of the unusual features of *Sa*PI-PLC is the very electropositive anion-binding pocket just below the rim of the barrel, adjacent to helix B. Neither the *Bacillus* enzymes nor *L. monocytogenes* PI-PLC have such a pocket and those enzymes cannot form the

helix B mediated dimer. In *Sa*PI-PLC the anion pocket is composed of the backbone amides of Lys38 and Asp39, as well as the cationic side chains of Lys42 and His86. Anion binding leads to a shift in the pocket residues, as well as pull the entirety of helix B (Val41 to Ala46) down, closer to the anion binding pocket, and thus away from the dimer interface. The shift in helix B would in turn increase the distance of valine pairs thus destabilize the dimer interface. Thus anion binding, probable at high salt concentrations, and dimer formation appear mutually exclusive.

4.9 Conclusions

We hypothesize that competition between anion binding and dimer formation is the key regulator of *Sa*PI-PLC activity (Fig. 4-10). Soluble anion binding weakens protein affinity for PI-rich vesicles, inhibiting *Sa*PI-PLC even at moderate salt concentrations. An anionic phospholipid might also bind to the pocket electrostatically, and thus aid in anchoring the protein to the surface. However, the presence of an anionic phospholipid such as PI near this site would favor the monomer and lead to low specific activity. High protein concentrations allow dimerization to compete with anionic lipid binding to the pocket leading to some dimers on the membrane, but activities are still low. PC then activates *Sa*PI-PLC, not by specific interactions, but by lowering the interfacial anionic lipid concentration and emptying the anion binding pocket, facilitating dimer formation. Occlusion of the pocket by the dimer interface makes the membrane-bound dimer much less sensitive to physiological salt concentrations. However, there is a trade-off: dimers have higher activity, likely because of a conformational change that enhances catalysis and/or because they optimize the protein orientation on the membrane for catalysis, but

Figure 4-10. A model for *Sa*PI-PLC-membrane interactions. Monomeric PI-PLC partitions from solution to the membrane via electrostatic interactions, and a molecule of PI diffuses to the active site (step 1). The enzyme then either binds a soluble anion, and falls off the membrane, or may bind an auxiliary anionic lipid in/near the anion binding pocket (step 2). PI-PLC dimerization is promoted by PC, which dilutes the interfacial concentration of the auxiliary anionic lipid, and releases it from the enzyme (step 3). Finally, in step 4, the dimer dissociates into monomers or dissociates from the membrane. PI-PLC is represented in semi-transparent space filling view, with the active site highlighted in red, and the anion binding pocket highlighted in cyan. PI lipids are modeled as blue spheres, the membrane is shown as a blue gradient, and a water-soluble anion is shown as a purple sphere.



they bind more weakly than monomers. On PC-rich bilayers they have lost the electrostatic interactions of the regulatory site.

S. aureus is known to cause a broad range of diseases in humans and is commonly found in skin infections and abscesses which can lead to toxemia and lethal bacteremia [20,22]. The association of an auxiliary anion binding site with SaPI-PLC but not with homologues from other Gram-positive bacterial species would argue that it is related to the biology of S. aureus. Since abscesses are acidic [24], the slightly more acidic optimum of SaPI-PLC enzymatic activity would make it more effective. In a study of methicillin-resistant S. aureus, expression of the PI-PLC, as well as other exoproteins and cytoplasmic proteins, was significantly increased [23]. Although this protein has a moderate affinity for anionic surfaces in the absence of salt, once the enzyme is secreted into the moderate ionic strength of the extracellular milieu, it will not interact with other S. aureus organisms. It is then poised to interact with the PC or sphingomyelincontaining membranes of eukaryotic target cells via the valine box of the dimer. The still weak interaction with pure PC surfaces may allow it to hone in on negatively charged regions (the cationic character of the anion binding pocket is still available), which include GPI-anchors, the targets of this exoprotein. Clearly, this small soluble phospholipase has evolved a complex, apparently unique way to control its access to the phosphatidylinositol moieties that are its substrates: (i) A cation- π latch allows it to work on membranes under acidic conditions and more easily release soluble product without dissociating from the surface. (ii) An anion-binding pocket near the region that binds to the membrane likely modulates the location of the protein, whether it is bound to a

negatively charged surface, one with significant zwitterionic lipid content, or in solution. (iii) High activity requires dimerization of the enzyme and dimerization is enhanced by PC content similar to that found in the external leaflet of mammalian plasma membranes. This interplay between protein dimerization and an anion sensing site could be a way of regulating other peripheral (or even integral) membrane enzymes. Juxtamembrane cationic regions, while favoring anionic membrane regions, could prohibit protein/protein interactions. However, an appropriately modified bilayer composition might change this balance. Chapter 5

Defining Cation- π Binding in Peripheral Membrane Proteins

5.1 Introduction

Peripheral membrane proteins often initially recognize specific lipids of their target membrane by the stereospecific features of phosphoinositide headgroups [45,46]. This interaction may be further modulated by insertion of hydrophobic and aromatic amino acids into the bilayer [31,48,49] and/or multivalent interactions [46]. Although there are numerous examples of specific binding to anionic lipids, less is known about specificity for zwitterionic lipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The trimethylammonium moiety of PC headgroups has been observed in the structures of various proteins [52-59] to have cation- π interactions with the aromatic residues. However, it is unclear whether the cation- π interaction by itself would provide sufficient binding energy to transiently bind a protein to the membrane.

To test the hypothesis that such a cation- π box might allow specific, but transient binding to a PC-rich membrane, I engineered such a box into *S. aureus* PI-PLC (*Sa*PI-PLC). Both *B. thuringiensis* PI-PLC (*Bt*PI-PLC) and *Sa*PI-PLC can target GPI-anchored proteins in the PC-rich outer membrane of eukaryotic cells [17,173]. Although *Bt*PI-PLC has a high affinity for PC vesicles [31], the similar PI-PLC from *S. aureus* has virtually no affinity for PC vesicles (refer to Chapter 4). High resolution field-cycling NMR experiments on *Bt*PI-PLC identified a discrete binding site for PC that is consistent with Tyr residues forming a cation- π box or sandwich [34]. However, this motif is not present in the *Sa*PI-PLC, which displays much weaker binding to PC-rich vesicles and virtually no binding to pure PC vesicles. Interestingly, unlike most other cation- π boxes, which are often made up of aromatic residues located on β strands and/or on loops with the separation required to sandwich a methyl ammonium between at least two π systems, the putative *Bt*PI-PLC cation- π box is proposed to be located on adjacent α helices on the outside of a β -barrel. Therefore, I added two more tyrosine residues to *Sa*PI-PLC to introduce a specific PC binding site and characterize this with several biophysical techniques including fluorescence correlation spectroscopy, high resolution field cycling NMR, as well X-ray crystallography.

5.2 Comparing B. thuringiensis and S. aureus PI-PLC

*Bt*PI-PLC binds to PC-rich vesicles with the residues located on the loop connecting helices F and G (notably Trp242), as well several tyrosine residues located on helix G (Tyr246, Tyr247, Tyr248, Tyr251) [30,34]. Sequence and structural alignments of *Bt*PI-PLC and *Sa*PI-PLC show that whereas Tyr246 and Tyr248 in the *Bacillus* enzyme correspond to Tyr253 and Tyr255 in *S. aureus*, the other two tyrosine residues (247 and 251) in the *Bacillus* enzyme have been replaced with Asn254 and His258 in the *S. aureus* enzyme (Fig. 5-1A). *Sa*PI-PLC binds to PC-rich membranes with much lower affinity than does *Bt*PI-PLC and has virtually no affinity for pure PC vesicles (refer to Chapter 4). This fact suggests that the higher affinity of *Bt*PI-PLC for PC is associated with the presence of an aromatic box that creates a binding site for the choline. We hypothesized that mutating Asn254 and His258 to tyrosines (N254Y/H258Y) could introduce a specific PC binding site in *Sa*PI-PLC. This expectation was based on the observation that the site could utilize cation-π interactions to bind the PC headgroup.

5.3 Vesicle Binding of N254Y/H258Y SaPI-PLC

5.3.1 Vesicle Binding Comparison of WT and Mutated Enzymes by FCS

FCS was used to measure binding of the WT and mutated enzymes to SUVs. All three proteins (WT, Y253S/Y255S, N254Y/H258Y) have similar affinities for $X_{PC} \le 0.2$ vesicles, but both WT and Y253S/Y255S have poor affinities once $X_{PC} \ge 0.5$, whereas N254Y/H258Y still has a millimolar affinity for these PC-rich vesicles (Fig. 5-1B). As the PC content increases the difference in binding between the double mutant protein and WT is quite pronounced. N254Y/H258Y has a 50-fold lower apparent K_d than WT for $X_{PC} = 0.8$ vesicles. Although WT shows virtually no binding (<8% is bound with 55 mM PC) to pure PC SUVs, N254Y/H258Y binds with an apparent K_d of 3.3±0.4 mM.

5.3.2 Testing PC Binding with a Filtration Assay

Moderate salt concentrations dramatically reduce *Sa*PI-PLC binding to vesicles (refer to Chapter 4), providing a simple assay to demonstrate whether PC is binding via one or both of the tyrosines that were introduced into the PI-PLC variant. Wild-type *Sa*PI-PLC, N254Y, H258Y, and N254Y/H258Y (0.2 mg/mL) were each incubated with 1 mM PG/PC (0.2 mM/0.8 mM) SUVs in 50 mM MES, pH 7.5, with 140 mM salt, followed by centrifugation to separate free protein from vesicle bound protein. The total phospholipid concentration of 1 mM was chosen to be close to the K_d for N254Y/H258Y measured by FCS. Under these conditions, no WT or N254Y protein was bound to the vesicles; however, 12% of H258Y and 59% of N254Y/H258Y were bound to the vesicles. This suggests that a tyrosine at residue 258 is required, and that a tyrosine at 254 significantly increases PC binding in the H258Y background. This same binding assay carried out with 1 mM PG/PE (0.2 mM/0.8 mM) to examine if N254Y/H258Y exhibited a preference for PC compared with PE. When N254Y/H258Y was incubated with either the PC or PE containing SUVs, 68% of the protein was bound to the PG/PC SUVs, whereas only 36% of the protein was bound to the PG/PE SUVs. The interactions of the protein with PC appear significantly stronger than with PE.

5.3.3 PC Binding Specificity by ³¹P NMR Line Broadening and Intrinsic Fluorescence Changes

Additionally, binding of *Sa*PI-PLC enzymes to PC micelles was explored by monitoring the ³¹P line width of diC₇PC in the presence of the protein (Fig. 5-1C). *Bt*PI-PLC induces formation of large protein-micelle complexes right around the critical micelle concentration (CMC), and mutations that weaken association with PC reduce this change in ³¹P line width [30]. *Sa*PI-PLC and the Y253S/Y255S variant had little or no effect on the diC₇PC line width, consistent with poor binding to a PC interface. However, N254Y/H258Y mimicked the behavior of the *B. thuringiensis* PI-PLC with a large increase in line width at the critical micelle concentration. The intrinsic fluorescence associated with the presence of aromatic residues in N254Y/H258Y also increased with the addition of micellar diC₇PC; this effect was not observed with the WT protein (Fig. 5-1C, inset).

Figure 5-1. Inserting the two missing Tyr residues generates PC specificity. (A) Alignment of the F/G helix region of *Bt* (PDB 1PTD) and *Sa* (PDB 3V18) PI-PLC. The secondary structure is shown above the alignment and the residues important for membrane binding are highlighted. (B) Apparent binding constants at pH 6.5 for WT (▲), Y253S/Y255S (○), and N254Y/H258Y (●) partitioning to SUVs as a function of mole fraction PC (X_{PC}). (C) ³¹P linewidth of diC₇PC in the absence (solid line) and presence of 3 mg/mL of *Bt*PI-PLC (○) and *Sa*PI-PLC variants: WT (▲) and N254Y/H258Y (●). The inset shows the relative change in intrinsic fluorescence of WT (▲) and N254Y/H258Y (●) as a function of the amount of micellar diC₇PC added (CMC of diC₇PC is ~1.5 mM at 25 °C). The line is a hyperbolic fit to the data.



5.4 Effect of the Added PC Site on Enzymatic Activity

The addition of two tyrosines to helix G in SaPI-PLC has apparently created a site on this enzyme that can bind one or more PC molecules. The question arises whether this enhanced PC binding influences enzyme activity. SaPI-PLC enzymatic activity toward PI in vesicles is sensitive to both pH and salt concentration, and PC in the interface both alters the optimal pH for activity and ameliorates the salt sensitivity of the WT enzyme. These kinetic effects are not due to specific PC binding but rather result from competition between anion binding to a specific pocket in SaPI-PLC and formation of a more active homodimer that occludes the anion binding site (Chapter 4). Like WT, N254Y/H258Y specific activity increases with increasing enzyme concentration indicating the active form is still a dimer. At pH 6.5 in the absence of salt, specific activity shows a large increase with PC content for both N254Y/H258Y and WT (Fig. 5-2A). If salt is present neither WT nor N254Y/H258Y exhibits much activity toward pure PI SUVs, and salt reduces the activity of WT SaPI-PLC even toward SUVs containing PC. However, once PC is present in the SUVs, the salt sensitivity of N254Y/H258Y enzyme is virtually abolished and the engineered enzyme exhibits activities in the presence of salt that are close to the values obtained without salt, whereas the activities of the WT are much lower in the presence of salt.

Specific activity toward PI in vesicles partially reflects how efficiently the protein partitions onto vesicles. For WT, the tightest binding is observed at pH 5.5, and added salt dramatically weakens binding to both pure PG and PG/PC (1:1) SUVs (Fig. 5-2, B and C). At pH>5.5 in the absence of PC (Fig. 5-2B), the binding of both WT and

N254Y/H258Y proteins are similarly inhibited by salt. However, once PC is present in the SUVs, N254Y/H258Y binding is not significantly affected by salt (Fig. 5-2C). The apparent K_d values for binding to $X_{PC} = 0.5$ SUVs, at pH 6.5 in the presence of 140 mM salt, were ~70 mM for WT and 2.6 ± 0.6 mM for N254Y/H258Y. Similarly, WT binding to pure PC SUVs was too low to measure, whereas N254Y/H258Y exhibited an apparent K_d of 5.4 ± 0.9 mM in the presence of salt (only about 50% higher than the apparent K_d in the absence of salt). Salt screens electrostatic interactions preventing WT *Sa*PI-PLC binding to these vesicles, but the engineered PC site in N254Y/H258Y allows binding to PC-containing interfaces even in the presence of salt.

