

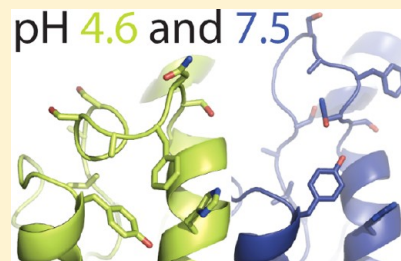
Structure of the *S. aureus* PI-Specific Phospholipase C Reveals Modulation of Active Site Access by a Titratable π -Cation Latched Loop

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ABSTRACT: *Staphylococcus aureus* secretes a phosphatidylinositol-specific phospholipase C (PI-PLC) as a virulence factor that is unusual in exhibiting higher activity at acidic pH values than other enzymes in this class. We have determined the crystal structure of this enzyme at pH 4.6 and pH 7.5. Under slightly basic conditions, the *S. aureus* PI-PLC structure closely follows the conformation of other bacterial PI-PLCs. However, when crystallized 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 loop displacement at acidic pH is the result of a titratable intramolecular π -cation interaction between His258 and Phe249. This was verified by a structure of the mutant protein H258Y crystallized at pH 4.6, which does not exhibit the large loop shift. The intramolecular π -cation interaction for *S. aureus* PI-PLC provides an explanation for the activity of the enzyme at acid pH and also suggests how phosphatidylcholine, as a competitor for Phe249, may kinetically activate this enzyme.



Phosphatidylinositol-specific phospholipase C (PI-PLC) is a virulence factor produced and secreted by many Gram-positive bacteria.¹ When secreted by extracellular pathogens (e.g., *Bacillus* and *Staphylococcus* strains^{2–5}), this enzyme catalyzes the cleavage of glycan-phosphatidylinositol (GPI) anchored proteins. PI-PLC removal of the protective GPI-anchored proteins on the exterior surface of mammalian cells allows cellular damage to occur, and the diacylglycerol, translocating across the bilayer,⁶ can interfere with cellular signaling processes.⁷ Phosphatidylinositol molecules are also good substrates for this enzyme and provide an easier system to explore mechanistic details of these enzymes.¹ PI-PLC cleaves its substrate via an intramolecular phosphotransferase reaction on the PI moiety to form a cyclic inositol phosphate molecule and diacylglycerol.⁸ Subsequent hydrolysis of the cyclic phosphodiester produces inositol 1-phosphate if PI is the substrate.^{9,10} The PI-PLC from *S. aureus* is unusual in exhibiting significant activity toward PI at acidic pH⁴—a property that may contribute to the virulence of the organism, which is often in an acidic milieu.¹¹

Crystal structures of the PI-PLC enzymes from *Bacillus* species^{12,13} and *Listeria monocytogenes*,¹⁴ an intracellular pathogen, show very similar distorted TIM barrels with an active site close to the barrel rim. Each has a short helix at the rim that contains at least one lysine, important for binding to negatively charged interfaces, and a tryptophan that, in the case of the *Bacillus* PI-PLC, has been proposed to insert into target membranes.^{15–17} Another key structural feature is a rim loop near the active site that contains one large hydrophobic residue (either a tryptophan or phenylalanine) amid many small flexible residues. This surface-exposed hydrophobic residue is also thought to aid in anchoring the protein to membranes.^{15,16}

Several of the bacterial PI-PLC enzymes exhibit kinetic activation by nonsubstrate phospholipids. Specifically, inclusion of PC in vesicles containing PI lead to large increases in the specific activity of these secreted PI-PLC enzymes.^{9,18,19} The detailed mechanism for PC activation is not the same for the different enzymes. *B. thuringiensis* PI-PLC has a discrete site for PC binding,²⁰ while the *L. monocytogenes* PI-PLC requires PC to dilute cationic enzyme/anionic lipid aggregates that trap the enzyme in a nonproductive state.¹⁹ The PI-PLC from *S. aureus* can also exhibit a significant increase in specific activity toward PI in vesicles containing PC (vide infra).

We have solved the structure of *S. aureus* PI-PLC strain FPR3757 at two different pH values: (i) pH 4.6, where the enzyme shows low activity (but significantly higher than other bacterial PI-PLC enzymes), and (ii) pH 7.5, where it is very active toward PI/PC (but not pure PI) vesicles. A large conformational change in the rim mobile loop between the acidic and basic pH structures depends on a titratable π -histidine cation interaction. π -Cation interactions are well-known in proteins; however, these interactions commonly exist in protein–protein interfaces, enzyme–drug interactions, helix stabilization, and protein–DNA interactions.^{21–23} π -Cation complexes with protonated histidine as the cation are also known.²⁴ Most of these interactions result in subtle structural changes; typically, they mediate ligand binding or helix stabilization.^{25,26} However, a recent structure of the transferrin receptor showed a large change in structure in the dimer interface region upon transferrin binding whereby a protonated

Received: January 13, 2012

Revised: March 5, 2012

Published: March 6, 2012



surface histidine flipped into the protein to engage in π -stacking with a phenylalanine and tyrosine.²⁷

Observation of the titratable intramolecular π -cation interaction in *S. aureus* PI-PLC suggests higher activity at acidic pH is connected with easier release of water-soluble product inositol 1,2-(cyclic)phosphate (cIP). This structural feature also provides an explanation for how PC may activate this bacterial PI-PLC. The presence of that zwitterionic phospholipid in a substrate-containing interface provides cationic choline moieties that can compete with cationic His258 for interactions with the π -system of Phe249, allowing the latter to partition into membranes. The higher activity once PC is added indicates that this membrane conformation must differ from the one where only PI is present.

