

The Cation- π Box Is a Specific Phosphatidylcholine Membrane Targeting Motif^[5]

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Background: Specific interactions of peripheral membrane proteins with phosphatidylcholine (PC) are poorly characterized.

Results: *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C has poor affinity for PC. Introduction of two tyrosines generates a specific PC binding site.

Conclusion: The PC choline cation interaction with amino acid π systems forms the PC-specific site.

Significance: Well defined choline cation-aromatic π interactions may be a general motif to anchor proteins to PC-rich bilayers.

Peripheral membrane proteins can be targeted to specific organelles or the plasma membrane by differential recognition of phospholipid headgroups. Although molecular determinants of specificity for several headgroups, including phosphatidylserine and phosphoinositides are well defined, specific recognition of the headgroup of the zwitterionic phosphatidylcholine (PC) is less well understood. In cytosolic proteins the cation- π box provides a suitable receptor for choline recognition and binding through the trimethylammonium moiety. In PC, this moiety might provide a sufficient handle to bind to peripheral proteins via a cation- π cage, where the π systems of two or more aromatic residues are within 4–5 Å of the quaternary amine. We prove this hypothesis by engineering the cation- π box into secreted phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*, which lacks specific PC recognition. The N254Y/H258Y variant selectively binds PC-enriched vesicles, and x-ray crystallography reveals N254Y/H258Y binds choline and dibutyrylphosphatidylcholine within the cation- π motif. 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.

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 (2, 3). 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 (2–4). Similarly, proteins specific to the anionic phospholipid phosphatidylserine (PS)² may coordinate Ca²⁺ ions for PS binding using conserved loop motifs as observed in annexin V (5) or directly bind PS via C2 domains as in coagulation factor V (6) and lactadherin (7) 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 (5, 6, 8) and/or multivalent interactions (3) via domain repeats (9) or specificity for more than one type of lipid (9, 10).

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 (11, 12) or choline (13, 14) as well as proteins that bind methylated lysine in histones (15–17) or methylated arginine (18). In these structures, the methylammonium 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, we have reviewed the Protein Data Bank database, and the only known structures containing PC with the choline held in a cation- π box are those for phosphatidylcholine transfer protein (supplemental Table S1 presents a list of crystal structures with choline and choline-related molecules bound). However, additional interactions with the acyl chains assist in PC binding by the transfer protein (19), and it is unclear

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[5] This article contains supplemental Tables S1 and S2 and Figs. S1 and S2. The atomic coordinates and structure factors (codes 4I9J and 4I8Y) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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² The abbreviations used are: PS, phosphatidylserine; diC₈PC, 1,2-dibutyryl-*sn*-glycero-3-phosphocholine; diC₇PC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; FCS, fluorescence correlation spectroscopy; PC, 1-palmitoyl-2-oleoylphosphatidylcholine; PE, 1,2-dioleoylphosphatidylethanolamine; PG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol); PI, L- α -phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PME, 1,2-dioleoylphosphatidylmethanol; SUV, small unilamellar vesicle; WT, wild-type recombinant *S. aureus* PI-PLC; X_{PC}, mole fraction PC; PDB, Protein Data Bank.

Engineering a Phosphatidylcholine Binding Site

whether the π -cation 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, we have engineered such a box into *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C (PI-PLC). *S. aureus* PI-PLC and the related PI-PLC from *Bacillus thuringiensis* are secreted virulence factors for extracellular bacteria that target glycosylphosphatidylinositol-anchored proteins in the PC-rich outer membrane of eukaryotic cells (20, 21). Although *B. thuringiensis* PI-PLC has a high affinity for PC vesicles (8), the similar PI-PLC from *S. aureus* has virtually no affinity for PC vesicles. High resolution field-cycling NMR experiments on *B. thuringiensis* identified a discrete binding site for PC that is consistent with Tyr residues forming a cation- π box or sandwich (22). However, this motif is not present in the *S. aureus* enzyme, which displays much weaker binding to PC-rich vesicles and virtually no binding to pure PC vesicles (23). 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 *B. thuringiensis* PI-PLC cation- π box is proposed to be located on adjacent α helices on the outside of a β -barrel. The addition of two tyrosine residues to the *S. aureus* PI-PLC introduces a specific binding site for PC that we characterize with several biophysical techniques including fluorescence correlation spectroscopy, high resolution field cycling NMR, as well x-ray crystallography. The molecular details of the engineered PC site in *S. aureus* PI-PLC suggest that it should be feasible to engineer similar PC headgroup-specific membrane binding modules into other proteins.