5.5 Defining the PC Binding Site on a Molecular Level

Two experimental approaches were used to confirm the expectation that PC binds in direct proximity to the two introduced tyrosines (N254Y/H258Y): (i) high-resolution field cycling ³¹P NMR analysis of the effect of spin-labeled protein on a mixed PC/PMe bilayer and (ii) X-ray crystallography structure determination for the N254Y/H258Y and H258Y *Sa*PI-PLC variants by Rebecca Goldstein. Comparisons with the WT structure that has been described previously (Chapter 4) offer insight not only into the conformational adaptability of the protein but also demonstrate that increased PC affinity results from PC binding mediated by the introduced tyrosine residues.

Figure 5-2. In the presence of PC, N254Y/H258Y SaPI-PLC is less salt-sensitive than WT. (A) Specific activity of S. aureus WT (triangles) and N254Y/H258Y (circles) PI-PLC at pH 6.5 toward different vesicle compositions (X_{PC} = mole fraction PC) in the absence (filled symbols) and presence (open symbols) of 140 mM salt. The concentration of PI was kept at 4 mM with increasing amounts of PC. B and C, the apparent fraction of protein bound to (B) pure PG (6.2 mM) or (C) PG/PC (8.2:8.2 mM) SUVs (extracted from FCS data) is shown as a function of pH: WT in the absence (\blacksquare) or presence (\boxtimes) of 140 mM salt; N254Y/H258Y in the absence (\square) or presence (\boxtimes) of salt. Error bars represent the variation in parameters from experiments using different protein and SUV preparations.



5.5.1 NMR Field Cycling Experiments with SUVs

Because the chemical shifts of different phospholipids are distinct, ³¹P field cycling NMR is useful for identifying specific phospholipid interactions with a spin-labeled protein in multicomponent vesicles [34]. In these experiments, differences in phospholipid ³¹P relaxation rates provide a direct measure of the proximity of different phospholipid species to the spin label site. In particular, we have shown that for PC/PMe SUVs, *Bt*PI-PLC spin labeled at D205C has a large effect on the ³¹P relaxation rate of PC with a much smaller effect on PMe consistent with a PC site near the spin label and a bound PC lifetime between 1 µs and 1 ms. PMe, an anionic lipid that also competes with PI substrate, was used in these experiments, because over the course of 24 h there is some hydrolysis of PG by the enzyme and generation of diacylglycerol that causes vesicle fusion. Asp205 in BtPI-PLC aligns with Asp213 in SaPI-PLC, and spin labeling at D213C has little effect on the PMe or PC resonances (Fig. 5-3A) under conditions (140 mM salt and 0.5 mg/mL of protein and 5 mM each phospholipid) where \sim 15–20% of this labeled WT protein is associated with the SUVs in the presence of salt. The field dependence data for D213C in the WT SaPI-PLC background appears equivalent to what is seen for the SUVs in the absence of protein [34].

However, a very different profile is observed for spin-labeled D213C/N254Y/H258Y under the same conditions, and the results for this variant resemble those for *Bt*PI-PLC (Fig. 5-3). A potential complication to the average distance determination arises from the fact that *Sa*PI-PLC forms transient dimers on vesicle surfaces via helix B side chains. However, based on the dimer X-ray crystal structure, a spin label at residue 213 should

be >35 Å from the active site of the opposing monomer, and 34–35 Å from H258Y on the opposing monomer. Because the distance dependence of the spin label falls off as $1/r^6$, the field cycling NMR should only report on an intramonomer PC site. The stronger effect exerted by the spin-labeled protein on the PC NMR relaxation rate compared with PMe therefore indicates that in the engineered protein (i) PC has a discrete binding site and (ii) it is near the region identified in the *B. thuringiensis* enzyme [34].

If we assume a single PC binds to the protein for the observed correlation time of the low field dispersion, then we can further use the ratio $\tau_{P-e} \Delta R_{P-e}(0)$ to estimate a distance for each of these phospholipids to the spin label at residue 213. These parameters are obtained from fitting the relaxation as a function of field that is specifically due to the introduction of the spin label on N254Y/H258Y at D213C (Fig. 5-3, inset). The correlation time for the 31 P-electron dipolar interaction is 2 ± 1 µs and the extrapolated $\Delta R_{P-e}(0)$ values for the two phospholipids are 8.5±1.3 s⁻¹ (PC) and 0.7±0.6 s⁻¹ (PMe). Although τ_{P-e} is not known precisely, what is relevant in determining r_{P-e} is the ratio τ_{P-e} $e/\Delta R_{P-e}(0)$, and this is very similar when fitting the data at 1 to 3 µs. For PC with $\tau_{P-e} = 2$ μ s, this yields $r_{P-e} = 15.2 \pm 0.4$ Å. If the plot of ΔR_1 versus field is fit at 1- or 3- μ s correlation times, there is at most a 0.5-Å variation in the estimated r_{P-e}. Interestingly, this value is a little longer than the r_{P-e} extrapolated for the BtPI-PLC with a spin label attached at the same site (13.5 \pm 0.2 Å [34]). The difference in r_{P-e} for PC binding to the two PI-PLC proteins may look small, however, it is real because $\tau_{P-e}/\Delta R_{P-e}(0)$ (which differs by a factor of two for the two proteins) is proportional to r_{P-e}^{6} . There is a small effect on PMe, which should only occupy the active site with this amount of salt in the buffer. If one uses the fit with 2 μ s r_{P-e}, bound PMe must be ~22–24 Å away, roughly about where the active would be from the spin label on residue 213. The key result is that the PC binding site we introduced in *S. aureus* N254Y/H258Y is located in the same general area of the protein as it is in the *Bt*PI-PLC.

5.5.2 Crystal Structures of N254Y/H258Y with Choline and diC₄PC

To further explore the location of the introduced PC site, Rebecca Goldstein (a graduate student in the laboratory) obtained crystal structures of the N254Y/H258Y double mutant. The overall structure of N254Y/H258Y closely resembles that of the *Bacillus* enzyme and differs only slightly from that of the *Sa*PI-PLC WT. Further evidence for the existence and the location of the binding site for a choline moiety was provided by crystal structures of N254Y/H258Y with choline and diC₄PC (Fig. 5-4). The structures revealed two discrete binding pockets – one between tyrosines on helix F and helix G and the second between helix G and a loop, which are formed by cation- π interaction of choline quaternary amines and the aromatic side chains of H258Y, Tyr212, Trp287 and Tyr290.

5.5.3 Do Both Choline Sites in N254Y/H258Y Bind PC?

A comparison of N254Y/H258Y structures in the absence or presence of trimethylammonium ligands provides insights into the nature of the specific choline and PC sites. To accommodate the cationic ligand there are clear rotations of side chains in choline binding site 1, relative to the unliganded enzyme (Fig. 5-4A). However, we only see soluble diC₄PC binding in choline site 2 and not in choline site 1. This could be the result of weak PC binding, at least in the absence of a bilayer (which could locally increase the choline headgroup concentration so that binding occurs more readily), or it could be attributed to the difficulty in moving side chains in site 1 to accommodate a choline moiety, which might be more difficult when the choline is part of a phospholipid molecule.

The field cycling ³¹P NMR experiments provide insight into where PC presented in a bilayer binds on the protein, for at least 2 µs [34]. For the same amount of protein and phospholipids, $\tau_{P-e}/\Delta R_{P-e}(0)$, which is proportional to r_{P-e}^{6} , is 2.35×10^{-7} s² for N254Y/H258Y, and 1.48×10^{-7} s² for *Bt*PI-PLC, consistent with PC binding closer to the spin label site in *Bt*PI-PLC than in the engineered *S. aureus* protein. The *Bacillus* protein only has choline site 1 available for PC binding. Furthermore, if a PC molecule occupied both choline sites when N254Y/H258Y was bound to PC/PMe SUVs, there should be a considerably stronger relaxation of the ³¹P nucleus that is roughly twice as effective as when a single PC binds to the protein. Because $\tau_{P-e}/\Delta R_{P-e}(0)$ is larger for the S. aureus N254Y/H258Y (meaning further away), only one PC binds well with a $\geq 2 \mu s$ lifetime. In turn, the observation that the averaged distance of the spin label to the bound PC ³¹P is larger in N254Y/H258Y than in *Bt*PI-PLC is consistent with PC binding to the *S. aureus* protein in choline site 2. Energy minimization of the crystal structure placed the isolated chains of diC₄PC against the protein, but the same orientation of the choline moiety could easily be occupied with a longer chain phospholipid anchored in a bilayer (Fig. 5-4B).

Figure 5-3. The spin label at D213C perturbs lipid signals for the N254Y/H258Y mutant but not WT. Effect of spin-labeled *Sa*PI-PLC (0.5 mg/mL) on PMe/PC (5:5 mM) SUVs: control PMe (\Box) and PC (\bigcirc) mixed with spin-labeled D213C; PMe (\blacksquare) and PC (\bigcirc) with the spin-labeled D213C/N254Y/H258Y. The inset shows the difference in R1 for each phospholipid ³¹P specifically attributed to the spin label on D213C/N254Y/H258Y; the data are fit with $\tau_{P-e} = 2 \ \mu s$.



Figure 5-4. SaPI-PLC structures with and without choline bound. (A) Overlay of the SaPI-PLC N254Y/H258Y structure without choline (dark blue, PDB 4I8Y) and with choline bound (teal, PDB 4I90) showing that choline site 2 (orange) is preformed, whereas site 1 (yellow) requires rotamer changes in side chains. (B) *In silico* models of PC and PI binding to discrete sites on SaPI-PLC N254Y/H258Y. The *S. aureus* mutant structure is shown as a dimer (mediated through helix B) with one molecules of diC₇PC in site 2 (orange) and one molecule of PI (dark gray) in the active site in views with the membrane interface at the top.



As further evidence for occupation of a single PC site in N254Y/H258Y, we generated Y211A/N254Y/H258Y/Y290A. Mutation of Tyr-290 to alanine should abolish choline affinity for site 2. Tyr-211 is not conserved in *B. thuringiensis* PI-PLC and must rotate by 77° to accommodate the choline cation. Its removal might be expected to enhance PC binding, if site 1 is significantly populated by a PC molecule. Using the centrifugation assay for partitioning of Y211A/N254Y/H258Y/Y290A onto PG/PC (0.2 mM/0.8 mM) SUVs, we found little protein associated with the SUVs with 1 mM total phospholipid. With 8 mM total phospholipid 7.7 \pm 3.8% of the protein was bound. In essence, abolishing choline site 2 prevents *S. aureus* N254Y/H258Y from binding to PC-containing SUVs. This confirms that although two choline sites were introduced into *S. aureus* N254Y/H258Y, only preformed site 2 interacts significantly with a PC headgroup.

5.6 Discussion

Sandwiches, cages, or boxes composed of aromatic amino acid π systems of aromatic amino acids are a facile way to bind trimethylammonium moieties via cation- π interactions. In so doing they provide a specific recognition motif for the headgroups of zwitterioinic phospholipids. *Bacillus* PI-PLC is activated by PC [10], in large part by enhancing vesicle binding [146]. The G helix region of *Bacillus* PI-PLCs has several Tyr residues that could form a sandwich or cage around a choline group [30,34], but how and whether PC binds to this box was unclear. Attempts to transplant this box into *S. aureus* PI-PLC by introducing two "missing" tyrosine side chains in helix G generated catalytically active protein that binds much more tightly to PC-rich interfaces. Field cycling NMR suggest there are similarities in the position of PC bound to *Bt*PI-PLC and to *S. aureus* N254Y/H258Y protein when the protein is transiently anchored on a vesicle. Although X-ray crystallography reveals two spatially close choline binding sites on the engineered protein, only choline site 2 between helix G and a loop is occupied by a PC molecule in the *S. aureus* protein. *Bt*PI-PLC does not have the key tryptophan residue needed for choline site 2. However, all the side chains are correctly oriented for binding a choline (or PC) in what is analogous to *S. aureus* N254Y/H258Y site 1.

Although the specific cation- π PC binding site in *Bt*PI-PLC (equivalent of choline site 1) and that in S. aureus PI-PLC (choline site 2) are different, the energetic contribution of these aromatic π -choline cation interactions to PC binding should be similar and different from values for partitioning a tyrosine side chain into a bilayer [174]. We can use the apparent K_d values as a way to assess this. The relative change in vesicle binding affinity when the cation- π site is abolished, K_d(no PC site)/K_d(intact PC site), should be similar for both enzymes. Data for $X_{PC} = 0.8$ SUVs were chosen for the comparison, because no K_d could be obtained for S. aureus WT binding to pure PC SUVs (refer to Chapter 4). For SaPI-PLC, $K_d(WT)/K_d(N254Y/H258Y) = 48$. For BtPI-PLC, the Y251A mutant was selected as one where the site analogous to choline site 1 should be abolished (Tyr251 is the equivalent of S. aureus H258Y). Binding data at the same mole fraction PC [175] yielded $K_d(Y251A)/K_d(WT) = 45$. Thus, the absence of a PC site, whether it is analogous to choline site 1 or choline site 2 in the S. aureus mutant, has essentially equivalent effects on vesicle binding. This translates to a change in free energy (at 22 °C, the temperature of the FCS experiments) of 9.5 and 9.3 kJ/mol for losing this cation- π interaction. For comparison, partitioning of a tyrosine or a tryptophan side chain from the interior of a bilayer to water is estimated as 3.9 or 7.7 kJ/mol, respectively [174]. Clearly, cation- π interactions between proteins and the PC headgroup can stabilize the transient membrane binding needed for peripheral proteins. Invoking these interactions provides an explanation for why *Sa*PI-PLC N254Y/H258Y binds to pure PC bilayers with significantly weaker affinity than *Bt*PI-PLC. Tallying up the tyrosine residues around the rim of the $\alpha\beta$ -barrel it is clear that the *Bt*PI-PLC has far more aromatic residues that could either (i) insert into the bilayer or (ii) form transient cation- π complexes.