■ EXPERIMENTAL PROCEDURES

Expression and Purification. The genomic DNA of *S. aureus* (strain FPR3757) was purchased from ATCC, and the gene coding for PI-PLC was amplified by PCR, cleaved by the restriction enzymes *EcoRI* and *XhoI* (New England BioLabs, Inc.), and inserted in to the vector (pET21a) with a C-terminal hexahistidine tag. The sequence of this gene, confirmed by Genewiz, was equivalent to that reported previously.⁴ For producing the H258Y mutant protein, QuickChange methodology with the site-directed mutagenesis kit from Stratagene and the complementary primer purchased from Operon were used. The sequence of the mutated gene was confirmed by Genewiz. The plasmids containing the two PI-PLC genes were transformed into *E. coli* BL21-Codonplus (DE3)-RIL cells. Overexpression of the proteins followed protocols used for the *B. thuringiensis* PI-PLC.¹⁵ The *S. aureus* PI-PLC proteins were purified in two steps: (i) lysed cell pellets were mixed with an Ni-NTA resin (Qiagen) and an imidazole gradient used to elute the PI-PLC; (ii) after overnight dialysis in 20 mM Tris, pH 8.3, the protein solution was further purified by elution from a Q-Sepharose fast-flow anion-exchange resin. The concentration of the PI-PLC enzymes, greater than 95% pure as monitored by SDS-PAGE, was measured by the absorption at 280 nm using an extinction coefficient calculated by the Web-based ProtParam software. The yield of purified protein was 60 mg/L cell culture.

Crystallography. Purified PI-PLC was concentrated to 20 mg/mL before being combined with *myo*-inositol. The protein solution was then diluted to 10 mg/mL using deionized water and incubated on ice for a minimum of 2 h before setting up crystallization trays. For determination of initial conditions, Qiagen JCSG core 1 and 2 screens were used. All PI-PLC samples were crystallized at 20 °C by vapor diffusion, using hanging drops of 3 μ L, at a final protein concentration of 10 mg/mL. Suitable crystals were mounted in nylon loops and frozen in liquid nitrogen.

For the basic pH structure, PI-PLC was crystallized in 10% isopropanol, 100 mM HEPES, pH 7.5, with 20% PEG 4000. Single crystals developed after ~1 week and grew to a final size of ~0.2 mm in length. For the acidic pH structure, the protein crystallized in 150 mM ammonium acetate, 100 mM sodium acetate, pH 4.6, with 10 mM magnesium nitrate, and 20% PEG 4000. Prior to crystallization, the protein was incubated with 100 mM *myo*-inositol and 5 mM diC₆PC (Avanti Polar Lipids). Suitable crystals presented as large plates (0.5–0.7 mm in length), which appeared in the drop after ~3 months. The H258Y structure was crystallized in 150 mM ammonium acetate, 100 mM sodium acetate pH 4.6, 100 mM magnesium

nitrate, and 26% PEG 4000. Prior to crystallization, the protein was incubated with 100 mM *myo*-inositol. Single crystals appeared after 2 days and grew to a size of 0.4 mm in length.

Data were collected at 100 K using an in-house Rigaku MicroMax-07 HF high-intensity microfocus rotating Cu anode X-ray generator, coupled with Osmic VariMax Optics and a R-Axis IV++ image plate area detector. Data were indexed and reduced using HKL2000.²⁸ The acidic pH structure was solved by molecular replacement in CCP4,²⁹ using PHASER³⁰ in CCP4, with the *B. cereus* structure IPTG¹² as a model. The basic and mutant structures were solved similarly using the acidic structure as a model. All models were initially refined using CNS,³¹ and then reanalyzed and refined with Refmac,²⁹ with manual model building in COOT.³² Maps generated in PHENIX³³ and manual model building in COOT.³² Ligands and ligand restraints were generated using ProDRG.³⁴ Structural comparisons were made using SSM superposition³⁵ in COOT and alignment in Pymol.³⁶ Adit³⁷ was used for structure validation.

PI-PLC Kinetics. The specific activity of the recombinant *S. aureus* PI-PLC enzymes was measured toward small unilamellar vesicles (SUVs) of bovine brain PI (4 mM) and PI/PC (4 mM:1 mM) using ³¹P NMR spectroscopy (Varian INOVA 600) to analyze product generation at fixed time points as described previously for the *B. thuringiensis* PI-PLC.¹⁶ The particular PC species used was 1-palmitoyl-2-oleoyl-PC (Avanti Polar Lipids). SUVs were prepared by sonicating the dispersed phospholipid solution on ice for 20 s and then off for 20 s, for a total time of 20 min. Vesicle sizes of PI containing SUVs prepared this way were quite similar for up to 50% PC³⁸ as estimated by light scattering. For pure PI SUVs in MES, H 6.5, the average radius was 133 Å with a 20.4% polydispersity; for PI/PC (1:1) SUVs, the average radius was 127 Å with 42.6% polydispersity. The enzyme (4 μ g/mL for pure PI SUVs and 1 μ g/mL for the PI/PC mixed SUVs) was incubated with the vesicles for fixed times (selected to ensure <20% PI cleavage) at 28 °C; the reaction was quenched with acetic acid (dropping the pH below 4) followed by the addition of the Triton X-100 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. The buffer was 50 mM MES/HEPES with the pH adjusted to pH 5.5, 6.5, or 7.5; all buffers included 1 mM EDTA and 0.1 mg/mL BSA (used to stabilize the protein at the very low concentrations used in these assays).

A qualitative turbidity assay was used to compare the ability of short-chain phospholipids with different head groups to alter PI (4 mM) cleavage when it was dispersed in Triton X-100 (8 mM) micelles in MES buffer at pH 6.5. The lipids added included 16 mM of diC₇PC, diC₈PE, diC₈PG, or diC₈PS.