EXPERIMENTAL PROCEDURES

S. aureus PI-PLC Expression, Purification, Modification, and Enzymatic Activity—Recombinant *S. aureus* PI-PLC was produced and purified as described previously (24). The gene for the N254Y/H258Y mutant was constructed from the wild-type recombinant PI-PLC gene using QuikChange site-directed mutagenesis methodology (Stratagene) and primers purchased from Operon. The sequence of the mutated gene was confirmed by Genewiz. The purity of the PI-PLC enzymes was above 95% as monitored by SDS-PAGE; concentrations were measured by the absorption at 280 using extinction coefficients of $\epsilon_{280} = 60280 \text{ M}^{-1} \text{ cm}^{-1}$ for WT and $\epsilon_{280} = 63260 \text{ M}^{-1} \text{ cm}^{-1}$ for N254Y/H258Y calculated by the web-based ProtParam software. Protein was stored at 4 °C until used. Other *S. aureus* PI-PLC variants constructed and verified in the same fashion include N254Y, D213C, D213C/N254Y/H258Y, and Y211A/N254Y/H258Y/Y290A; H258Y was characterized previously (24).

The fluorescent dye Alexa Fluor 488 carboxylic acid, succinimidyl ester from Invitrogen was used to modify the N terminus of PI-PLC proteins for FCS studies. For the field cycling NMR experiments, both wild-type and N254Y/H258Y proteins with D213C were modified with the spin labeling reagent 2,2,5,5-tetramethyl-L-oxyl-3-methyl methanethiosulfonate, obtained

from Toronto Research Chemicals. Excess dye or spin labeling reagents were removed with three Bio-Spin 6 columns.

PI-PLC cleavage of PI in small unilamellar vesicles (SUV), prepared by sonication, with varying mole fractions of PC, X_{PC} , was monitored by ^{31}P NMR using a Varian VNMRS 600 spectrometer as described previously (23, 24). Phospholipids were obtained from Avanti Polar Lipids, Inc., and used without further purification. Most enzymatic assays were carried out in 50 mM MES with 1 mM EDTA and 0.1 mg/ml of BSA, pH 6.5, using 0.2 to 8 $\mu\text{g/ml}$ of the PI-PLC enzymes. Buffers used in assays at other pH values have been described previously (23). Assays for each X_{PC} were run in duplicate.

Vesicle Binding Measured by Fluorescence Correlation Spectroscopy (FCS)—The partitioning of both wild-type recombinant *S. aureus* PI-PLC and the N254Y/H258Y mutant was measured by FCS as described previously (22, 25). The fluorescence was monitored at 22 °C with samples placed in chambered coverglass wells (Lab-Tek, Nunc), containing 10 nM labeled *S. aureus* PI-PLC protein and 1 mg/ml of BSA in 300 μl of 50 mM MES, pH 6.5 (the same buffer as used for enzymatic assays). SUVs of the anionic phospholipid dioleoylphosphatidylglycerol (used as the substrate analog) with different mole fractions of 1-palmitoyl-2-oleoyl-PC were prepared by sonication. FCS data were analyzed as previously described (23, 26). The fitted diffusion coefficient of free, Alexa Fluor 488-labeled *S. aureus* PI-PLC (D_{free}) was $50 \pm 2 \mu\text{m}^2 \text{ s}^{-1}$; D_{bound} , determined using vesicles containing fluorescent labeled lipids, was in the range of 12–15 $\mu\text{m}^2 \text{ s}^{-1}$. K_d values for each X_{PC} were determined in duplicate using different protein and SUV preparations. Once the binding profiles were established for WT and N254Y/H258Y proteins, a quicker centrifugation assay (27) to separate vesicle bound and free protein, with quantification of free protein with the Bio-Rad DC protein assay, was used to compare the fraction of other related protein variants bound to 1 mM PG/PC (1:4) in 50 mM MES, pH 6.5, with 140 mM salt added.