5.7 Conclusions

These results, combined with structural studies of proteins that bind methyl-Lys, methyl-Arg, or choline using similar cation- π cages/boxes suggest that this cation- π motif has evolved in a variety of secondary structural contexts ranging from β barrels to β propellers to α helices and loops, anywhere that aromatic side chains can be separated by 8–10 Å to allow space for binding a methylammonium moiety. In the case of membranes, such π system motifs could provide a general way to introduce specific yet transient interactions of peripheral membrane proteins with PC headgroups.

Chapter 6

Identification of a Novel Mammalian cis-Aconitate

Decarboxylase

6.1 Introduction

ITA was first discovered as a product of pyrolytic distillation of citric acid in an osmophilic strain of green *Aspergillus species* [79]. It was postulated to be synthesized via aconitase and *cis*-aconitate decarboxylase (cADC) activities [81,82]. As an important step in the clarification of ITA biosynthesis, a 55 kDa protein was purified from the high ITA producing strain *A. terreus* TN484-M1 [87]. The corresponding cADC gene was then identified to further verify the ability of this enzyme to produce ITA [85,88]. This cADC enzyme contains a conserved domain of the MmgE/PrpD family of proteins of bacteria and fungi [85], which include several 2-methylcitrate dehydratases of bacteria that are involved in propionate catabolism.

Very recently, itaconic acid (ITA) was detected in mammalian cells including a macrophage-like tumor cell line (VM-M3) [114]. The ITA was shown to be synthesized via decarboxylation of *cis*-aconitate, indicative of a novel mammalian cADC [114]. This unusual solute is secreted by a variety of mammalian cells [115-118]. However, up to the beginning of our project, there was no specific gene encoding the mammalian cADC. The discovery of ITA metabolite in a metastatic tumor cell line [114] hints that it may also have a role in tumor biology. Thus, identification of this novel mammalian cADC will help to clarify the physiological function of ITA in the mammalian immune system and in cancer.

6.2 Optimization of ITA Production in the Macrophage Stimulation

The optimal working pH value for the mammalian cADC activity in protein extracts of RAW264.7 cells was determined to be pH 7.5 (Fig. 6-1A). Macrophage stimulation conditions, including time after LPS addition (1 to 24 h) as well as LPS concentration (0.5 ng/mL to 50 μ g/mL), were optimized. Relative cADC activities were calculated by the following equation:

$E_r = (P_n / A_{280,n}) / (P_{max} / A_{280,max})$

where E_r , is the relative activity at a specific condition determined by ITA production ratio and the estimated total protein concentration. The ITA production ratio, P_n , is determined by integrals of ¹H NMR peaks for both –C=CH₂ protons in ITA (either of the two separate peaks, at 5.82 and 5.34 ppm, can be used) and -C=CH- proton in *cis*aconitic acid (at 5.61 ppm). The highest ratio of ITA production was chosen as the maximum, P_{max} . The absorbance at 280 nm ($A_{280, n}$) was used to estimate the total protein concentration of cell lysates in order to minimize the differences in the amount of cells between samples. The mammalian cADC activity was detected ~1 h after adding LPS to the cells and reached half of the maximum value in ~4 h. The cADC activity in the extracts was constant after 8 h of stimulation and maintained up to 24 h post-LPS stimulation (Fig. 6-1B). The optimal LPS concentration range was determined to be from 2 ng/mL to 10 μ g/mL (Fig. 6-1C); use of higher LPS concentrations (>10 μ g/mL) did not generate more cADC activity in the cells. Thus, for the following cell culture experiments, the stimulation time was always fixed at 8 h and LPS concentration was 10 ng/mL unless specifically mentioned.

Figure 6-1. Optimization of ITA production in RAW264.7 cell extracts. The relative enzymatic activity was monitored as a function of (A) pH, (B) time course after stimulation, and (C) LPS concentration.



6.3 RAW264.7 Cell Stimulation by Toll-like Receptor (TLR) Agonists

LPS is a common used reagent in macrophage stimulations. It activates immune cell responses by binding to Toll-like receptors (TLRs). Since ITA production and the amount of target mammalian cADC were highly enhanced in macrophage stimulations by LPS, it is important to elucidate the molecular mechanism involved in TLR signaling. TLRs are a class of receptors that share common structures and signaling that leads to NF-κB activation. Stimulation with every agonist except for ssRNA40 (TLR7 agonist) resulted in ITA production (Fig. 6-2). By comparison, there was no detectable ITA production for the unstimulated cells. The cell lysate after LPS (TLR4 agonist) stimulation exhibited the highest cADC activity toward substrate *cis*-aconitic acid and was used as the standard. Polyinosinic-polycytidylic acid (Poly(I:C)), a TLR3 agonist, showed a comparable stimulation effect of cADC activity. At the concentration used, the agonists, Pam3CSK4 (a synthetic lipopeptide (LP) that targets TLR1/2) and FSL-1 (a synthetic LP that targets TLR6/2), generated ~60% of the cADC activity observed in LPS stimulation. HKLM (bacterial L. monocytogenes, a TLR2 agonist) and FLA (S. typhimurium flagellin, a TLR5 agonist) were slightly less effective in inducing cADC activity producing <40% of the activity generated with LPS. These results show that mammalian cADC production is involved in TLR signaling pathways. It is noteworthy that all the material used to engage TLRs were based on bacterial triggers except for ssRNA40, which is associated with viruses. This target enzyme may be a product under the synergistic effect of various TLR signaling pathways excluding TLR7. The level of target cADC expression is closely related to TLR4 signaling pathway considering the highest activity in LPS stimulation.

Figure 6-2. Toll-like receptor (TLR) agonists stimulate cADC enzymatic activity in the RAW264.7 cells. The specific target TLR for each TLR agonist is shown on the top of each bar. The control is the sample without any stimulation. The error bars are the S.D. determined from three independent agonist stimulation tests.



6.4 Actinomycin D Assay Differentiates de novo Synthesis of cADC Activity from Post-Translational Modification

The basic strategy for identification of the enzyme exhibiting cADC activity was to compare the proteins profiles of unstimulated and stimulated cell lysates. However, prior to trying to fish out the enzyme, I first explored the source of cADC activity upon stimulation. It was unknown whether the increased activity was due to post-translational modification of an enzyme pool already in cells or caused by de novo protein synthesis. Actinomycin D (ActD) inhibits transcription in cells by binding to DNA at the transcription initiation complex and preventing elongation of RNA chain by RNA polymerase. However, ActD also shows toxicity to mammalian cells. Thus, after coincubating LPS and ActD in the samples, the RAW264.7 cells were monitored under a light microscope from 1 h to 8 h to check cell viability. A time point of 6 h was chosen as the co-incubation time for LPS and ActD since at this time point there is no obvious cell death. Different ActD concentrations (from 10 ng/mL to 1 μ g/mL) and pre-incubation times (from 0 to 1 h) were used. Without pre-incubation with ActD, the cADC activity decreased as the ActD concentration increased (Fig. 6-3 No. 1-3 and 6) but the cell death ratio also increased when ActD concentration reached 1 μ g/mL. An ActD concentration of 100 ng/mL was optimal since it led to less than 5% cell death. There was no difference in the inhibitory effect of ActD toward cADC activity for 20 min and 1 h (Fig. 6-3 No. 4 and 5). But the pre-incubation of ActD before LPS stimulation caused a further 20% reduction of cADC activity at the same ActD concentration (Fig. 6-3 No. 3-5). Increasing the ActD pre-incubation time to 1 h had about the same effect in reducing cADC activity.

These results indicate that in stimulated RAW264.7 cells, the cADC enzyme is produced by *de novo* protein synthesis, which means the comparison of unstimulated protein profile to stimulated one can be utilized to identify the target enzyme.

6.5 Enrichment of Target Enzyme from the Stimulated Cell Extract

Several experiments were designed to simplify the proteins backgrounds and maximize the target enzyme concentration in the matrix, in order to finally identify the target enzyme. The designed identification scheme of mammalian cADC is shown in Fig. 6-4. After the LPS stimulation, the cells were harvested and disrupted by sonication for better separation of the proteins from other organelles for use of centrifugation techniques later. One of the various purification methods, ion-exchange column was tried first. Fractions eluted with different salt gradients were assayed for cADC activity. The fractions that exhibited the highest relative cADC activity were collected, as well as the same salt fractions generated by chromatography of unstimulated cell extracts. These two samples could then be examined by MS proteomics methods to identify a group of proteins that only existed or were significantly overexpressed in the stimulated sample. Based on the further sequence alignment to fungal cADC, the potential mammalian cADC enzyme could be identified. Taking this one step further, possible cADC candidate proteins can be tested by siRNA knockdown experiment of specific genes and then assaying for cADC activity once these cells were stimulated with LPS.

<u>Figure 6-3.</u> Actinomycin D differentiates post-translational modification from *de novo* protein synthesis of mammalian cADC activity. Conditions are listed the Table (right). The error bars are the S.D. determined from three independent tests.



No.	Conditions
1	LPS only 6h
2	LPS & 10 ng/mL ActD co-incubate 6h
3	LPS & 100 ng/mL ActD co-incubate 6h
4	20 min ActD (100 ng/mL) incubation before 6h stimulation
5	1 h ActD (100 ng/mL) incubation before 6h stimulation
6	LPS & 1 µg/mL ActD co-incubate 6h
7	1 h ActD (1 μg/mL) incubation before 6h stimulation



Figure 6-4. Scheme for identification of mammalian cADC.

6.5.1 Centrifugation

The crude cell lysate after sonication was further separated by centrifugation (14,000 rpm, 30 min) and both the supernatant and pellet were assayed for cADC activity. Over 95% of the activity was found in the supernatant, indicating that the enzyme was a soluble protein. Ultracentrifugation was also used for better separation of the soluble proteins from those associated with small membrane fragments of other large complexes. The sample was centrifuged at 100,000×g for 90 min. Since >90% of enzyme activity was in the supernatant, this was the fraction used for different purification techniques.

6.5.2 Ion Exchange, Hydrophobic, and Gel-filtration Chromatography

Several protein purification methods were used to further separate the most active fractions from the soluble fraction obtained from stimulated RAW264.7 cells. An ion-exchange column (QFF) was used along with a NaCl gradient from 0 to 0.6 M (in 20 mM Tris-HCl, pH 8.0). Protein, monitored by A₂₈₀, was compared to cADC enzymatic activity (Fig. 6-5A). The mammalian cADC was eluted at the beginning of the salt gradient in a broad peak. This indicates that the mammalian cADC is negatively charged at this specific pH value since it can bind well with the cationic matrix of the QFF resin (the functional group is quaternary amine). It also indicates that the isoelectric point (pI) value for the cADC protein should be slightly lower than 8. Based on multiple characterizations of cADC activity in several stimulated cell extract samples from different preparations, the most active fractions was eluted from 0.09 to 0.24 M salt (Fig. 6-5B). These fractions were collected for further purification.
Hydrophobic interaction chromatography (HIC) separation is based on the reversible interaction between a protein and the hydrophobic ligand attached to the chromatography matrix. Several HIC resins with different hydrophobic characteristics were used, including phenyl sepharose, butyl sepharose and octyl sepharose. However, all of the active proteins accumulated on the resin since the cADC activity was only detected after mixing the resin after elution with the *cis*-aconitic acid substrate. This indicates that this mammalian cADC has rather high hydrophobicity and HIC purification is not useful.

Gel-filtration was the last method that we used to further purify the cADC protein (Fig. 6-6). With two different types of gel-filtration matrix, Superdex 75 and 200, the most active fractions were associated with proteins that have a molecular weight around 67 kD (by comparing with standard gel-filtration curve), which also give the estimated molecular weight range of the target cADC protein.

6.6 1D and 2D SDS-PAGE for Comparison of Stimulated and Unstimulated Samples

The stimulated and unstimulated protein mixtures after same steps of purification were further concentrated and lyophilized for 1D and 2D SDS-PAGE. However, the large number of proteins remaining made it impossible to identify the extra bands (1D SDS-PAGE) or spots (2D SDS-PAGE) in the stimulated compared to the unstimulated protein samples. This procedure was abandoned.

Figure 6-5. Partially purification of stimulated RAW264.7 cell extracts by QFF. (A) A typical QFF purification plot for (\bullet) salt gradient, (\blacktriangle) UV signal and (\Box) relative cADC activity versus fraction number. (B) The dependence of cADC activity on the salt gradient. The highlighted region represents the salt gradient chosen for the fraction collection. The data were collected from six discrete purification processes.



<u>Figure 6-6.</u> Gel-filtration purification of protein mixtures after QFF by using (A) Superdex 200 and (B) Superdex 75. Inset: the activity profiles of each fraction. The highlighted area by dashed lines indicated where the most active fractions are.



6.7 Mass Spectrometry Proteomics Analysis of Stimulated and Unstimulated Samples

The same amount of stimulated and unstimulated cell extracts subjected to the same steps of purification (sonication, centrifugation, ion-exchange purification with collection of fractions from 0.09-0.24 M NaCl), were concentrated for the MS proteomics analysis. By comparing the protein profiles of stimulated and unstimulated samples, the immunoresponsive gene 1 (IRG1) appeared to be the most likely candidate since it was not found in the unstimulated sample but was the major component in the stimulated one (Table 6-1). The protein encoded from IRG1 was reported to be involved in the immune system, which is also consistent with the finding that mammalian cADC only appears during the macrophage stimulation.