■ RESULTS AND DISCUSSION

Structure of *S. aureus* PI-PLC. All three forms of *S. aureus* PI-PLC (wild type, acidic and basic conditions, and H258Y) crystallized as monomers in the P2₁2₁2₁ space group. Data for the acidic structure were collected at a resolution of 2.3 Å and refined to a final R value of 15.6% (R_{free} = 21.3%); the unit cell, with dimensions *a* = 104.16, *b* = 43.61, and *c* = 62.22 Å, contained *myo*-inositol in the active site and one chloride ion. Electron density contoured to 1 σ for the rim loop with the model superimposed is shown in Figure 1. Although the acidic form of PI-PLC was cocrystallized with 5 mM dihexanoyl-PC, no discrete

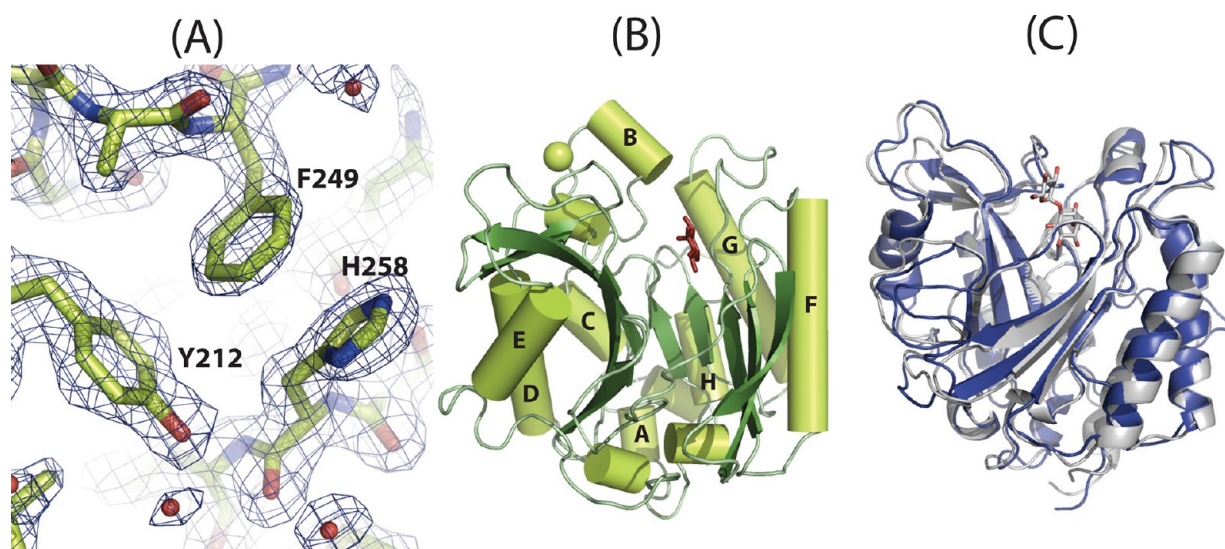


Figure 1. (A) Representative electron density of the rim loop of the acidic model, view of the π -cation interaction residues. Electron density of part of the rim loop in the acid pH structure, shown in dark blue and contoured at 1σ , is shown with the model superimposed. (B) Overall structure of the acidic form of *S. aureus* PI-PLC with the helix notation used in the structure of *L. monocytogenes* PI-PLC (PDB 2PLC).¹⁴ *myo*-Inositol in red is shown bound to the active site; the chloride ion is shown as a green sphere occupying the cationic rim pocket. (C) Comparison of basic form of *S. aureus* PI-PLC (blue) and *B. cereus* PI-PLC (silver), PDB 1GYM. The glucosamine- α 1,6-*myo*-inositol ligand seen in 1GYM is shown in stick representation; the inositol ring marks the active sites of the enzymes.

density for phospholipid was observed in the crystal structure. However, there was a significant improvement in crystal stability when that short-chain phospholipid was present in the crystallization medium. Data for the basic pH structure were collected at a resolution of 2.3 Å and refined to a final R value of 18.2% ($R_{\text{free}} = 25.6\%$); this unit cell, with dimensions $a = 85.47$, $b = 57.95$ and $c = 61.74$ Å, contained one sulfate ion. The H258Y mutant structure, with data collected at a resolution of 1.9 Å and refined to a final R value of 18.7% ($R_{\text{free}} = 23.7\%$), was found to contain *myo*-inositol and two acetate anions. Interestingly, H258Y, though crystallized under acidic conditions, had a unit cell almost identical to that of the basic structure of wild-type recombinant PI-PLC. Table 1 summarizes crystallographic data and refinement statistics for the three different crystals.

Similar to the other bacterial PI-PLC crystal structures^{12–14} in the database, the enzyme from *S. aureus* folds into an imperfect ($\beta\alpha$)₈-barrel. Notation of the helices, following the other bacterial PI-PLC structures, is shown in Figure 1B. The mature *S. aureus* PI-PLC begins with Ser1, as noted previously.⁴ The overall structure of the *S. aureus* PI-PLC obtained at pH 7.5 is most similar to *B. thuringiensis* (3EA2) and *B. cereus* PI-PLC (IPTG) structures.^{12,13} Figure 1C compares the basic structure of the *S. aureus* protein to the *B. cereus* PI-PLC. Density for Ser1 was observed in the acidic structure, while density began at Asp2 in both the basic and H258Y structures. In two of these *S. aureus* PI-PLC structures, a portion of the uncleaved C-terminal His₆ tag was visible extending from the C-terminus of the protein. In the acidic structure, density extended to Leu303, in the basic pH structure, density extended to His305, and in H258Y, it extended to His308. Active site residues (and electrostatic maps of the active site) in all three *S. aureus* PI-PLC structures are well conserved, with all catalytic residues remaining relatively unchanged in both position and rotamer. Additionally, *myo*-inositol binding remains the same in both the acidic and H258Y mutant *S. aureus* PI-PLC structures.