PI-PLC Line Broadening of diC_7PC ^{31}P Resonance— diC_7PC (Avanti Polar Lipids, Inc.) was titrated into a solution of 3 mg/ml (0.085 mM) of PI-PLC in the same MES buffer with 1 mM EDTA. Protein-induced line broadening at 242.76 MHz was measured on a Varian VNMRS 600 spectrometer. For other bacterial PI-PLCs with significant PC affinity, the lipid linewidth increases dramatically around the critical micelle concentration (1.5 mM (10)), as protein-lipid micelle complexes form, then decreases as more diC_7PC is added to reach a limiting line width. Proteins with weakened affinity for PC have very little effect on the diC_7PC ^{31}P line width (28). Line widths at a given diC_7PC concentration were measured in duplicate samples.

Intrinsic Fluorescence of PI-PLC—Intrinsic fluorescence measurements of PI-PLC (0.2 μM) were carried out on a Fluorolog spectrometer (Horiba Jobin Yvon FL3–22). Samples were excited at 282 nm, and changes in the fluorescence intensity at the emission maximum, 337 nm, upon the addition of diC_7PC 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 diC_7PC . A small amount of background signal from pure buffer solution or buffer with different concentrations of diC_7PC was sub-

tracted 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 (10, 28).

High Resolution ³¹P Field Cycling NMR Spectroscopy—High resolution ³¹P field cycling NMR spin lattice (R_1) relaxation measurements, using a custom-built high resolution field cycling system on a Varian UnityPlus 500 spectrometer (29), were carried out with *S. aureus* PI-PLC spin-labeled at D213C. This position is comparable with D205C in *B. thuringiensis* PI-PLC, where a spin label had the largest effect on ³¹P relaxation (22). 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_1 caused by the spin label for each lipid (ΔR_1) in a vesicle compared with 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/spin-labeled PI-PLC interaction. A constant residual R_1 at higher fields, c , is likely to reflect a limiting chemical shift anisotropy 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_0 , are related to r_{p-e} , the distance between the phospholipid ³¹P and the nitroxide when the ligand is bound (22). 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 5 mM of the ligand. We also assume that we are looking at a single PC or phosphatidylmethanol (PMe) binding in a given site for a time approaching 1–2 μ s, long enough to suggest a specific complex as opposed to non-specific 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 the outer monolayer. The average distance of the bound phospholipid on the protein at a specific site is calculated from the following expression.

$$r_{p-e}^6 = [\text{PI-PLC-SL}]/\{(2/3)[L]_0\} \times (S^2\tau_{p-e}/R_{p-e}(0))(0.3 \mu^2(h/2\pi)^2\gamma_p^2\gamma_e^2) \quad (\text{Eq. 1})$$

S^2 , the order parameter of the electron spin-³¹P dipolar interaction, is approximated as 1 because of the long r_{p-e} compared with the size of local picosecond motions; μ , γ_p , and γ_e are well defined physical constants.

Crystallization and Structure Determination—*S. aureus* PI-PLC N254Y/H258Y was crystallized in three different conditions in 150 mM ammonium acetate, 100 mM sodium acetate, pH 4.6, and 100 mM magnesium nitrate with the following additives and treatments. (i) Crystals in the absence of choline compounds were grown with 26% PEG 4000; prior to crystallization, the protein was incubated with 100 mM *myo*-inositol for 2 h. (ii) Crystals with choline bound to the protein were grown with 26% PEG 4000; prior to crystallization, N254Y/H258Y was incubated with 30 mM glycerophosphocholine and 25 mM choline chloride. Before data collection, the crystals were soaked

for 2 h in the crystallization buffer with the addition of 33% PEG 400 and 500 mM choline chloride. (iii) Crystals with diC₄PC bound to the protein were grown with 27% PEG 4000; protein was pretreated with glycerophosphocholine and choline chloride. Before data collection, the crystals were soaked in the crystallization buffer with 90 instead of 100 mM magnesium nitrate, 30% PEG 4000 and 100 mM diC₄PC (below its CMC ~250 mM (30)).