6.8 siRNA Transfection to Confirm IRG1 as a Mammalian cADC

In order to further confirm the cADC activity of IRG1 protein, the siRNA transfections (using ON-TARGETplus SMARTpool of mouse IRG1 genes) of RAW264.7 cells were carried out. Changes in the cADC activity between cells untransfected, cells transfected with control siRNA and cells transfected with targeting siRNA of various concentrations were monitored. As higher concentration of IRG1 siRNA were used, cADC activity in cell extracts was decreased. At 90 nM siRNA transfection, the residual cADC activity was ~20% of extracts from the untreated cells or cells transfected with scrambled siRNA (Fig. 6-8). This provides another strong evidence that IRG1 codes for the cADC in mammalian cells.

No	Protein	Un.	Sti.	Ratio
1	IPI00111285 - Irg1 - immunoresponsive gene 1	<u>0</u>	<u>338</u>	-
2	PI00221614 - Arf3 - ADP-ribosylation factor 3		196	-
3	IPI00667787 - LOC545592 - similar to heterogeneous nuclear ribonucleoprotein A3	0	114	-
4	IPI00466185 - 33 kDa protein	0	114	-
5	IPI00409405 - EG620772 - similar to cofilin-1 isoform 1	0	35	-
6	IPI00853668 - LOC100040634;LOC100046224 - similar to glyceraldehyde-3- phosphate dehydrogenase (GAPDH) isoform 1	0	34	-
7	IPI00622795 - LOC100042427 - similar to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 1	0	34	-
8	IPI00828490 - Hcfc1 - host cell factor C1	0	18	-
9	IPI00761442 - 12 kDa protein	0	16	-
10	IPI00114065 - Cfb - complement factor B precursor (fragment)	0	16	-
11	IPI00117379 - Atox1 - copper transport protein ATOX1	0	13	-
12	IPI00754445 - OTTMUSG00000016644 - hypothetical protein LOC667373 isoform 1	0	13	-
13	IPI00109142 - Esd - S-formylglutathione hydrolase	0	12	-
14	IPI00129180 - Slfn1 - Schlafen1	0	12	-
15	IPI00117730 - Gbp3 - guanylate-binding protein 4	2	51	25.5
16	IPI00226188 - Camk1d - calcium/calmodulin-dependent protein kinase isoform 1	2	25	12.5
17	IPI00120113 – Tyki - thymidylate kinase family	53	508	9.6
18	IPI00130624 - Pld3 - phospholipase D3	2	17	8.5
19	IPI00319509 - Anpep - aminopeptidase N	2	15	7.5
20	IPI00136858 - Il1rn - Interleukin-1 receptor antagonist protein precursor isoform 1	16	118	7.4
21	IPI00224740 - Pfn1 - profilin-1	4	27	6.8
22	IPI00776096 - Ifit1 - bone marrow macrophage cDNA	8	52	6.5
23	IPI00123570 - Ifi47 - activated spleen cDNA	29	180	6.2
24	IPI00187512 - Nt5c3 - isoform 2 of cytosolic 5'-nucleotidase 3	5	31	6.2
25	IPI00130118 - Rab10 - Ras-related protein Rab-10	5	30	6.0
26	IPI00169925 - Ndufv2 - isoform 1 of NADH dehydrogenase	2	12	6.0
27	IPI00263863 - Hspe1 - 10 kDa heat shock protein, mitochondrial	23	136	5.9
28	<code>IPI00113992</code> - <code>Gmppb</code> - <code>mannose-1-phosphate</code> guanyltransferase subunit eta	2	11	5.5
29	IPI00229517 - Lgals1 - galectin-1	5	23	4.6
30	IPI00225634 - LOC672959;Rps12 - 40S ribosomal protein S12	8	35	4.4
31	IPI00127501 - Grcc10 - protein C10	6	26	4.3
32	IPI00124223 - Psme1 - proteasome activator complex subunit 1	3	13	4.3
33	IPI00653158 - Acaa2 - 14 days embryo liver cDNA	9	37	4.1
34	IPI00222515 - Psmd11 - 26S proteasome non-ATPase regulatory	3	12	4.0

<u>Table 6-1</u>. Relative increase in proteins in partially purified RAW264.7 extracts of LPSstimulated and unstimulated cells as identified by MS proteomics analyses. Figure 6-7. Effect of IRG1 siRNA transfection on cADC activity. Controls are from either untransfected cells (left) and cells transfected with control (scrambled) siRNA (right). IRG1 siRNA^a concentration are 30 nM, 60 nM and 90 nM from left to right. The error bars are the S.D. determined from three independent siRNA transfection tests.



^{*a*}The IRG1 siRNA is ON-TARGETplus SMARTpool (Mouse IRG1), a mixture of 4 siRNA, provided by Dharmacon with undisclosed sequence information.

6.9 Discussion

The ITA biosynthetic pathway in fungi occurs via decarboxylation of *cis*-aconitate by cADC [81,82]. The ITA detected as a novel metabolite in mammalian cells, especially in macrophage-derived cells, was also attributed to decarboxylation of *cis*-aconitate by a specific decarboxylase [114]. Fungal cells secrete large amounts of ITA (presumably as an antibacterial agent); ITA is also secreted in significant amounts by stimulated RAW264.7 cells and by the metastatic mouse VM-M3 tumor cells. However, the role of ITA in these mammalian cells is not clear. The speculation that ITA may act as an antibacterial agent [120,121] suggests a relationship between ITA and the immune response. Macrophage activation processes usually stimulate numerous gene products that are significant to the immune response, some of which may be highly related to the ITA production and its biological roles.

In my work, the observation that actinomycin D (ActD) can highly inhibit the cADC activity eliminated the possibility of post-translational modification (PTM) of an existing protein and showed that mammalian cADC was produced by *de novo* protein synthesis during macrophage stimulation. The expression of mammalian cADC is induced by immune response, which also addressed the significance of ITA and the related enzyme in this process.

Knowing that the protein is synthesized upon cell stimulation provides a way to purify the increased cADC protein from all the background proteins in cells. Classical biochemistry approaches would be tried to purify the protein based on its unique biophysical properties, such as relatively low or high pI values, hydrophobicity or different molecular weight. Various purification techniques can assist in enriching cADC activity and potentially yielding the protein responsible. However, after several purification steps the cADC activity was sufficiently diluted as to be almost undetectable by our ¹H NMR assy. Furthermore, the high hydrophobicity of mammalian cADC made it impossible to find an optimal hydrophobic resin for further purification. Gel-filtration did not separate the protein sufficiently to determine its identification, but it did provide a native mass range of target enzyme (around 67 kDa, near the BSA protein eluting position according to the standard gel-filtration curve).

Proteomics using mass spectrometry can provide global analyses of proteins in a proteome, as well as focus on post-translational modification of proteins. For us, this technique identified which soluble mammalian proteins were overexpressed when RAW264.7 cells were stimulated. This led to the hypothesis that IRG1 was the source of the cADC activity.

Recently, the IRG1 gene was identified from an LPS-activated RAW264.7 cell line [91] and suggested to be one of mammalian cADC candidates. In macrophages, IRG1 gene is highly involved in the regulation of immune responses, and closely related to the TLR signaling pathways [135-137]. In our TLR agonists experiments, the target mammalian cADC was also synthesized in response to specific engagement of various Toll-like receptors. TLRs are a type of non-catalytic receptors that are expressed in macrophages and usually activate the immune response by recognizing structurally conserved molecules in microbes. One of the major signals for TLR is the MyD88(myeloid differentiation factor-88)-dependent response that activates NF-κB and mitogen-activated

protein kinase. The fact that the IRG1 gene is highly related with MyD88 [135-137] further links IRG1 production to TLR signaling.

The subcellular localization of IRG1 in murine macrophages, as a prototypic TNF- and IFN- γ -coregulated gene, is associated with mitochondria, which are known to play crucial roles in the regulation of cellular processes, such as apoptosis and innate immune activation [140,141]. Clearly, further study on the localization of mammalian cADC is important in understanding its roles in mitochondrial functions as well as in the entire immune system.

What my work shows is that the recombinant protein encoded by IRG1 is 54 kDa, a 13 kDa difference from the ~67 kDa molecular weight range from the gel-filtration result. The native protein may have an unusual shape, or it could be interacting with a smaller binding partner. Alternatively, the protein could have some post-translation modifications that alter it significantly. The identification of the IRG1 protein from RAW264.7 cell lysate via the specific antibody targeting is a possible way to prove the existence of post-translation modification. MS-based proteomics may also be used to identify any modification sites on cADC. Such modifications could provide insights into how this protein catalyzes the unique decarboxylation reaction of *cis*-aconitate.

6.10 Conclusions

Itaconic acid (ITA) is a metabolite produced by primary macrophages and cell lines derived from macrophages that is dramatically increased upon stimulation. ITA is synthesized by a *cis*-aconitate decarboxylase (cADC) enzyme. Sequence searches with a known fungal cADC show little homology to ORFs in mammalian cells. The macrophage cell line RAW264.7 was studied to show that cADC activity upon stimulation is dependent on *de novo* protein synthesis. MS analyses of partially purified RAW 264.7 protein extracts from stimulated cells show a large increase for immunoresponsive gene 1 protein (IRG1). IRG1 synthesis follows the time course of ITA generation (measured by ¹H NMR) and siRNA knockdown of the IRG1 reduces cADC activity upon stimulation. However, the function of IRG1 is still worthy of further investigation in macrophages and its up-regulation in stimulated macrophages.

Chapter 7

Expression of IRG1 and Comparison to Fungal cADC

7.1 Introduction

The IRG1 cDNA was first identified by screening a cDNA library from an LPSactivated RAW264.7 cell line by Lee G. G., et. al (1995) [91]. This gene was identified as a marker and target for many cellular processes. In primary macrophages, the IRG1 gene was also identified and found to be highly involved in regulating innate and adaptive immune responses. The function of the IRG1 gene is related to the myeloid differentiation factor-88 (MyD88), the key adaptor molecule of the Toll-like receptor (TLR) signaling pathway [135-137]. The subcellular localization of IRG1 in murine macrophages, as a prototypic TNF- and IFN-γ-coregulated gene, is associated with mitochondria, which are known to play crucial roles in the regulation of cellular processes, such as apoptosis and innate immune activation [140,141]. Clearly, the function of IRG1 and its upregulation in stimulated macrophages are worth studying in detail.

Generation of recombinant cADC can help to clarify the unique decarboxylation mechanism. It also opens up an array of future studies in the mammalian immune system and cancers. To that end I cloned IRG1 into an *E. coli* expression system, purified and characterized the enzyme and compared it to the fungal cADC. Suspected active site residues were identified by mutagenesis studies based on a homology structure model of fungal cADC.

7.2 Secondary Structure and Thermostability of IRG1 and Fungal cADC

The secondary structure content and thermal stability of IRG1 and fungal cADC were measured using far-UV circular dichroism (CD). The CD spectrum for IRG1 protein is typical of a protein with mixed secondary structure (Fig. 7-1A). The analysis of secondary structure content with the CDNN program is shown in Table 7-1; it matches the CD spectrum and hence secondary structure content of the fungal cADC.

The loss of secondary structure with increasing temperature defines an operational melting temperature (T_m) of around 50.5 °C for the mammalian cADC (Fig. 7-1B). This indicates that in normal cells at 37 °C, the IRG1 protein is well folded and has no tendency to unfold. Interestingly, the MBP-tagged fungal cADC protein shows a plot with two distinct transitions (Fig. 7-1C), one with a mid-point of 37.9 °C and the other at 57.4 °C. By testing the thermal stabilities of both cleaved MBP tag and cADC tag-off protein, these two temperatures were attributed to each of the two components. The melting temperature (T_m) was 38.2 °C for WT fungal cADC with the MBP tagged removed, significantly lower than the mammalian enzyme. Mutant proteins of the fungal cADC showed little change in this effective T_m (the range was 35.7-39.3 °C). These mutant proteins also exhibited the same secondary structures as the WT fungal cADC (Table 7-1, the data are shown for MBP tagged mutant proteins). Similar behavior was observed for mammalian IRG1 mutants. Compared with WT IRG1 protein, there were no significant changes in structure and thermal stability (T_m is within 3 °C of the T_m for WT) for enzymes bearing a single altered amino acid (data not shown). Therefore, any difference in activity of both IRG1 and fungal cADC variants is not due to significant alterations in protein structure or stability.

<u>Figure 7-1.</u> Comparison of CD spectra and thermostability for IRG1 and fungal cADC. (A) CD spectra of (\bigcirc) IRG1 and (\square) fungal cADC. (B) The ellipticity of (\bigcirc) IRG1 (solid line) and (\square) fungal cADC (dashed line) at 222 nm as a function of temperature. (C) The ellipticity of (\bigcirc) MBP tagged fungal cADC (solid line), (\square) tag-off fungal cADC (dashed line) and (\triangle) MBP tag (dotted line) as a function of temperature.



	Secondary Structure				- (0.0)	
Protein	α-Helix	β-Sheet	<i>β</i> -Turn	Random Coil	T _m (℃)	
			%			
IRG1	23.7	24.1	17.2	35.1	50.5	
Fungal cADC	21.6	26.5	17.5	34.4	38.2	
MBP tagged fungal cADC mutant						
WT	23.7	24.7	17.5	34.1	37.9	
E100A	25.4	23.5	17.4	33.7	37.7	
Y104A	22.0	26.1	17.4	34.4	37.9	
H111A	22.1	25.7	17.5	34.7	38.1	
H168A	22.6	25.6	17.5	34.3	36.4	
K217A	22.7	25.6	17.5	34.1	36.2	
K288A	21.5	26.3	17.4	34.6	39.1	
H396A	26.1	23.2	17.6	33.0	39.3	
K428A	24.9	24.2	17.6	33.4	35.7	

Table 7-1. Comparison of secondary structure content and T_m values of IRG1 and fungal cADC.