Two key structural features of the *Bacillus* enzymes are short helix B and a mobile rim loop, both of which have been

Table 1. Full Refinement and Model Statistics

	acidic (PDB 3V16)	basic (PDB 3V18)	H258Y (PDB 3V1H)
<i>diffraction data</i>			
resolution range (Å)	39.9–2.3	48.0–2.34	34.9–1.9
no. of reflections	17 112	12 534	23 280
no. of reflections in free set	923	648	1246
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
<i>unit cell</i>			
a (Å)	104.16	85.35	87.95
b (Å)	43.61	58	57.22
c (Å)	62.22	61.72	61.14
completeness	97.1	98.4	97.7
R_{merge}	0.045	0.119	0.093
<i>refinement</i>			
R_{cryst}^a	0.1555	0.1820	0.1867
R_{free}^b	0.2131	0.2566	0.2374
no. of residues	303	304	306
no. of non-hydrogen protein atoms	2445	2431	2476
no. of non-hydrogen inositol atoms	12	0	12
no. of H ₂ O molecules	225	156	265
no. of ions	1	1	2
rmsd bonds (Å)	0.022	0.018	0.023
rmsd angles (deg)	1.7	1.7	1.8
<i>Ramachandran plot (%)</i>			
most favored	89.0	89.4	90.9
additionally allowed	11.0	13.6	9.1
generously allowed	0	0.4	0
disallowed	0	0	0
av B-factor (Å ²)	28.5	41.0	27.1

^a $R_{\text{cryst}} = \{ \sum (|F_o| - |F_c|) / |F_o| \}$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. ^bBrunger (1992).

implicated in membrane binding.^{15–17,20} Helix B in *S. aureus* PI-PLC (41V-K-S-V-W-A-K) is one residue longer than in the

B. cereus structures. It also has two lysine residues making a more positively charged feature. This, in conjunction with the other rim positively charged residues (Lys209, Lys113, Lys114, Lys121, and Arg69), forms an extended region of positive charge along the barrel rim and would suggest that membrane binding (at least to substrate membranes with moderate negative charge) is largely driven by electrostatics (Figure 3). Additionally, a bound anion is seen in each structure in a conserved position in a cationic pocket on the rim, composed of the side chains of His86 and Lys42 and the backbone amides of Lys38 and Asp39. The identity of the bound ligand changes with each structure: the pH 7.5 structure contains a sulfate, the pH 4.6 structure contains a chloride, and the H258Y structure contains an acetate anion. However, in all *S. aureus* PI-PLC crystal structures there is an anion present in this highly electropositive pocket. The position of this bound anion near at the rim surface is where one might expect the headgroup of an anionic phospholipid to bind.

The mobile rim loop contributes a key hydrophobic residue, Trp242 in *Bacillus* PI-PLC and Phe237 in the *L. monocytogenes* structure) to membrane binding.^{12–14} The analogous residue in the basic *S. aureus* PI-PLC structure, Phe249, is pointed toward the membrane interface, slightly above the lip of the ($\beta\alpha$)₈-barrel (Figure 2 suggests the membrane-binding orientation by

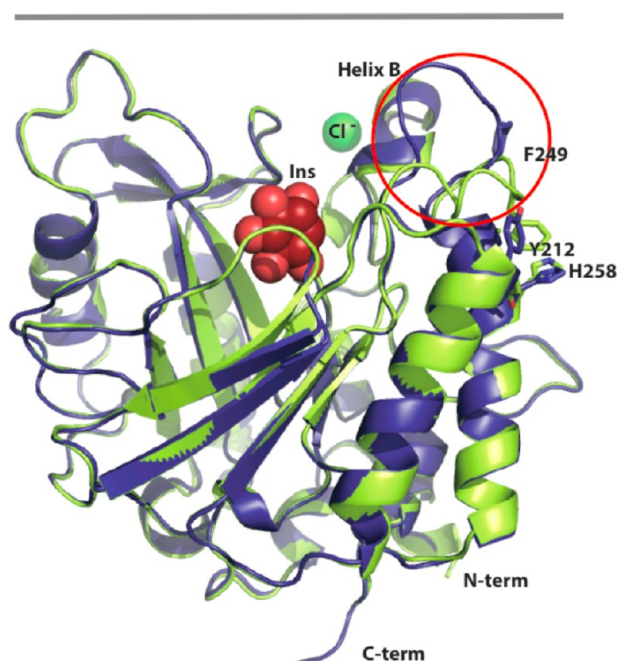


Figure 2. Overlay of the acidic pH (green) and basic pH (blue) structures of *S. aureus* PI-PLC. The red circle highlights the mobile rim loop and the large conformational change in it with pH. Key residues forming the π -cation interaction are labeled. *myo*-Inositol is depicted in red space-filling representation and the chloride ion as a green sphere. The C- and N-terminals and helix B are labeled. A gray bar represents the membrane interface.

a gray line). However, in the *B. cereus* structure, that side chain is pointed down, along the axis of the ($\beta\alpha$)₈-barrel between helices G and H, and away from the membrane interface.¹² Similarly, in *L. monocytogenes* PI-PLC the equivalent residue (Phe237) is again pointed away from the membrane interface, though in that structure it flanks helix G on the opposite side.