For all crystallizations, purified PI-PLC proteins were diluted to a concentration of 10 mg/ml and crystallized at 20 °C by vapor diffusion, using hanging drops of 3 μ l. Single large crystals (0.5–0.7 mm) appeared overnight. Suitable crystals were mounted in nylon loops and frozen in liquid nitrogen. Data were collected at 100 K using an in-house Rigaku MicroMax-07 HF high-intensity microfocussing 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 (31). All structures were solved by molecular replacement in PHENIX (32) using PHASER (33), with the previous H258Y structure (24) as a model. All models were refined in PHENIX with manual model building in COOT (34). Ligands and ligand restraints were generated using sketcher in CCP4 (35). Structural comparisons were made using SSM superposition (36) in COOT and alignment in PyMOL (Schrodinger). PROCHECK was used for structure validation (37).

RESULTS

Comparing *B. thuringiensis* and *S. aureus* PI-PLC—Bacterial PI-PLCs are single domain enzymes with active sites located at the center of a TIM-like barrel folding motif (24, 38, 39). PI-PLC from *B. thuringiensis* binds to PC-rich vesicles; residues located on the loop connecting helices F and G (notably Trp-242), as well several tyrosines located on helix G (Tyr-246, Tyr-247, Tyr-248, Tyr-251) were implicated in binding to PC interfaces (22, 28). Sequence and structural alignments of the *B. thuringiensis* and *S. aureus* PI-PLC enzymes show that whereas Tyr-246 and Tyr-248 in the *Bacillus* enzyme correspond to Tyr-253 and Tyr-255 in *S. aureus*, the other two tyrosine residues (247 and 251) in the *Bacillus* enzyme have been replaced with Asn-254 and His-258 in the *S. aureus* enzyme (Fig. 1A). *S. aureus* PI-PLC binds to PC-rich membranes with much lower affinity than does the *Bacillus* enzyme and has virtually no affinity for pure PC vesicles (23). This fact suggests that the higher affinity of the *Bacillus* enzyme for PC is associated with the presence of an aromatic box that creates a binding site for the choline. We hypothesized that mutating Asn-254 and His-258 to tyrosines (N254Y/H258Y) could introduce a specific PC binding site in *S. aureus* PI-PLC. This expectation was based on the observation that the site could utilize cation- π interactions to bind the PC headgroup.

Vesicle Binding and Enzymatic Activity of N254Y/H258Y *S. aureus* PI-PLC—FCS was used to measure binding of the WT and mutated enzymes to SUVs. We used low (~10 nM) enzyme concentrations similar to those used for enzymatic assays. WT, Y253S/Y255S, and N254Y/H258Y proteins were fluorescently labeled at the N terminus with a succinimidyl ester of Alexa Fluor 488, and apparent K_d values for PG/PC SUVs were measured as a function of the mole fraction PC, X_{PC} (Fig. 1B). All