7.3 Cofactors

Decarboxylation reactions are usually catalyzed by enzymes with the assistant of various cofactors [98] such as biotin, flavin, NAD⁺/NADP⁺, pyridoxal 5'-phosphate (PLP), thiamine diphosphate (TPP), metal ions (Fe²⁺, Mg²⁺, Mn²⁺). The active sites of decarboxylases can bind these cofactors to stabilize the carbanion upon elimination of carbon dioxide from the substrate. However, there is also one type of decarboxylase, which can activate the decarboxylation that does not use an exogenous cofactor [98]. Orotidine 5'-monophosphate decarboxylase (OMPDC) and malonate semialdehyde decarboxylase (MSAD) are examples of this type of decarboxylase enzyme. To that end, we added a series of cofactor candidates to both the fungal cADC and IRG1 decarboxylation reaction system to test for any activation. No obvious enhancement of activity was observed, and some metal ions cofactors, like Fe²⁺ and Mn²⁺ had an inhibitory effect on the cADC activity (Fig. 7-2). This indicates that *cis*-aconitate decarboxylase might share the enzymatic mechanism with OMPDC or MSAD, which catalyze decarboxylation reactions without any cofactors.

<u>Figure 7-2.</u> Effect of exogenous cofactors on the activity of (\blacksquare) IRG1 and (\Box) fungal cADC. The error bars are the S.D. from independent experiments.



7.4 Kinetic Behavior of IRG1 and Fungal cADC

The pH optimum of these two enzymes was determined. The fungal cADC showed optimal activity between pH 5.8-6.2 while for IRG1 the pH optimum was pH 7.5. This indicates that this mammalian cADC works under physiological conditions but the fungal cADC prefers slightly acidic environment. This is consistent with the result in the cADC activity evaluation of RAW264.7 cell lysate (see Chapter 6), which also exhibited the highest activity around pH 7.5.

From the measurements of the cADC activity versus substrate (*cis*-aconitic acid) concentration, the K_m for *cis*-aconitate was determined to be 2.8±0.6 mM for fungal cADC at pH 6.0 (Fig. 7-3B). The equation used for fitting the kinetic curve, $v = V_{max}[S]^n/(K_m^n + [S]^n)$, tries to account for any cooperativity. However, neither enzyme showed significant cooperativity ($n = 1.3\pm0.4$ for the fungal enzyme and 0.8 ± 0.2 for IRG1) indicates that the cADC enzymes are active as monomers. The V_{max} for the fungal enzyme was 17 ± 2 µmol min⁻¹ mg⁻¹ (Fig. 7-3B). IRG1 exhibited a two-fold lower V_{max} (8.2 ± 0.1 µmol min⁻¹ mg⁻¹) with a K_m of 0.38 ± 0.08 mM. It should be noted that the data for both IRG1 and fungal cADC were each normalized to the highest observed activity in this plot (Fig. 7-3B). Enzyme efficiency, k_{cal}/K_m , was 5.5 ± 0.6 mM⁻¹ s⁻¹ for the fungal enzyme and 20 ± 1 mM⁻¹ s⁻¹ for IRG1. Thus, the *cis*-aconitic acid is more efficiently converted to itaconate by IRG1 than by the fungal cADC, at least as measured at the pH optimum for each enzyme.

The high value of K_m for fungal cADC might suggest the possibility of other better substrates for this enzyme. According to the sequence alignment result, the fungal cADC

contains a conserved domain of the MmgE/PrpD family of proteins that includes several 2-methylcitrate dehydratases. Trisodium (2RS, 3RS)-2-methylcitrate was used as the substrate to test the 2-methylcitrate dehydratase activities for both IRG1 and fungal cADC. Since a carbon-carbon double bond is formed in this specific reaction, the comparison of UV-vis spectra between substrate and product was used to monitor activity [176]. However, no obvious 2-methylcitrate dehydration activity was observed either by IRG1 or by fungal cADC. Therefore, both the fungal and mammalian cADC are specific catalysts for the decarboxylation of cis-aconitic acid. The K_m values reflect only a moderate affinity for the substrate. Working at V_{max} would require a *cis*-aconitate concentration of over 3 mM (IRG1) or 15 mM (fungal cADC). However, enzyme activity at a lower rate could occur producing a basal level of ITA. Interestingly, in previous studies of VM-M3 and RAW264.7 cell extracts using NMR did not detect any cisaconitate. However, metabolites would need intracellular concentrations of >0.5 mM to be detected by this method. ¹³C-labeled *cis*-aconitate was detected but not quantified by mass spectrometry. IRG1 is rapidly produced upon activation of RAW264.7 cells (Chapter 6) and it is possible that other factors affect the activity of this enzyme.

Figure 7-3. Comparison of kinetic behaviors of IRG1 and fungal cADC. (A) pH dependence for (\bullet) IRG1 and (\Box) fungal cADC. (B) Relative specific activity of (\bullet) IRG1 at pH 7.5 and (\Box) fungal cADC at pH 6 as a function of total substrate concentration. For each system the observed activity was normalized to the maximum observed activity, 8.2±0.1 µmol min⁻¹ mg⁻¹ for IRG1 and 17±2 µmol min⁻¹ mg⁻¹ for fungal cADC. The error bars are the S.D. from independent experiments.



7.5 Inhibitor Screening for Both IRG1 and Fungal cADC

cADC catalyzes an unusual decarboxylation reaction without a cofactor. Presumably the cis-aconitate structure is optimized for decarboxylation. If so, investigation of a variety of related compounds might provide hints as to what is critical for binding. To that end, we screened a variety of substrate analogues (di- and tri-carboxylates) as inhibitors of ITA production. As shown in Fig. 7-3, with 1:1 ratio of *cis*-aconitic acid to added compound, most di- and tri-carboxylates were not inhibitory. The two inhibitory compounds for both fungal cADC and IRG1 protein were maleic acid and citraconic acid. Under these conditions, maleic acid showed around 25% inhibition on the activities of both fungal cADC and IRG1, and itaconic acid (product) and citraconic acid showed more significant inhibition of IRG1 (~65%) when mixed 1:1 with substrate. Interestingly, itaconic acid is not an inhibitor of the fungal cADC activity. As for citraconic acid, by pre-incubating this substrate analogue with substrate 30 min (at room temperature) prior to reaction, the cADC activity of IRG1 can be reduced less than 20% of original value. This compound has the highest structure similarity to *cis*-aconitic acid. The citraconic acid can be further employed as a stabilizing reagent in the crystallization of IRG1 protein.

Figure 7-4. Inhibitor screening for IRG1 and fungal cADC. (A) The chemical structures of di- and tricarboxylates used in the inhibitor screen compared with itaconic acid and *cis*-aconitic acid. (B) The inhibition effect of maleic acid, itaconic acid, citraconic acid on (\blacksquare) IRG1 and (\Box) fungal cADC. The error bars are the S.D. from independent experiments. *Citraconic acid: pre-incubate citraconic acid with IRG1 enzyme for 30 min (r.t.).



7.6 Mutagenesis Study Based on a Homology Model of Fungal cADC

In a structure similarity search for fungal cADC, several proteins were found (Fig. 7-5), including a HUMAN Immunoresponsive gene 1 protein homolog, which is the HUMAN homolog of the IRG1 that was identified in murine macrophage stimulation. Based on the structure and active site composition of iminodisuccinate epimerase (PDB ID: 2HP0) [90], the only protein with known crystal structure, a predicted model structure (assisted by Boguslaw Stec, Sanford-Burnham Medical Research Institute, La Jolla, CA) for the fungal cADC was generated and several mutants (E100A, Y104A, H111A, H168A, K217A, K288A) were designed (Fig. 7-6A, highlighted in red). These residues are highly conserved among the proteins found in the structure similarity search (Fig. 7-5, highlighted in yellow and cyan). The only exception is Tyr104, which was replaced by serine, threonine or cysteine in other proteins. However, considering that the side chain may act as a hydrogen bonding donor for Ser92 in iminodisuccinate epimerase, these residues with hydroxyl or thiol side chains (tyrosine, threonine, cysteine) are also worth replacing to assess their function in forming active site.

In enzymatic assays of mutant protein, using longer reaction time (100 min compared to the regular 20 min) and higher protein concentration (200 μ g/mL compared to the regular around 10 μ g/mL), no production of ITA was observed for all of these predicted active site mutant proteins. This indicates that mutating these residues to alanine totally deactivates the cADC enzyme and these residues play important roles in the active site. As controls, His396 and Lys428 were converted to alanine to test the effect of deleting a specific residue on enzyme function. These residues in the cADC model are located far

away from proposed active site (Fig. 7-6, highlighted in blue). These two mutant proteins (H396A and K428A) showed the same kinetic behavior as WT fungal cADC.

Based on the sequence alignment of IRG1 with fungal cADC (Fig. 7-6B), the homologous residues (D93A, H103A, H159A, K207A, K272A) were chosen to verify the same active site composition of these two cADCs. These mutant proteins were also inactive (Table 7-2), which suggests that IRG1 protein might share the same enzymatic mechanism for the decarboxylation of *cis*-aconitic acid with fungal cADC.

7.7 Discussion

The recombinant mammalian cADC exhibits slightly different properties compared to fungal cADC. However, both of them have a conserved domain of the MmgE/PrpD family of proteins. There is one type of MmgE/PrpD proteins that only has less than 25% sequence identities compared to *E.coli* PrpD. Most of these proteins are of unclassified functions. Both the IDS epimerase that was used to build up the fungal cADC structure model and the IRG1 protein are included in this minimal sequence similarity subgroup of MmgE/PrpD family. The typical fold of IDS epimerase can be used as the representative structure for the whole MmgE/PrpD family, which is conserved for most of the MmgE/PrpD proteins even though they only have limited sequence similarity. Thus, this structure can be utilized as a scaffold to support different enzymatic functions, such as dehydration, epimerization, and so on. This may also provide some structural based evidence on how the IRG1 and fungal cADC catalyze the decarboxylation reaction to produce ITA.

The epimerization of IDS under IDS epimerase is cofactor-independent. Similar to

other epimerases, the IDS epimerization of IDS was shown to catalyze by IDS epimerase via a single or two-base mechanism (Fig. 7-7, Scheme I, II and III) [90]. Based on the crystal structure and in silico docking models of IDS epimerase, His99 (conserved resides are His103 in IRG1 and His111 in fungal cADC) and Tyr145 (this residue is less conserved) are identified as possible candidates that act as a general base or acid in deprotonation/reprotonation of IDS [90]. By using one or two histidine residues for IRG1 or fungal cADC, the decarboxylation of *cis*-aconitic acid may also be catalyzed by a similar one or two-base mechanism as IDS epimerization (Fig. 7-7, Scheme IV). Abstraction of the α -proton, facilitated by the adjacent carboxyl group, could result in an enolic intermediate that may be stabilized by interactions with electrophilic groups of the enzyme [90]. This could be facilitated by His150 (His159 in IRG1 and His168 in fungal cADC), Lys198 (K207 in IRG1 and K217 in fungal cADC) or Lys266 (K272 in fungal cADC and K288 in fungal cADC). The loss of decarboxylation activity of both IRG1 and fungal cADC variants by mutating these predicted key active site residues to alanine strongly suggests that the *cis*-aconitate decarboxylase shares mechanistic similarity with the catalytic mechanism of IDS epimerase. However, the missing of Tyr145 counterpart in the decarboxylases and the more complicated features of *cis*-aconitate decarboxylation imply that the two mechanisms are still quite different.

According to the enzymatic mechanisms of other cofactor-independent decarboxylases that catalyze non-oxidative decarboxylation, the reactions of substrate are either via bimolecular electrophilic substitution or stepwise decarboxylation and protonation [98]. However, none of these decarboxylation reactions can remove a

carboxyl group and produce a carbon-carbon double bond simultaneously. Thus, this unique decarboxylation mechanism of cADC needs to be clarified based on the further structure alignment of IRG1 (or fungal cADC) with the other types of decarboxylases to prove the possibility of similar active site shape and space arrangement of key residues. However, considering that the non-oxidative decarboxylases only cleave the bond between leaving carboxyl group and the other parts of the substrate molecules while cADCs cleave this bond by introducing a C=C bond, these decarboxylases may share a very different catalytic mechanism.

The inhibitor screen found a substrate analogue – citraconic acid – that may prove useful in stabilizing IRG1 for crystal formation. To date, we only tried crystallization of the fungal cADC. Unfortunately, no crystals were obtained. The difficulty in the crystallization of this decarboxylase may be due to its low stability or structural flexibility. Thus, by adding an inhibitor in the active site, the structure may be more organized. If crystals are obtained, the interactions between substrate analogue and proposed active site residues should provide strong evidence for the decarboxylation mechanism. Further docking model of substrate into the structure *in silico* should also help to elucidate this unique mechanism. By trapping the carbanion-like intermediate in to the structure, it can be verified whether the decarboxylation is via carbanion intermediate or not. Figure 7-5. Sequence alignment of fungal cADC with most similar proteins: (1) Fungal *cis*-aconitate decarboxylase [*Aspergillus terreus*]; (2) Iminodisuccinate epimerase [*Agrobacterium tumefaciens* BY6, PDB 2HP0]; (3) 2-Methylcitrate dehydratase [*Bacillus anthracis Ames:* BA-2349]; (4) 2-Methylcitrate dehydratase [*Escherichia coli* str. K-12 substr. MG1655]; (5) Pdh1p [*Saccharomyces cerevisiae* S288c]; (6) Possible methylcitrate dehydratase PrpD [*Mycobacterium tuberculosis* H37Rv]; (7) HUMAN immunoresponsive gene 1 protein homolog [*Homo sapiens*]. The highlighted residuces are the predicted active site based on the crystal structure of (2) iminodisuccinate epimerase with the conserved ones in yellow and the less conserved one in cyan.