This stresses the flexibility of the rim loop and the Trp/Phe side chain position in the different bacterial PI-PLC proteins.

It is this loop of the *S. aureus* protein that undergoes the largest change in structure with pH (Figures 2 and 4). In the acidic form of PI-PLC, the π -system of Phe249 forms a π -cation complex with protonated His258. This draws Phe249 down between helices F and G and leads to a condensed conformation of the rim loop—the π -cation complex acts as a latch to restrain the loop close to the protein surface. This position for the Phe side chain is similar to that of Phe237 in *L. monocytogenes* PI-PLC. However, *L. monocytogenes* PI-PLC has an alanine in the position corresponding to His258; thus, no π -cation complex can be formed. Although Phe237 is pointed down between two helices, it is shifted upward 3.1 Å from its *S. aureus* PI-PLC equivalent. Without the π -cation complex, the loop is held in an extended conformation in the *L. monocytogenes* PI-PLC. Additionally in *S. aureus* PI-PLC, the loop is most stable in the acidic form, with excellent coverage of electron density (see Figure 1) and relatively low *B*-factors. In the basic form, the rim loop is significantly more mobile, with higher *B*-factors and less complete electron density. This trend is mirrored in the H258Y mutant, where the loop is again in its extended and significantly more mobile position. Crystal packing has no effect on the mobile loop. In the basic form of the structure, the closest crystallographic neighbors are about 6–8 Å away. The H258Y form of the structure shows one crystallographic neighbor 4.5 Å from Asn250, though this neighbor does make contact within the structure. Rather, it hydrogen bonds with Asn254 and does not affect the loop. Finally, in the acidic structure, one hydrogen bond is made between the backbone carbonyl of Ala248 and the side-chain amide of neighboring Asn10. However, crystal packing would not have sterically hindered this conformation if the loop had adopted the extended position in this structure. Therefore, the difference in loop conformations is not an artifact of crystal packing but a conformational change in response to the protonation state of the protein.

pH-Dependent π -Cation. Aside from the mobile loop and a few aromatic residues with different rotamers, (Tyr211, Tyr212, and Tyr255 move to accommodate Phe249, and Trp45 in helix B moves to accommodate the mobile loop in the acidic pH structure) the enzyme side chains are largely unchanged between the acidic and basic pH structures. The rim loop undergoing the pH-dependent movement consists of 11 residues (²⁴¹S-V-A-S-G-G-S-A-F-N-S). It shows a maximum backbone displacement of 9.4 Å between the acidic and basic forms of *S. aureus* PI-PLC, as measured at the α -carbon of Ser247. At pH 4.6, the cationic nitrogen on His258 is positioned 3.5 Å from the π -system of Phe249, which itself is 3.5 Å from that of Tyr212 in a face-edge interaction. The π -cation interaction holds Phe249, and thus the rest of the mobile loop in a position where Phe249, the lone hydrophobic residue in the mobile rim loop, is unable to partition into membranes (Figure 4A). At pH 7.5, His258 will be deprotonated. With the cation no longer present, the rim loop reverts to the more extended loop position, ~10 Å away (Figure 4B), where His258 is 11.7 Å away from the π system of Phe249. Similarly, His258 is 4.6 Å from Tyr212.

Interestingly, the large pH-dependent structural change we see is reminiscent of what is seen in the homodimeric transferrin receptor (TFR) when its ligand, the human transferrin molecule (hTF), binds the apo-homodimer at biological pH. Binding of hTF to TRF at pH 7.4 causes two

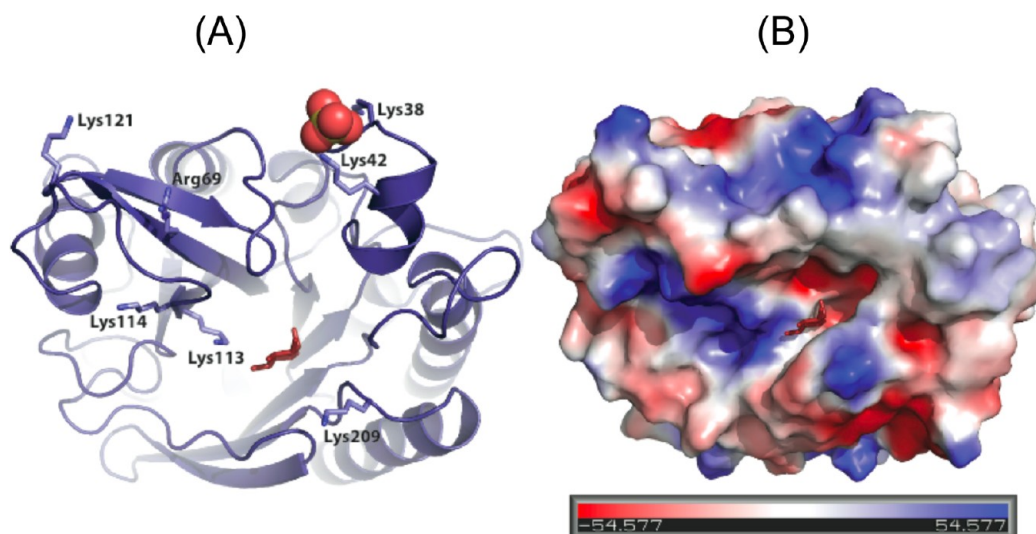


Figure 3. (A) Cationic residues around the rim of the $\beta\alpha$ barrel of *S. aureus* PI-PLC at pH 7.5, as seen from the membrane interface. These residues create an electropositive region, contributing to electrostatic binding of the protein to the membrane. *myo*-Inositol shown in red stick orientation indicates the active site, and the sulfate ion is shown as red spheres. (B) Electrostatic map of the unliganded protein as seen from the same orientation. Regions of positive charge around the barrel rim can be seen in blue.