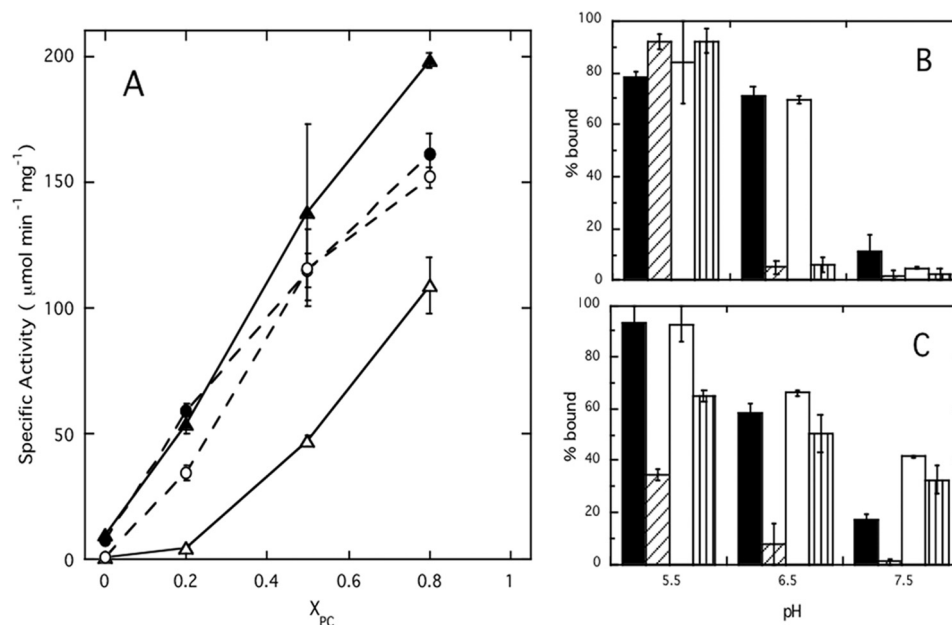


FIGURE 2. In the presence of PC, N254Y/H258Y *S. aureus* PI-PLC is less salt-sensitive than wild-type. A, specific activity of *S. aureus* WT (triangles) and N254Y/H258Y (circles) PI-PLC at pH 6.5 toward different vesicle compositions (X_{PC} = mole fraction PC) in the absence (filled symbols) and presence (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 (■) or presence (□) of 140 mM salt; N254Y/H258Y in the absence (□) or presence (▣) of salt. Error bars represent the variation in parameters from experiments using different protein and SUV preparations.

N254Y/H258Y and WT (Fig. 2A). If salt is present neither WT nor N254Y/H258Y exhibits much activity toward pure PI SUVs, and salt reduces the activity of WT *S. aureus* PI-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 (24). 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. 2, B and C). At pH > 5.5 in the absence of PC (Fig. 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. 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 *S. aureus* 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.

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 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 *S. aureus* PI-PLC variants. Comparisons with the WT structure that has been described previously (24) 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.

NMR Field Cycling Experiments with SUVs—Because the chemical shifts of different phospholipids are distinct, ^{31}P field cycling NMR is useful for identifying specific phospholipid interactions with a spin-labeled protein in multicomponent vesicles (22). In these experiments, differences in phospholipid ^{31}P 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, *B. thuringiensis* PI-PLC spin labeled at D205C has a large effect on the ^{31}P 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. *B. thuringiensis* Asp-205 aligns with *S. aureus* Asp-213, and spin labeling *S. aureus* PI-PLC at D213C has little effect on the PMe or PC resonances (Fig. 3A) under conditions (140 mM salt and 0.5 mg/ml of protein and 5 mM each phospholipid) where ~ 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 *S. aureus* PI-PLC background appears equivalent to what is seen for the SUVs in the absence of protein (22).

However, a very different profile is observed for spin-labeled D213C/N254Y/H258Y under the same conditions, and the

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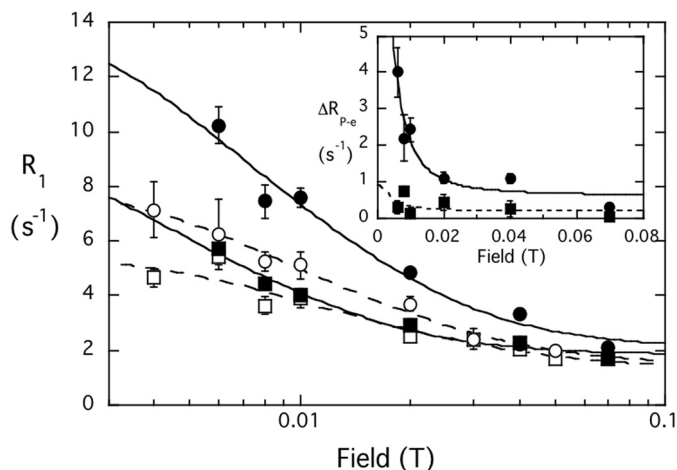


FIGURE 3. The spin label at D213C perturbs lipid signals for the N254Y/H258Y mutant but not WT. Effect of spin-labeled *S. aureus* PI-PLC (0.5 mg/ml) on PMe/PC (5:5 mM) SUVs: control PMe (□) and PC (○) mixed with spin-labeled D213C; PMe (■) and PC (●) with the spin-labeled D213C/N254Y/H258Y. The inset shows the difference in R_1 for each phospholipid ^{31}P specifically attributed to the spin label on D213C/N254Y/H258Y; the data are fit with $\tau_{p-e} = 2 \mu\text{s}$.

results for this variant resemble those for *B. thuringiensis* PI-PLC (Fig. 3). A potential complication to the average distance determination arises from the fact that *S. aureus* PI-PLC forms transient dimers on vesicle surfaces via helix B side chains (23). However, based on the dimer x-ray crystal structure, a spin label at residue 213 should be $>35 \text{ \AA}$ from the active site of the opposing monomer, and $34\text{--}35 \text{ \AA}$ 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 (22).