	100 104 111	
[1]	MTNSAFIOATELDDYHSEAPLHSASIVLPAVFAASEVLAEOGKTISGIDVIL	141
[2]	FVNGMAAHAIDFDDSFPVMRGHPSSSLVPAIFAVGEHVGANGHNCLK	124
[3]	FNIGCMIRWL <mark>D</mark> YND <mark>T</mark> WLAAEWG <mark>H</mark> PSDNLGGILAVADYISRV-RISEGKEPLKVREVLE	144
[4]	FNIGAMIRWL <mark>D</mark> FND <mark>T</mark> WLAAEWG <mark>H</mark> PSDNLGGILATADWLSRN-AVASGKAPLTMKQVLT	146
[5]	FAIGTLIRWLDYNDCWLAAEWGHPSDNLGGILAVADHLSRLNKATHGKNGKQFLVKDVLE	174
[6]	WANGVAARELDFHDTFLAADYSHPADNIPPLVAVAQQLGVCGAELIR	166
[7]	FVNGVAIHSMDFDDTWHPAT-HPSGAVLPVLTALAEALPRSPKFSGLDLLL	132
	· · · · · · · · · · · · · · · · · · ·	
	168	
[1]	AAIVGFESGPRIGKAIYGSDLLNNGW <mark>H</mark> CGAVYGAPAGALATGKLLGLTPDSMEDALGIAC	201
[2]	SYVLGIEVVATLG-RAVGKGHYLAGW <mark>H</mark> PTSTLGVFGATTAAALLLGADEEQLRNAWGIAA	183
[3]	MMIKAHEIQGVLALENSLNRVGLD <mark>H</mark> VLYVKVATTAVVAKMLGGTREEIFNALSHAW	200
[4]	AMIKAHEIQGCIALENSFNRVGLD <mark>H</mark> VLLVKVASTAVVAEMLGLTREEILNAVSLAW	202
[5]	AMIKAHEIQGIIALENSFNKVGLD <mark>H</mark> VVLVKVATAGVVSKMLGLSQEQTIEALSQAF	230
[6]	GLVTAYEIHIDLTRGICLHEHKID <mark>H</mark> VAHLGPAVAAGIGTMLRLDQETIYHAIGQAL	222
[7]	AFNVGIEVQGRLLHFAKEANDMPKRF <mark>H</mark> PPSVVGTLGSAAAASKFLGLSSTKCREALAIAV	192
	· * · · · · · * · *	
r 1 1	217	257
[1]		207
[2]	SNSCGIIKNFGTMTKPMHTGSAARNGVLSAWLSMQSFTGCQTVFDDAEGILAMY	237
	IDNSSLRTYRHAPNTGSRKSWAAGDATSRGVHLAMTALKGEMG-YPTALSAPGWGFQDVL	259
	VDGQSLRTYRHAPNTGTRKSWAAGDATSRAVRLALMAKTGEMG-YPSALTAPVWGFYDVS	201
[2]		290
[0]	HLTTSTRUSRKG-AISSWKAFAPAHAGKVGIEAVDRAMRGEGSPAPIWEGEDGVIAWL	2/9
[/]	SHAGAPMANAATQT <mark>K</mark> PLHIGNAAKHGIEAAFLAMLGLQGNKQVLDLEAGFGAFY	246
	· · · · · · · · · · · · ·	
	200	
[1]	TKGNGREPPYKEEEVVAGI.GSFWHTFTIRT <mark>K</mark> I.YACCGI.VHGPVEATEKI.ORRYPEI	313
[2]	GAOPGPEI.FNAMOKEGTPWATIAPGI.YK <mark>K</mark> SWPSCYANHKPI.AGI.FAIMKEHGI.T.	291
[2]	FNKOEIKLARPLESYVMENULFKUSYPAEFHAOTAAECAVKLHPETKER	308
[4]	FKGESFRFORPYGSYVMENVLFKISFPAEFHSOTAVEAAMTLYEOMOAA	310
[5]	FKGKPFSFOORSKYDSYVMENVLF <mark>K</mark> ISFPAEFHAOTAVEAAVKAYRTLAKO	341
[6]	LAGPEHTYRVPLPAPGEPKRAILDSYTKOHSAEYOSOAPIDLACRI.RER	328
[7]	ANYSPKVLPSTASYSWLLDOODVAF <mark>K</mark> RFPAHLSTHWVADAAASVRKHLVAF	297
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Figure 7-6. Mutagenesis design for IRG1 and fungal cADC. (A) The active site residues chosen based on the protein structure predicted by Boguslaw Stec on the crystal structure of iminodisuccinate epimerase (2HP0). The active site is highlighted in red and the control residues are in blue. (B) Sequence alignment between IRG1 and fungal cADC. The active site residues are highlighted in red and the control residues are in cyan.





Protein	[Protein] (µg/mL)	[<i>cis</i> -Aconitic acid] (mM)	Reaction time (min)	Product (%)		
IRG1 mutant						
WT	12.5	10	20	20%		
D93A	200	10	100	<2%		
H103A	200	10	100	<2%		
H159A	200	10	100	<2%		
K207A	200	10	100	<2%		
K272A	200	10	100	<2%		
Fungal cADC mutant						
WT	10	10	20	20%		
E100A	200	10	100	<2%		
Y104A	200	10	100	<2%		
H111A	200	10	100	<2%		
H168A	200	10	100	<2%		
K217A	200	10	100	<2%		
K288A	200	10	100	<2%		
H396A	10	10	20	23%		
K428A	10	10	20	18%		

<u>Table 7-2.</u> Activity table for active site residues mutant protein compared with WT IRG1 and fungal cADC.

Figure 7-7. The proposed cADC decarboxylation mechanism based on epimerization mechanism of minodisuccinate (IDS). (I) Epimerization of the stereoisomers of iminodisuccinate catalyzed by IDS epimerase; (II) One-base mechanisms for IDS epimerisation; (III) Two-base mechanism for IDS epimerization; (IV) Proposed decarboxylation mechanism of *cis*-aconitic acid to produce itaconic acid based on the two-base mechanism of (III).



7.8 Conclusions

The IRG1 was cloned into the *E. coli* expression system for further purification. The pH profile and kinetic properties of this recombinant enzyme were characterized and compared to the fungal enzyme. This unique decarboxylase does not use cofactors and was strongly inhibited by citraconic acid. Suspected active site residues were identified by mutagenesis studies based on a homology structure model built from fungal cADC. The identification of this enzyme can help to clarify the unique decarboxylation mechanism and also open up future studies into the specific role of ITA in the mammalian immune system and cancers.

Chapter 8

Future Directions

The first aim of my thesis was to understand the membrane modulation of one type of peripheral protein, bacterial PI-PLC, using recombinant enzymes from tow different types of bacteria (*Bt*PI-PLC and *Sa*PI-PLC). Most of my effort was focused on defining membrane interactions of *Sa*PI-PLC. Different techniques were applied to study the interaction of this protein with vesicles composed of both the substrate lipid PI (or substrate analogues PG or PMe) and the activator lipid PC. Based on these results, a surface dimerization and activation mechanism was proposed for this PI-PLC (Chapter 4, Fig. 4-10). The enzyme activity is modulated by competition between binding of soluble anions or anionic lipids to the cationic sensor and transient dimerization on the membrane depleted in anionic phospholipids.

As part of the proof for an active dimer, I tried to construct a disulfide linked dimer based on the V44C *Sa*PI-PLC variant. However, no active dimer was obtained, presumably because of a misaligned arrangement of the dimer interface residues. A possible explanation is that the dimer interface was disrupted by the hydrogen bonding of cysteine side chains (DTT was initially present to keep the protein reduced). It would be interesting to see if we can find a better position to introduce cysteine resides and form a disulfide-linked dimer. The prediction is that it would possess higher PI cleavage rates, particularly towards low substrate concentrations at high X_{PC}. Since the conformation of the covalent dimers may be different from the WT *Sa*PI-PLC dimer crystal structure (PDB ID: 4F2B), it is also worth crystallizing this protein assuming we can separate disulfide-linked from monomeric protein. A detailed view of this artificial dimer interface may help explain the unique membrane modulation of *Sa*PI-PLC enzymatic activity.

Both *Bt*PI-PLC and *Sa*PI-PLC can target GPI-anchored proteins in the PC-rich outer membrane of eukaryotic cells. However, unlike to *Bt*PI-PLC that has a high PC binding affinity, SaPI-PLC has virtually no affinity for PC vesicles (Chapter 4). In order to verify the cation- π box in *Bt*PI-PLC to be a specific PC recognition motif, we engineered such a box into SaPI-PLC by adding two more tyrosine residues. This generated a catalytically active protein that binds much more tightly to PC-rich interfaces (N254Y/H258Y variant, Chapter 5). However, the binding affinity of SaPI-PLC N254Y/H258Y towards pure PC bilayers is still significantly weaker than *Bt*PI-PLC, which may be due to the fact that *Bt*PI-PLC has more aromatic residues around the $\alpha\beta$ -rim facing the membrane and these can either form other transient cation- π complexes with PC or insert into the bilayer. How can we experimentally distinguish cation- π complex formation from membrane insertion of aromatic side chains in both BtPI-PLC and SaPI-PLC? One option is the application of fluorinated tyrosine and phenylalanine. Fluorine substitutions can deactivate cation- π interactions due to the diminished negative electrostatic potential of the aromatic ring caused by the high electronegativity of fluorine [177]. By incorporating this kind of unnatural amino acid into the PI-PLCs and measuring the energetic contributions of the two types of interactions (aromatic π -choline cation of PC interaction versus aromatic residues side chain partitioning into a bilayer) based on apparent K_d values, we should be able to identify which protein aromatic groups form complexes with the PC cation. This would complement a molecular dynamics simulation of *Bt*PI-PLC docking to a bilayer where quite a few cation- π complexes could be distinguished. Added to the biochemical work is the need to generate crystal structures for the fluorinated proteins. These are important to rule out changes in side chain orientations that might affect PC binding and activation. This would be most critical for incorporation of di-3,5-fluorotyrosine where the hydroxyl pK_a is significant reduced (to \sim pH 6.5) [178].

Bacterial PI-PLCs are known to target GPI-anchored proteins in the external leaflet of a host cell. Release of the tethered protein and generation of diacylglycerol in the membrane is thought to contribute to the pathogenicity of the bacterium. Future studies on the binding affinity of these proteins to membranes need to use membrane vesicles containing GPI anchors. While recombinant generation of specific GPI-anchored proteins is not feasible at the moment, one can often enrich membrane preparations with GPIanchored proteins. To that end, we should be able to directly observe how PC composition or other factors affects the enzymatic cleavage of the PI moiety from the rest of the carbohydrate tether plus protein. Understanding how the different bacterial PI-PLCs interact with target membranes may provide specific ways to selectively inhibit them, and hence provide insights into novel methods to control the bacterial infections.

Endoplasmic reticulum (ER) membranes are continuous with the nuclear envelope outer membrane in eukaryotic cells. These kinds of membranes are also rich in PC with an even higher ratio of it compared to the outer cell membrane phospholipid composition. The ER provides a site for lipid and protein biosynthesis. Along with the major phospholipid PC, ER membranes also have phosphatidylethanolamine (PE) and phosphatidylserine (PS), as well as smaller amounts of PI. Bacterial PI-PLCs are usually extracellular proteins, but some are secreted from intracellular pathogens. *Listeria monocytogenes* produces a PI-PLC that among other things contributes to escape from primary and secondary vacuoles [179,180]. This might be the best candidate to explore binding to ER-like membranes.

The second part of my thesis was to identify and characterize a novel mammalian cADC. Based on the structural model of fungal cADC, a possible active site was proposed. However, this unique mechanism suggested for this enzyme is worth further investigation. The enzyme appears to simultaneously remove a carboxyl group and introduce an unsaturated bond. What is the spacial arrangement of key active site residues? These questions would be answered by the crystallization of mammalian or/and fungal cADC with or/and without a substrate analogue (e.g., citraconic acid).

The biological role of ITA is also unknown. Based on our experiments to identify the mammalian cADC, ITA production is shown to be highly related to the TLR signaling pathway. The accumulation of ITA upon macrophage stimulation indicates its significant role in protecting the host organism. ITA does inhibit some bacterial TCA enzymes, but it is not a very strong inhibitor [120,121]. Since so much of it is secreted by the RAW264.7 cells, perhaps it has another function. It is also possible that the mammalian cADC carries out another activity as well. IRG1 has been implicated in a wide variety of immune responses, not all involving bacteria. Could the molecule be an extracellular signal? Perhaps intracellular pools of ITA modulate host TCA activity in a way useful. The cADC could also form complexes with other proteins and these might be relevant in the immune response. Certainly the function of ITA in immune system is still worth of further studies.
Appendix I

Phosphatidylcholine Activation Varies on the Changes of

Surface Cationic Residues in *B. thuringiensis* PI-PLC

AI.1 Introduction

There are several types of noncovalent interactions that play critical roles in maintaining cell membrane functions and facilitating protein-membrane interactions. These include hydrogen bonding, van der Waals contacts, electrostatic attractions, and ion-dipole interactions. For interfacial interactions electrostatic forces often initiate the binding of many soluble proteins to the membrane surface. This occurs from the attraction between the anionic lipid head groups and cationic amino acids on the protein surface. Once the protein is in the vicinity of the membrane, other interactions enhance protein binding via more specific contacts (hydrophobic partitioning, hydrogen bonding, or cation- π interactions) of key residue side-chains. Specific electrostatic driving forces are crucial for phospholipases such as the human group IIa secreted phospholipase A₂ (PLA₂) to bind to phospholipid membranes [37]. The importance of these electrostatic interactions for PLA₂ binding to anionic membranes was shown by charge-reversal mutants.

For a peripheral protein, such as *Bt*PI-PLC, interfacial interactions usually influence the enzymatic activity of the protein. A recent model of *Bt*PI-PLC membrane binding and activity indicated the importance of some key residues around protein surface. The membrane binding is mediated by the electrostatic interactions between Lys44 in helix B and anionic phospholipids as well as the hydrophobic plug containing two tryptophan residues (Trp47 in helix B and Trp242 on an adjacent 240s'loop). This initial interaction correctly orients the protein to the membrane and could induce a transient dimerization if it occurred. A previous crystal structure of the W47A/W242A protein exhibited a dimer formed by the Tyr zipper involving Tyr residues 246-248 and 251, which will activate PI-PLC toward both membrane-bound (PI) and soluble substrates (cIP) [44].