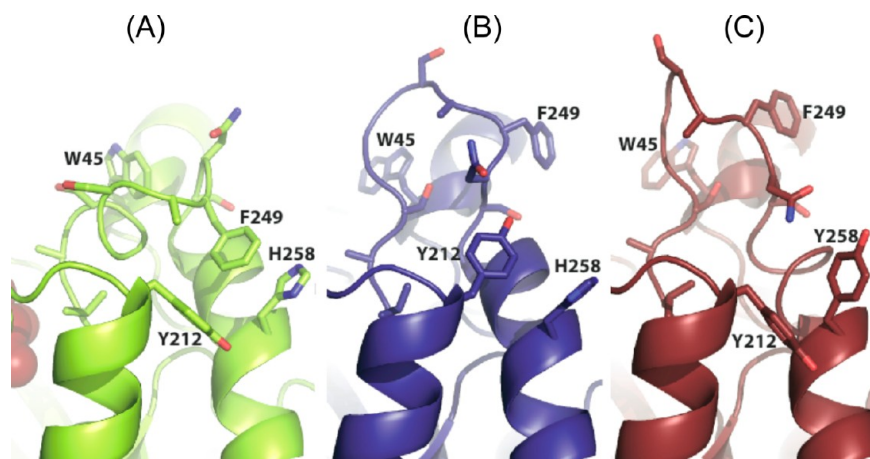


Figure 4. A close-up of the *S. aureus* PI-PLC mobile rim loop conformation near helices F and G in the wild-type enzyme at (A) pH 4.6 and (B) pH 7.5, compared to the same region in the H258Y structure at pH 4.6 (C). The proposed Phe249/His258 π -cation interaction and relevant side chains are indicated in each panel.

dimer interface tryptophan residues per monomer to switch from π - π stacking interactions to formation of π -cation interactions. The switch in these interactions causes rotation of the dimer interface, which primes the homodimer for chain reaction at the interface when endosomal pH is encountered, resulting in the rapid release of the iron held by the hFT/TFR complex.²⁷ Though not specifically an intraprotein movement, this dimer rotation further emphasizes the importance of these increasingly encountered π -cation interactions.

H258Y: Abolishing the Intramolecular π -Cation Interaction. To confirm that the mobile loop conformational switch is dependent on a π -cation interaction between Phe249 and His258, the mutant protein H258Y was prepared and crystallized. The H258Y mutation eliminates any possibility of a titratable π -cation interaction in *S. aureus* PI-PLC. The crystal structure obtained at pH 4.6 (Figure 4C) shows the mobile loop in an extended position similar to that of the native PI-PLC structure under basic conditions. Additional

crystal structures of native *S. aureus* PI-PLC at both pH 4.6 and 7.5 with or without *myo*-inositol and other ligands (unpublished data) show a strong trend. This enzyme will crystallize in one of two unit cells in a pH-dependent manner in a very consistent basis. At pH 4.6, *S. aureus* PI-PLC crystallizes with an average unit cell of $a = 104.01$ Å, $b = 43.57$ Å, and $c = 62.17$ Å; at pH 7.5, it crystallizes with an average unit cell of $a = 85.68$ Å, $b = 58.22$ Å, and $c = 61.65$ Å. This trend with pH is only broken by the H258Y structure. Although crystallized at pH 4.6, it has a unit cell consistent with the basic form of native enzyme. Thus, the determining factor between the two unit cells appears to be the position of the mobile loop, rather than pH alone. The H258Y mutation prevents the π -cation interaction and the mutant crystallizes with the mobile rim loop in an extended conformation. The H258Y structure confirms that the mobile loop conformational change is the result of the protonation state of His258 and the resulting π -cation interaction.

Activity of *S. aureus* PI-PLC. Given the two discrete conformations adopted by *S. aureus* PI-PLC, two questions need to be addressed. (i) How are the two conformations related to enzymatic activity? (ii) Does this pH-dependent change have a physiological role in *S. aureus* infectivity? To explore the first, we have measured PI-PLC activity toward small unilamellar vesicles composed of either pure PI or PI/PC (4:1) at pH 5.5, 6.5, and 7.5. The enzyme binds tightly to vesicles with phosphatidylglycerol replacing PI and small amounts of the nonsubstrate PC [Cheng, J., unpublished results]. The PI-PLC enzyme mechanism involves two histidine residues acting as a general base and general acid.^{1,10} If the π -cation interaction in *S. aureus* PI-PLC modulates activity, it may be difficult to sense since it also involves a histidine residue. However, we can examine activity of H258Y as a control that cannot form the intramolecular π -cation interaction. As shown in Figure 5, a comparison of activities