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. 3, inset). The correlation time for the ^{31}P -electron dipolar interaction is $2 \pm 1 \mu\text{s}$ and the extrapolated $\Delta R_{p-e}(0)$ values for the two phospholipids are $8.52 \pm 1.28 \text{ s}^{-1}$ (PC) and $0.71 \pm 0.61 \text{ s}^{-1}$ (PMe). Although τ_{p-e} is not known precisely, what is relevant in determining r_{p-e} is the ratio $\tau_{p-e}/\Delta R_{p-e}(0)$, and this is very similar when fitting the data at 1 to 3 μs . For PC with $\tau_{p-e} = 2 \mu\text{s}$, this yields $r_{p-e} = 15.2 \pm 0.4 \text{ \AA}$. If the plot of ΔR_1 versus field is fit at 1- or 3- μs correlation times, there is at most a 0.5- \AA variation in the estimated r_{p-e} . Interestingly, this value is a little longer than the r_{p-e} extrapolated for the *B. thuringiensis* PI-PLC with a spin label attached at the same site ($13.5 \pm 0.2 \text{ \AA}$ (22)). 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

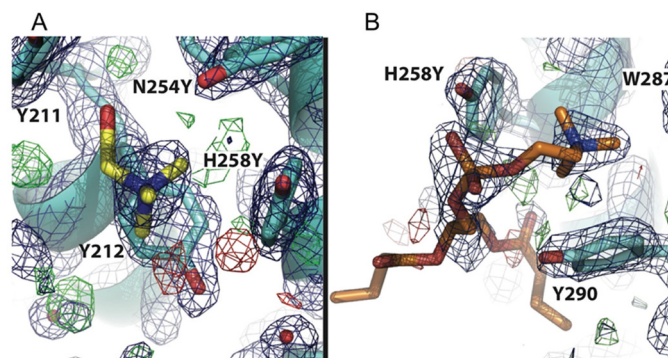


FIGURE 4. Representative electron density for the choline binding site. Electron density, in dark blue and contoured at 1σ , is shown with the model superimposed: A, binding site 1 with a choline molecule refined (PDB 4190); B, binding site 2 showing a molecule of diC₄PC fit to the electron density (PDB 419J). Residues that make up the binding pocket are indicated.

with this amount of salt in the buffer (23). If one uses the fit with $2 \mu\text{s}$ r_{p-e} bound PMe must be $\sim 22\text{--}24 \text{ \AA}$ 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 area of the protein as it is in the *B. thuringiensis* PI-PLC.

Crystal Structures of N254Y/H258Y with Choline and DiC₄PC—To further explore the location of the introduced PC site, we obtained crystal structures of the N254Y/H258Y double mutant and compared it with those previously obtained for WT. The overall structure of the double mutant follows closely that of the native structure in basic conditions (24), with only slight deviations in the positioning of the mobile loop. The refined model of *S. aureus* PI-PLC N254Y/H258Y closely resembles that of the *Bacillus* enzyme especially in the region spanning the top halves of helices F and G. In this region, the structure of the N254Y/H258Y (PDB entry 4I8Y) differs only slightly from the basic form of the *S. aureus* native structure, aside from the mutations and the rotation of Tyr-212 and Tyr-255 by 83° and 22° , respectively. These rotamer differences are necessary to accommodate the larger side chains of the mutated residues. The rest of the structure shows only small variations associated with the inherent mobility of the enzyme and different amino acid content.