In this work, several cationic residues identified by MD simulation as aiding in electrostatic interactions of the protein to the anionic lipids bilayer, Lys44, Lys38, Arg71, Lys 201 and Lys 279, were changed to alanine to measure the effect on vesicle binding and catalytic activity. Meanwhile, another mutation, V46K was designed to see if an additional positive charge could enhance enzymatic activity and vesicle binding. The enzymatic activity and membrane binding profile of different mutant proteins were measured. It was found that many cationic residues contribute to the binding of *Bt*PI-PLC to vesicles with PI and this aids in the hydrolysis of PI in the absence of PC. These cationic residues can be fairly distant from the active site. Some of these mutants also affected the PC binding affinity. For example, V46K exhibits a totally different behavior with no improvement on PI binding but large impairment of PC binding.

AI.2 Materials and Methods

All the chemicals and methods to construct *Bt*PI-PLC mutants, characterize PI-PLC enzymatic activity and binding affinity towards SUVs are described in Chapter 2, 2.1-2.10.

AI.3 MD Simulations of WT BtPI-PLC and Construction of the Mutant Protein

In this MD simulations model (done by Prof. Nathalie Reuter, University of Bergen, Norway), only three of the six tested orientations of PI-PLC led to binding to the implicit model membrane. The anchoring was monitored by calculating the binding energy along the simulation and the result showed that all bound PI-PLC had very similar orientations with the same membrane interacting residues. It is possible to further evaluate the energy of these residues by using the conformations from the last 1.5 ns of simulation. The "energy decomposition" (Fig. AI-1B) plotted for all the involved residues can be separated into two groups: (i) one group dominated by hydrophobic interactions and (ii) another group with electrostatic interactions. This result can be better visualized by anchoring the protein into the implicit membrane, which was composed of two simplified layers – the aqueous layer and a gradient of dielectric with overall negative charge as the membrane model (Fig. AI-1A). Several cationic residues were identified by MD simulation as aiding in electrostatic interactions of the protein to the anionic lipid bilayer: Lys44, Lys38, Arg71, Lys 201 and Lys 279. Residues that contributed in the hydrophobic component of binding were Pro42, Ile43, Val46 and Trp47, which were already studied in our previous work [33]. These residues belong to helix B, one of the two interesting features at the barrel rim (helix B and 240s' loop) that can penetrate into the membrane by insertion of Trp47 and Trp242. These hydrophobic amino acids can be clustered together to facilitate the Trp47 penetration into membrane. Previous mutagenesis studies showed that Pro42 could more or less fix the conformation of helix B (which is really flexible). This residue was also found to be involved in the dimer stabilization in the crystal structure of W47A/W242A [44]. Ile43 and Val46 were proposed to provide hydrophobic contacts that stabilize the outward pointing Trp47 conformation. During the interaction with the anionic head group of phospholipids, the electrostatic force is thought to orient the protein and thus initiate the binding. Therefore, in my work, the cationic

residues Lys38, Lys44, Lys201, Lys279 and Arg71 were mutated to alanine and further used to probe their contributions to membrane binding and enzymatic activity. For a control, Lys122 was also mutated to alanine since it is not near the active site or membrane binding surface. The electrostatic attraction between anionic phospholipids and cationic amino acids usually facilitates membrane binding of peripheral membrane proteins, as is the case for K44A and K44E [146]. Both of these mutants reduce the binding of *Bt*PI-PLC to membranes. They also severely impaired the binding synergy provided by the combination of PG and PC in the vesicles. It was proposed that Lys44 might drive the motion of the protein to the membrane allowing the hydrophobic interactions of PI-PLC with anionic phospholipids-rich vesicles.

AI.4 Secondary Structure and Thermal Stability Characterized by CD Spectroscopy

All of the mutant proteins were well-folded as monitored by CD wavelength scanning; thermal stabilities were essentially the same during the denaturation assay (Table AI-1). The similar secondary structure content indicates that removing or introducing one cationic residue did not have a dramatic effect on the protein structure. Thermal stabilities were all very similar (average $T_m=57.3\pm1.4$ °C), although K44A was even more stable than the other proteins ($T_m=60.3$ °C). Thus, any difference for activity or binding effect should not be due to the altered structures of mutant proteins compared to WT *Bt*PI-PLC.

Figure AI-1. MD simulation results shown by structural and energy decomposition models. (A) View of the insertion of PI-PLC into the implicit membrane. The limit of the interior region of the membrane is represented in brown, and the position of the negative charge in magenta. A color gradient going from blue to red is used to describe the residues having respectively a favorable or an unfavorable hydrophobic contribution to binding. (B) Plot representing the same energy decomposition (in kcal/mol), the Gouy-Chapman terms that account for electrostatic interactions are represented with black bars, while boxes represent the hydrophobic contacts.



Protein	Secondary Structure				– (%a)
	α-Helix	8-Sheet	<i>β</i> -Turn	Random Coil	Т _т (°С)
			%		
WT	20.6	32.2	17.2	30.0	57.1
K38A	20.2	32.5	17.1	30.1	57.3
K44A	21.6	30.9	17.4	30.1	60.3
R71A	21.4	30.9	17.2	30.3	56.8
K44A/R71A	21.9	30.9	17.4	29.8	58.8
K201A	21.6	30.9	17.4	30.1	56.6
K279A	20.8	31.8	17.2	30.1	56.0
K122A	21.3	31.4	17.3	29.9	57.2
V46K	20.7	31.6	17.2	30.6	55.4

<u>Table AI-1.</u> Comparison of secondary structure contents and T_m values of WT and mutant *Bt*PI-PLCs.

AI.5.1 Phosphotransferase Reaction Activity

Long chain lipid PI dispersed in neutral lipid Triton X-100 (TX-100) micelles is a good substrate system for *Bt*PI-PLC. The concentration of PI used was 4 mM with 8 mM TX-100. Removal of one cationic residue led to reduction of the PI cleavage activity for K38A, R71A and K201A, which exhibited 30~50% of the activity of native *Bt*PI-PLC (Fig. AI-2A). However, other positional mutants (K44A, K122A and K279A) showed more or less the same PI cleavage activity as the control. The double mutant K44A/R71A with two surface cationic residues are removed had the lowest activity. V46K with an added surface cationic residue exhibited the same specific activity as the control in PI/TX micelles, but very little PC activation was observed for this enzyme. Most of the other mutant enzymes (K38A, K44A, R71A and K122A) except for V46K, showed higher specific activities comparable to the native protein when the PI was dispersed in diC₇PC micelles. Only the activities of double mutant K44A/R71A, K201A and K279A were lower than the WT *Bt*PI-PLC.

The activation ratio, specific activity in the presence compared to the absence of PC can be used to measure the extent of PC activation. All the mutants except V46K behaved comparably to WT with higher activation ratios for K38A, R71A and K44A/R71A. This suggests that the PC binding site is undisturbed if removing one or two cationic residues from the surface of the protein in micellar lipid aggregates system. The interaction between PI-PLC and the anionic lipid in the neutral micelles was impaired since the electrostatic attraction between anionic head group of lipids and cationic amino acids was

reduced, especially for R71A and K201A, which are near the $\alpha\beta$ -barrel rim of the protein and could play important roles in helping the substrate bind in the active site.

SUVs are a better mimic of the natural target membrane surface that the enzymes attack. Pure PI SUVs are not a good substrate for *Bt*PI-PLC – they exhibit less than 10% of the PI cleavage obtained in PI/TX micelles [10,31,38,43]. A good part of this is impaired binding of the protein to SUVs as shown in Chapter 3. All the mutant PI-PLCs with the exception of V46K had low activities towards PI SUVs that were even lower than WT enzyme, even the control mutant K122A (Fig. AI-2B, inset). There were two classes of lower activity mutants K44A, R71A, K44A/R71A and K122A had only 10-15% residual activity compared to WT, while K38A, K201A and K279A roughly had half of the WT activity. However, all the mutants could be activated by PC. Like WT, K44A, R71A, K122A, K201A and K279A show substantial surface dilution inhibition comparing activities at $X_{PC} = 0.8$ to $X_{PC} = 0.5$, indicating that the tighter binding in PCrich vesicles reduces opportunities for the enzyme to dissociate and find the next substrate. The only exception is the double mutant K44A/R71A, which did not show any surface inhibition at $X_{PC} = 0.8$. That may be due to weak binding at this mole fraction PC, which would make it easier for this mutant enzyme to find substrate PI in another vesicle and catalyze its cleavage. However, the most interesting mutant protein is V46K, which had very little PC activation in a micellar assay system but was always more active than WT in SUVs (2-3 fold). Thus, introducing one more cationic residue can significantly increase the activity toward pure PI substrate in vesicles as well as those with PC. This is a very different behavior compared to the micellar assay system.

AI.5.2 Cyclic Phosphodiesterase Reaction Activity

cIP hydrolysis in the absence and presence of diC₇PC provides another view of the importance of those positive side chains. Therefore, the second step of the *Bt*PI-PLC reaction, cIP hydrolysis to I-1-P, was also examined for these mutant enzymes. As a water-soluble substrate, cIP will not bind to the lipid micellar surface. All mutant enzymes except K38A exhibit similar activity (within a factor of two compared to WT). Again V46K is anomalous and is more active than WT). Moreover, all the mutants can be activated by diC₇PC to approach the specific activity of WT PI-PLC although V46K and K44A/R71A show a lower extent of activation (only about 5-6 fold compared to the 20fold for WT). That observation suggests that there may be two PC binding regions for enzyme activation – one for when the enzyme is processing long chain PI substrate (the region on helix G that is involved in cation- π interactions with PC) and the other for when cIP is hydrolyzed. For K38A, cIP hydrolysis was really impaired (with specific activity only 20% of WT) but could be almost completely recovered when diC₇PC was added. The large recovery if activity with PC present might suggest that this mutant enzyme has weak binding to anionic compounds but that when PC binds to the protein, they enzyme works as well as WT. These data indicate that of all the cation residues near the membrane surface and around the active site, the position of Lys38 is optimal for interacting with the cIP negative charge in the absence of PC.

Figure AI-2. Specific activities of *Bt*PI-PLC variants in different systems. (A) Specific activities of WT *Bt*PI-PLC and mutant *Bt*PI-PLCs for cleavage of 4 mM PI in 8 mM neutral detergent Trion X-100 (black bars) and in 16 mM diC₇PC (hatched bars) micelles. (B) Specific activities towards 2 mM PI in pure PI SUVs (black bars), PI/PC (4:1) SUVs (hatched bars), PI/PC (1:1) SUVs (white bars) and PI/PC (1:1) SUVs (grey hatched bars). Inset: Enlarged figure for pure PI SUVs. (C) Specific activities for cleavage of 8 mM cIP in the absence (black bars) and presence (hatched bars) of 8 mM diC₇PC. Error bars reflect S.D. from multiple assays.



Val46 is important for transmitting the PC activation signal in assay systems with micelles. In SUVs, it appears less important. Presumably the extra positive charge added by V46K overcomes some of the surface dilution inhibition, possibly by enhancing vesicle binding. The Val46 side chain is still critical for PC activation since activity for PI/diC₇PC is the same as PI/TX-100. The kinetic assays suggest that PC activation varies depending on which step of the reaction is examined. It was further hypothesized that *Bt*PI-PLC might have two (or more than two) PC binding sites since the diC₇PC may bind close to the active site with cIP as substrate (no acyl chains) but occupies other site(s), notably the helix G cation- π in the presence of long-chain PI.

AI.6 Line Broadening of diC₇PC Induced by PI-PLC

As suggested in the previous studies, *Bt*PI-PLC can interact with diC₇PC micelles and induce their aggregation. This can be monitored by the increased ³¹P line width of diC₇PC [30]. The phosphodiester resonance width at half-height, $\Delta v_{1/2}$, is broadened in the presence of protein when the diC₇PC concentration is around and just above the CMC (1.5 mM) (Fig. AI-3). This large increase in line width is due to the slower exchange rate among the more complex components in the system (monomer diC₇PC into the bulk micelle or onto the enzyme at a discrete site(s)). After the diC₇PC concentration exceeds 20 mM, the ratio of diC₇PC/*Bt*PI-PLC is high enough that the weight averaged contribution of protein bound diC₇PC should be small and the values of $\Delta v_{1/2}$ should be close to the pure diC₇PC micelles at the same concentration. The results of these diC₇PC titrations carried on different mutant proteins together with the enzyme kinetics suggest that there are three groups of mutant proteins. All the cationic mutants except K38A caused the same line width broadening of diC₇PC, indicating that the PC interaction is unaffected by removing the cationic residues at those specific positions. This is consistent with the same PC activation ratios between PI/TX and PI/diC₇PC, as well as cIP with or without diC₇PC (Fig. AI-2, A and C). Interestingly, the slightly higher activation rate of K38A in both PI micelles and cIP assays was not related to the tighter binding of that protein to PC species. This suggests that the micellar aggregate structure is not responsible for diC₇PC activation of PI cleavage or cIP hydrolysis. As for the opposite type of mutant, V46K, removing one of the hydrophobic residues of helix B really hinders the interaction of protein with the PC lipids (Fig. AI-3, open circles). This correlates with lower activation rates in both PI cleavage and cIP hydrolysis. Not surprisingly, the impaired interaction of protein with the PC activator leads to a loss of the PC activation. Moreover, this also indicates the importance of hydrophobic residues of helix B in the interaction with PC lipids. However, this mutant enzyme exhibits PC activation in the SUV assay system. All these results need information of SUV binding of the mutant enzymes to generate a complete model for what is involved in initial binding of the protein to a surface and what is needed for PC activation.

Figure AI-3. The interactions between *Bt*PI-PLC variants (85 μ M) and diC₇PC by line width broadening experiments. ³¹P line width (Hz) of the diC₇PC phosphorus resonance by increasing lipid concentration in absence of any protein (solid line) and the presence of 3 mg/mL WT (solid square), K38A (open square), K44A (dashed line), R71A (short dashed line), K201A (solid circle), K279A (short dotted line), K122A (dotted line) and V46K (open circle). Error bars reflect S.D. from multiple readings of the line width. Inset: Enlargement of the peak region. The CMC of pure diC₇PC is 1.5 mM.