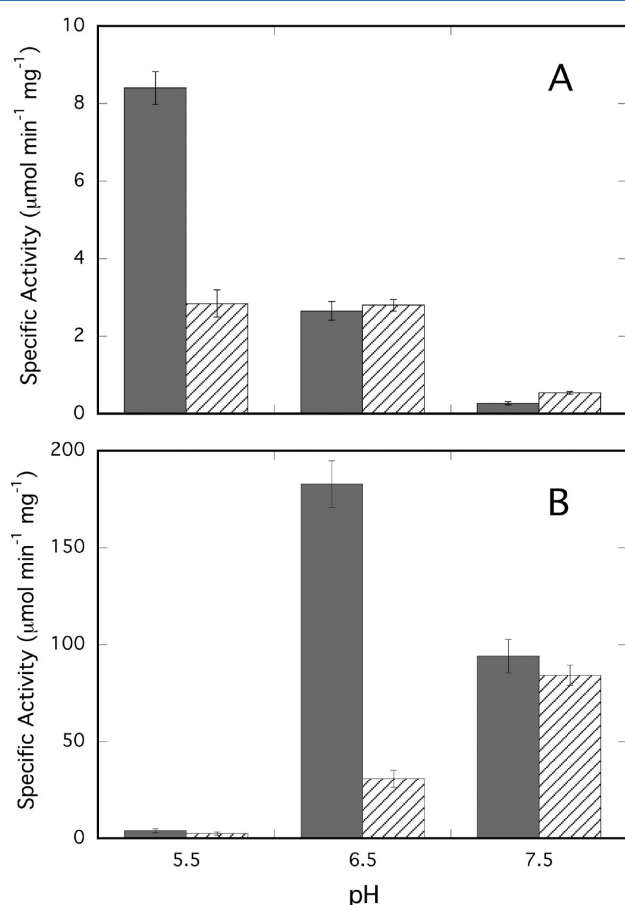


Figure 5. The pH dependence of the specific activity of *S. aureus* recombinant PI-PLC and the H258Y mutant toward (A) 4 mM PI and (B) PI/PC (4 mM:1 mM) small unilamellar vesicles in 50 mM MES/HEPES, 1 mM EDTA with 0.1 mg/mL BSA adjusted to the indicated pH. Wild-type enzyme activities are the gray bars with the H258Y mutant shown as the hatched bars.

toward PI vesicles at pH 5.5, 6.5, and 7.5 shows that the recombinant enzyme has higher activity at pH 5.5, where the π -cation interaction and inward-folded orientation of the rim loop occur, than H258Y. Specific activities are similar and lower for both enzymes as the pH increases (and presumably the π -His–cation interaction is broken). When 20 mol % PC is present in the vesicles, the activities are low at pH 5.5 but

increase as the pH is raised. Wild-type enzyme shows higher activity at pH 6.5 than H258Y. If the only difference is the formation of the π -cation interaction, it appears that the presence of PC may alter the pK_a values for the catalytic His residues and possibly the His in the π -cation interaction as well. Regardless of the presence of PC, the inward-folded rim loop position appears to be associated with higher enzymatic activity at acidic pH.

Previous work has shown that this PI-PLC, with an acidic pH optimum, is extremely sensitive to added salt.⁴ Such behavior is consistent with electrostatic forces dominating partitioning of the protein onto membranes and binding of an individual substrate in the active site. Our structural analysis emphasizes the cationic character of the rim and active site that would be responsible for such behavior (Figure 3). However, we can obtain insight into the activity at acidic pH by looking at space-filling models of the *S. aureus* PI-PLC at the two pH values (Figure 6). Access of substrate to the active site from the membrane face is apparent in both structures. However, a side view, that should detect an exit path for the water-soluble product cIP with the protein still attached to the membrane, shows access to the active site *only* in the acidic pH structure. In the basic structure, the loop is extended, but active site access via other than the membrane interface is restricted. The two conformations of Trp45 appear to be linked to restricting access to the active site (Figure 7). This suggests the one function of the π -cation latch is to ensure water-soluble product can diffuse from the active site and into bulk solution. It allows for at least some degree of processive catalysis for this enzyme bound to membranes under acidic conditions.

The shift in the PI-PLC activity profile with 20 mol % PC present in the bilayer suggests that the phospholipid choline moiety, among other effects, can compete with His258 for interaction with Phe249. At pH 5.5 both wild-type and H258Y exhibit low specific activities. We now have an extended loop, which may allow membrane partitioning of the protein, but not easy release of the water-soluble product. Increasing the pH enhances activities above what is seen in the absence of PC—a result that suggests other more subtle conformational changes in the protein occur with PC added. The pK_a values for the general base and general acid histidines may be shifted when PC is present. Whatever the discrete changes, the structure suggests that PC could compete for Phe249 and the extended loop structure can 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 diC₇PC and diC₈PE activate the enzyme above what is observed with the PI dispersed in TX-100, while diC₈PG and diC₈PS 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 pK_a values or lead to a more productive conformation involving that rim loop.