Initial crystallization trials to produce a crystal with a PC molecule bound to the N254Y/H258Y protein used solutions with 100 mM HEPES. Serendipitously, we found that rather than the PC, two HEPES molecules could be refined in the vicinity of the introduced tyrosines (supplemental Fig. S1 and Table S2). Because HEPES is cationic, the thought was that we might be able to get choline-containing molecules to bind if we soaked crystals with high enough concentrations of the potential ligands. 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. These crystals were initially grown in 30 mM glycerol phosphocholine and 25 mM choline chloride. Under these conditions, no density was observed for either ligand in the vicinity of the new tyrosine residues. However, when these crystals were soaked for 2 h in a solution containing 500 mM choline or 100 mM diC₄PC, distinct density for these choline ligands was

TABLE 1
Crystallographic data for critical *S. aureus* PI-PLC crystals examined

Crystal	N254Y/H258Y	N254Y/H258Y + choline	N254Y/H258Y + diC ₄ PC
PDB ID	4I8Y	4I90	4I9J
Diffraction data			
Resolution range (Å)	2.10–30.69Å	1.65–30.08	1.85–37.76
No. of reflections	18,210	36,554	24,758
Reflections in free set	933	1840	1279
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell			
<i>a</i> (Å)	85.55	85.98	85.65
<i>b</i> (Å)	57.78	57.58	57.47
<i>c</i> (Å)	61.38	61.69	61.75
Completeness	99.2%	97.2%	92.5
<i>R</i> _{merge}	9.9	5.8	8.4
Protein molecules in A.U.	1	1	1
Refinement			
<i>R</i> _{cryst} ^a	0.1781	0.1772	0.1627
<i>R</i> _{free}	0.2312	0.2136	0.2076
No. residues	303	301	301
No. non-hydrogen protein atoms	2441	2449	2465
No. H ₂ O molecules	138	195	239
No. acetate molecules	1	2	1
No. chloride ions	1	1	0
Root mean square bonds (Å)	0.009	0.007	0.019
Root mean square deviation angles (°)	1.11	1.05	1.94
Ramachandran plot (%)			
Most favored	98.02	98.69	98.70
Additionally allowed	1.98	1.31	1.30
Generously allowed	0	0	0
Disallowed	0	0	0
Average <i>B</i> -factor (Å ²)	33.74	29.40	28.998

^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.

^b Brunger (1992).

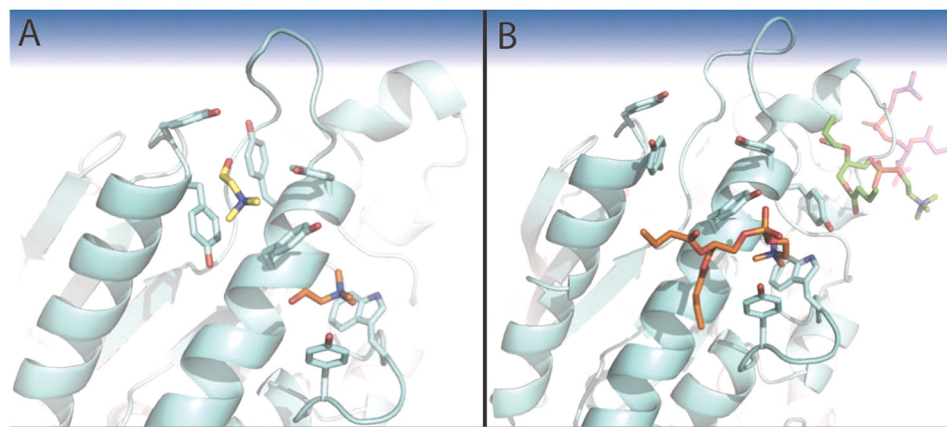


FIGURE 5. Cationic ligand binding pockets on *S. aureus* PI-PLC N254Y/H258Y. View of the two choline binding pockets (A) occupied by choline shown in yellow in site 1 and orange in site 2 (PDB entry 4I90) or (B) diC₄PC (PDB entry 4I9J) with the lipid in choline site 2 in orange, diC₄PC below helix B in green, and diC₄PC associated with the anion binding pocket in magenta.

observed (representative density with the models superimposed is shown in Fig. 4). Statistics for the different crystal structures are presented in Table 1.

In the N254Y/H258Y structure with choline (PDB entry 4I90), two molecules of that ligand could be identified (Fig. 5A). The quaternary amines of each choline were observed to bind on either side of H258Y, with the quaternary amine of choline 1 making cation- π interactions with the aromatic side chains of H258Y and Tyr-212, whereas choline 2 makes similar cation- π interactions with Trp-287 and Tyr-290. Comparison with an unliganded structure of N254Y/H258Y shows that the side chains of Tyr-211 and H258Y are rotated 77° and 100°, respectively (Fig. 6A). The rotation of Tyr-211 creates binding pocket 1, whereas the rotation of H258Y forms the right side of binding pocket 1, as well as the left side of binding pocket 2.