AI.7.1 K38A

In the crystal structure of *B.cereus* PI-PLC (PDB ID: 1PTD), Lys38 is in a loop right before helix B placing it near the rim of the $\alpha\beta$ -barrel and above the active site. In the kinetic studies, K38A exhibited lower activity toward pure PI substrate (PI/TX micelle or PI SUVs) and also towards cIP (it is the lowest among all the mutants enzymes), which indicates that the PI binding site is impaired by substituting that lysine residue with an alanine. The kinetic result is consistent with weak binding affinity of this mutant protein (Fig. AI-4A, dashed line, open square) towards pure PG SUVs - the apparent K_d is around 3-fold higher than that of WT PI-PLC. As for PC activation, all the kinetic studies show that with PC species involved, both the PI cleavage and cIP hydrolysis are activated to nearly the same specific activity as WT. Moreover, in PI/PC SUVs, when the PC mole fraction is above 0.5, K38A has higher activity than WT (Fig. AI-4A). However, based on the binding profile and line width broadening assay, the PC binding site is somewhat impaired due to the removing of the cationic residue at this position. The K_d values for SUVs with PC included are all about 10-fold higher than those for WT protein, which suggests a weakened affinity of the mutant protein for PC. However, when the total phospholipid concentration used in the kinetic assay is above the K_d values at that X_{PC} , the enzymatic activity is the same.

AI.7.2 K44A

As observed in the surface dilution assay (Fig. AI-4B), the specific activity of K44A for pure PI SUVs and PI/PC (4:1) SUVs was very low, only about 10-20% of the WT activity. This corresponds to the results reported before [146] and is in agreement with the very high apparent K_d of this mutant protein for pure PG SUVs. The dependence of the relative activity on X_{PC} is similar to that seen for WT (Fig. AI-4B). The typical surface dilution inhibition of enzyme activity was also observed due to the tight binding with vesicles at $X_{PC}=0.8$. As the only charged residue in both helix B and the 240s' loop, Lys44 is nearby but directed away from Trp47 according crystal structure of Y247S/Y251S (which is kinetically similar to wild-type PI-PLC). This position should be important for electrostatic interactions with anionic membranes according to both the reduced activity and weak binding in pure PI (or PG as an analogue) system. However, this lysine does not have a large influence on the PC activation since K44A does not change the enzyme activity toward PI/diC₇PC and PI/PC SUVs (the PC activation ratio is >20%), and the binding to pure PC SUVs was just slightly weaker than WT. These results confirmed the importance of this cationic residue for protein binding to surfaces rich in negatively charged phospholipids. Clearly, PC facilitates the binding of K44A protein with lipid aggregates, resulting in activities comparable to wild-type PI-PLC in PC-rich assay systems.

Figure AI-4. Membrane binding and activity of *Bt*PI-PLC variants towards SUVs. (A) Binding of WT *Bt*PI-PLC and mutant proteins (10 nM) to DOPG/POPC SUVs as a function of X_{PC} . The bold dashed line indicated the total phospholipid concentration used in kinetic assays. (B) Variation of the specific activity of *Bt*PI-PLC variants towards PI/POPC SUVs with 2 mM PI and varying X_{PC} . WT (solid square), K38A (open square), K44A (solid circle), R71A (open circle), K44A/R71A (solid triangle), K279A (open triangle), and V46K (cross). Error bars reflect S.D. from multiple assays.



AI.7.3 R71A and K44A/R71A

Arg71 is at top of β -strand but away from the membrane binding surface, and this arginine as well as Lys44 are conserved in the bacterial PI-PLCs of various species (e.g., Staphylococcus aureus, Enterococcus faecalis, Erwinia amylovora). Except for Lys44, Arg71 is the only other cationic residue near the barrel rim that is likely to contribute a large positive potential for attracting the protein to the negatively charged interface. Based on the results of enzymatic activity in various assays and the binding curve of R71A compared with WT *Bt*PI-PLC, this arginine was shown to contribute to the total electrostatic interaction of the protein with an anionic membrane. The lower activity in pure PI dispersed in Triton X-100 or pure PI SUVs for R71A was due to the weaker binding effect of the mutant to anionic lipid surface in binding and kinetic assay conditions (the physiological salt concentration in the buffer will weaken purely electrostatic binding). However, because of unimpaired PC binding, enzyme activities were comparable to WT BtPI-PLC in PC-rich assay systems (PI/diC7PC and PI/PC SUVs). A similar surface dilution inhibition and the synergistic effects between anionic lipids and PC activator are still observed, indicating that PC still facilitates the interaction of the R71A mutant protein with lipid aggregates. This can also include promotion of an optimized protein conformation for both binding of substrate and subsequent catalysis.

The double mutant K44A/R71A had a significantly lower activity and is the only mutant that does not exhibit surface dilution inhibition effect. The binding profile of K44A/R71A is quite similar to K44A but with the slightly higher K_ds from X_{PC} =0.2 to 1.0. The K_d at X_{PC} =0 (pure PG SUVs) is too high to be estimated indicated the really

impaired anionic lipid binding after removing the two important surface cationic residues. Meanwhile, the PC binding affinity is much weaker in K44A/R71A compared to WT and other mutant *Bt*PI-PLCs, which may cause the absence of surface dilution inhibition in this double mutant protein.

AI.7.4 K279A

This lysine is pointed away from the active site and also not near the region previously proposed to be important for membrane interaction (helix B and 240's loop). Except in the pure PI substrate system, all the other activities of K279A are nearly the same as WT. This is confirmed by the SUV binding profile showing that only the binding to pure anionic phospholipid vesicle is hindered. The PC activation of this mutant is comparable to WT and the binding synergy between substrate analogue (PG) and activator (PC) is unimpaired. This result also shows that not all surface cationic residues have large effects on binding or PC activation. However, missing one of these residues will highly weaken the PI binding affinity even if these residues are far away from the active site. On the other hand, this mutant can work as a control to further verify that the different PC activation observed for K44A and K38A is not just due to the reduced positive charge of the surface.

AI.7.5 V46K

Val46 is part of the hydrophobic face in helix B and was proposed to stabilize the conformation with Trp47 side chain poised to interact with the membrane. As seen in kinetic studies, V46K is more active than WT enzyme towards pure PI or PI/PC SUVs

but exhibits reduced PC activation in PI/diC₇PC micelles and towards cIP. These kinetics results along with the line width broadening experiments suggest that Val46 is important for mediating the hydrophobic interaction with PC-rich interface. This mutation that changes a hydrophobic side chain to a cationic one should reduce PI-PLC affinity for PC vesicles. The FCS experiments show that the affinity of V46K for PC SUVs is decreased significantly (the apparent $K_d = 21.7 \pm 0.5$ compared to the $K_d = 0.0298 \pm 0.0034$ mM for WT binding to pure PC SUVs). However, introducing one more cationic residue did not strengthen the binding of protein to the anionic substrate in the buffer used (PBS containing 139 mM salt). The apparent K_{ds} at X_{PC} from 0.2 to 0.8 are nearly the same (around 1 mM) and are all lower than the K_{ds} for pure PG or pure PC, which indicates that PC and PI still exhibit some synergism for V46K, but for this mutant enzyme the tightest binding occurs between $X_{PC}=0.7$ and $X_{PC}=0.9$. These results confirm the importance of this hydrophobic residue for protein binding to surfaces rich in PC lipids. Once bound to a vesicle, the relative weak affinity of V46K for PC still facilitates the interaction of the protein with lipid aggregates, resulting in activities nearly double of wild-type PI-PLC in SUVs. In this system the added positive charge may enhance substrate binding into the active site. In PI/diC₇PC and cIP/diC₇PC assay system, the impaired PC binding interaction has a negative effect on the activity that affects PC activation. It appears that substitution of this hydrophobic residue with a charged one hinders the interaction between the helix B region and the substrate-containing vesicle (or micelle) as well as the subsequent promotion of the protein conformation optimization for both binding and catalysis.

Since Val46 was not expected to be a key residue (like Trp47) for membrane insertion, these results indicate that introducing a second cationic residue into helix B is likely to destabilize either the conformation or stability of this small helix. The dependence of the relative activity on X_{PC} was, however, similar to that seen for WT PI-PLC (Fig. AI-5A-C). With fixed PI, increasing PC caused a substantial increase in activity followed by surface dilution inhibition at high X_{PC} (e.g., fixing PI = 2 mM and increasing the total concentration of PC). However, by increasing the fixed PI to 4 mM, the surface dilution inhibition is dramatically reduced (compare the V46K activity at X_{PC} =0.5 and 0.8). This strongly suggests that in WT enzyme this surface dilution inhibition is strong binding of the protein to PC and perhaps slower dissociation to find another vesicle with substrate. V46K shows higher activity towards all PC-containing SUVs because it has a weaker interaction with activator-rich domain. If the substrate concentration is not observed.

Recent field cycling ³¹P NMR studies of wild-type PI-PLC interacting with phosphatidylmethanol (PMe)/PC vesicles (Pu) suggest that enzyme-induced demixing of PMe (substrate analogue) and PC (activator) leading to sequestration of the enzyme in PC-rich regions resulting in reduced accessibility to substrate. Thus, another comparison of V46K and WT was tested by fixing total phospholipid concentration at 1 mM (the similar values as the K_{ds} at X_{PC} =0.2, 0.5, 0.8 of V46K) but altering X_{PC} (Fig. AI-5). V46K activity is still twice that of WT, which likely reflects that weak binding can help the protein to easily dissociate from PC-rich region to access the next substrate.

AI.8 Effect of Salt on the Enzymatic Activity

Since increasing salt partially shields electrostatic interactions, around 100 mM salt (137 mM NaCl, 2 mM KCl, mimic the PBS buffer condition) was added into the assay buffer to further explore the roles of these cationic residues. K38A was chosen as the example of the Lys mutant group and K279A was used as the control. In this specific experiment, the SUVs system with different composition of lipids (PI was fixed at 2 mM and PC increased from 0, 0.5, 2 to 8 mM) was used. The activity data (Fig. AI-6) with or without salt were plotted together to emphasize the salt effect. All the proteins show reduced activity (3-4-fold) towards PI SUVs with salt present (Fig. AI-6A, inset). For WT and K279A, the PI cleavage rate was about 40% of that without salt, but for K38A and V46K, it was less than 20%. Therefore, in the pure anionic phospholipid system, the dominant factor is the electrostatic interaction that binds the protein to the SUV. This will be weakened by moderate salt concentrations. A similar rate reduction for K279A and WT BtPI-PLC suggested that Lys279 was not the key residue contributing to electrostatic interactions. Compared with WT BtPI-PLC, the enzyme activity profile for K279A as a function of X_{PC} with salt present is not altered by mutating Lys279 to alanine.

<u>Figure AI-5.</u> Specific activities of WT *Bt*PI-PLC (black bars) and V46K *Bt*PI-PLC (hatched bars) for cleavage of PI in the different SUV system: (A) fixed [PL]=1 mM; (B) fixed [PI]=2 mM; (C) fixed [PI]=4 mM. [PL] stands for total phospholipid concentration. The table shows the K_ds at different X_{PC} . Error bars reflect S.D. from multiple assays.



For K38A and V46K, there was a higher reduction in activity toward pure PI with salt present. Therefore, along with the different electrostatic interactions (K38A is weaker and V46K is stronger), the binding of protein to PI substrate is affected by other factors, for example the correlated conformation changes as noted in MD simulations of the isolated protein (Chapter 3). With PC incorporated into the vesicles, adding salt usually activates the enzyme since the dominant interaction between protein and PC-rich surface is the hydrophobic partitioning of Trp and Tyr. This effect can be observed for WT (Fig. AI-6A, solid bar and Fig. AI-4B, solid square) with the slightly higher activity in the presence of salt at $X_{PC}=0.2$ and 0.5. For $X_{PC}=0.8$, the surface dilution inhibition is the major effect, so the activity with or without salt was similar. As for K38A, in PI/PC mixed vesicles ($X_{PC}=0.2$, 0.5 and 0.8), addition of salt enhanced the activity so that specific activities were always higher than WT (Fig. I-6A, solid bar and shaded bar as well as in Fig. AI-4B, open square). Removal of that cationic residue weakens vesicle binding – but that may promote easy dissociation from one vesicle onto another. V46K exhibited a similar activation profile but with even less surface dilution inhibition with slat present.

Figure AI-6. Effect of salt on PI cleavage activity of selected *Bt*PI-PLC variants in SUVs. (A) Salt effect on specific activities of WT (black bars), K38A (hatched bars), K279A (white bars) and V46K (grey hatched bars). The SUVs conditions are the same as Fig. I-2B. (B) The comparison of rates with and without salt among selected *Bt*PI-PLC variants: WT (solid square, solid line), K38A (open square, dashed line), K279A (solid circle, dotted line) and V46K (solid triangle, dash dotted line). Error bars reflect S.D. from multiple assays.



AI.9 Conclusions

MD simulations of *Bt*PI-PLC docking to an implicit membrane identified several basic residues – K38, K44, K201 and K279 – that could contribute to electrostatic attraction of enzyme to a target bilayer. In this work, these cationic residues were substituted by alanine to assess the effect on enzyme activities and vesicle binding. Another mutant protein, V46K, was constructed to see if an additional positive charge in helix B (a region that does contact the bilayer) would enhance enzymatic activity or vesicle binding. For most of these mutations, the activity towards pure PI vesicles was substantially decreased with removal of one of the cationic residues, even if some of them were fairly distant from the active site. In terms of the binding profiles of mutant proteins compared with WT, Lys38 and Lys44 were found to be particularly important for PC binding. Introducing a cationic residue instead of the hydrophobic residue in short helix B (V46K), really impaired the PC binding. However, at high lipid concentration, PC could still activate the enzyme. Therefore, almost all of surface charges aid in binding to negatively charged vesicles, but for PC activation the effect of removing a cationic residue is more complex since weaker binding could facilitate exchange of the protein among vesicles.

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