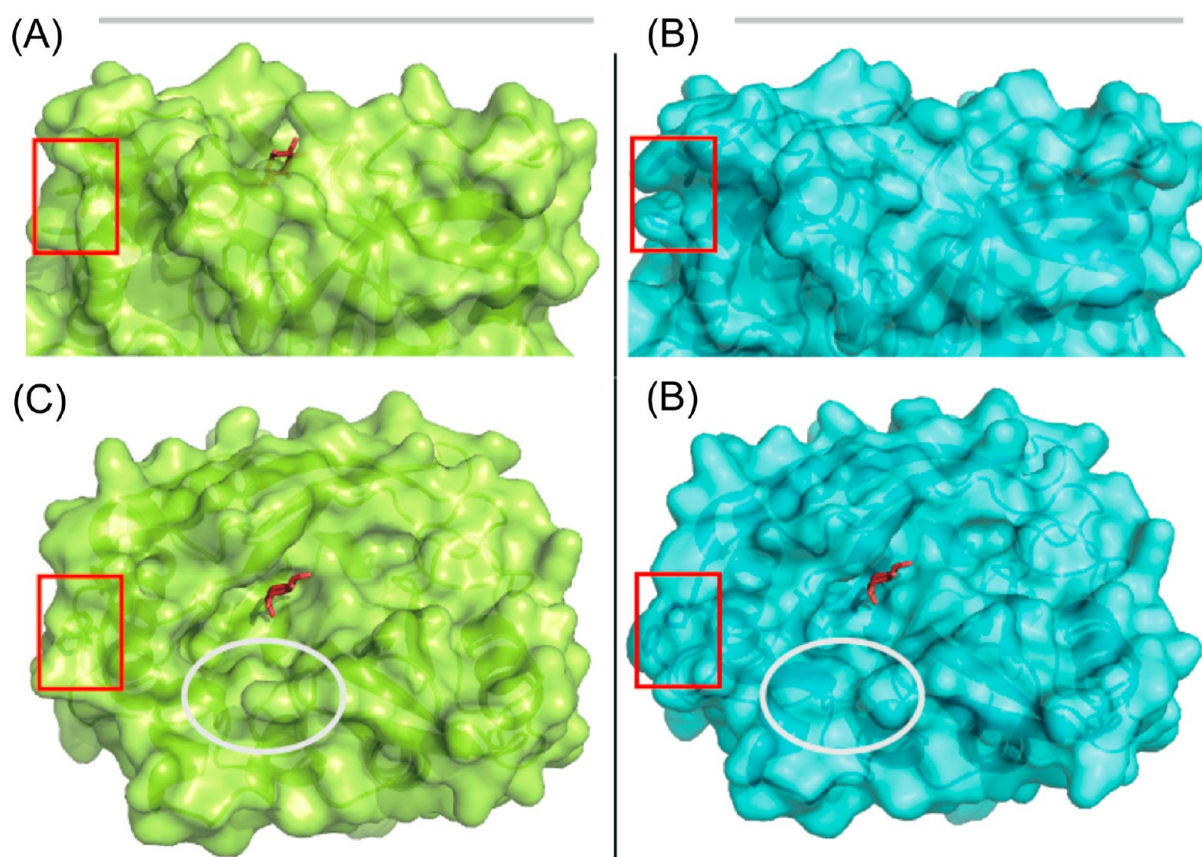


Figure 6. Space-filling models depicting the active site wall channel for native structures. Structures at pH 4.6 are shown (A) juxtaposed to the membrane, which is represented by a gray bar, and looking down into the $\alpha\beta$ barrel, as seen from the membrane interface (C), with the active site channel location marked with a gray oval. The same set of orientations are shown for the pH 7.5 structure, with the view (B) facing the membrane and (D) looking down from the membrane interface. *myo*-Inositol, red stick representation, is visible in both views from the membrane, but not in the basic structure seen from the side. The location of the key π -cation residues is shown as a red box.

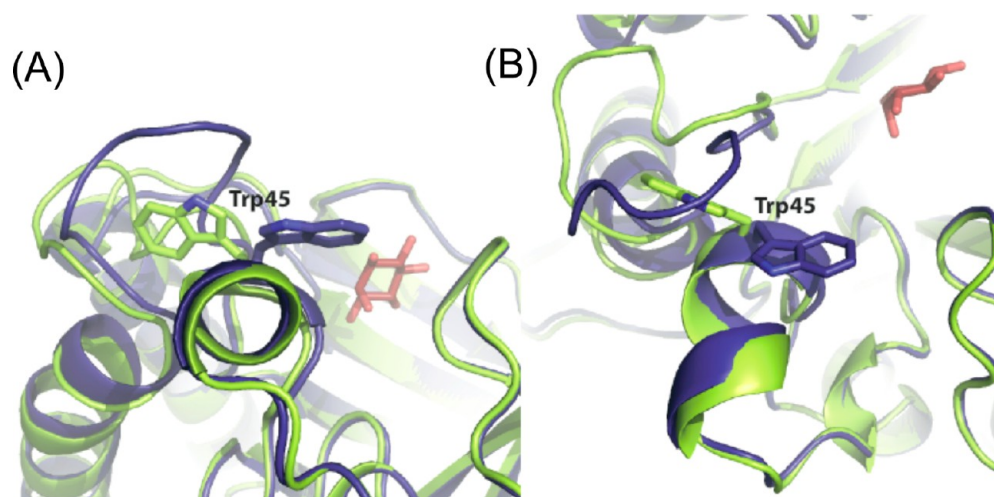


Figure 7. Trp45 gate shown as a cartoon with Trp45 side chain shown as sticks. Both top down (A) and sideways (B) views are shown. The position of the mobile loop in the acidic structure (green) allows the Trp45 side chain to open, while the position of the mobile loop in the basic structure (blue) forces the Trp45 side chain into the closed position, sealing off the active site. Inositol is seen in red in the background.

Connecting the π -Cation Latched Loop with *S. aureus* Infection? *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.^{7,39} Secreted *S. aureus* PI-PLC may aid in colonization and replication of the

microorganism by generating diacylglycerol from GPI-anchored proteins. It is one of the exoproteins shown to be upregulated upon infection in community-associated methicillin-resistant *Staphylococcus aureus*.⁴⁰ This may be part of the response of *S. aureus* in coopting the immune response of host cells. The pH of

an *S. aureus* abscess decreases to 6.6—the pH optimum of *S. aureus* PI-PLC.^{4,11} Our kinetic studies also show that in bilayers with PC as well as PI the pH optimum for this enzyme is around this value.

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Funding

This research was supported by NIH Grant GM60418 (M.F.R.).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

cIP, inositol 1,2-(cyclic)phosphate; diC₆PC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; diC₇PC, diheptanoyl-PC; diC₈PE, 1,2-dioctanoyl-*sn*-glycero-3-phosphoethanolamine; diC₈PG, 1,2-dioctanoyl-*sn*-glycero-3-phosphoglycerol; diC₈PS, 1,2-dioctanoyl-*sn*-glycero-3-phosphoserine; GPI, glycan-phosphatidylinositol; PC, phosphatidylcholine; PEG, poly(ethylene glycol); PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

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