The structure for the mutant protein in the presence of the short-chain lipid diC₄PC (PDB entry 4I9J) showed three molecules of diC₄PC bound (Fig. 5B). One molecule of diC₄PC (refined with 80% occupancy) was located in choline binding pocket 2. The quaternary amines of the choline and diC₄PC ligands are completely superimposable. The phosphate moiety of diC₄PC makes additional polar contacts to the side chain hydroxyls of Tyr-290 and Tyr-258. Density for this ligand (Fig. 4B) is quite strong for the phosphocholine portion of the molecule and the glycerol moiety. However, there is substantial disorder at the lipid chains. No diC₄PC lipids were seen occupying choline binding site 1. However, the high concentration of lipid led to the observation of two additional diC₄PC molecules in the structure at sites not occupied by choline. One diC₄PC, found at 70% occupancy, was bound through polar

Engineering a Phosphatidylcholine Binding Site

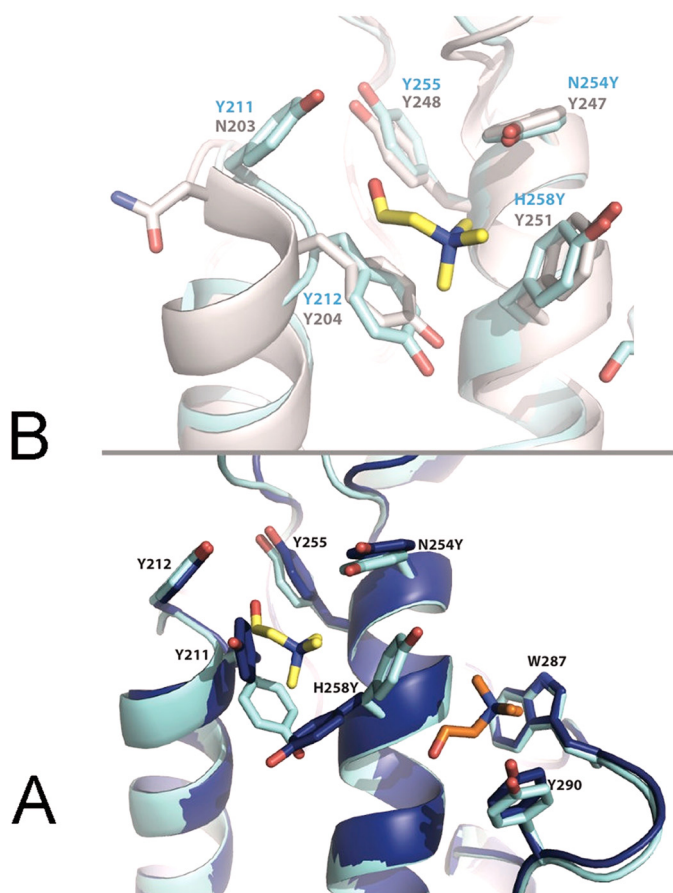


FIGURE 6. *A*, overlay of the *S. aureus* PI-PLC N254Y/H258Y structure without choline (dark blue, PDB entry 4I8Y) and with choline bound (teal, PDB entry 4I9O) showing that choline site 2 is preformed, whereas site 1 requires rotamer changes in side chains. *B*, overlay of choline site 1 in *S. aureus* N254Y/H258Y (teal) with the (gray) *B. thuringiensis* PI-PLC (PDB entry 1T6M). Residues interacting with the cholines are identified.

contacts of the phosphate to the backbone carbonyls of Leu-37 and Lys-38 and the side chain hydroxyl of Ser-43 (supplemental Fig. S2A). An additional molecule of diC₄PC (80% occupancy) was observed (supplemental Fig. S2B) with the phosphate occupying an anion binding pocket (23, 24). Under the conditions this crystal was formed, an acetate ion would be a typical occupant of the anion pocket. The high levels of diC₄PC in the soaking liquor could easily outcompete the acetate ion. The observation of a diC₄PC phosphate interacting at the anion binding pocket is consistent with the proposal that anionic phospholipids (including substrate PI) in a vesicle can bind in this region (23), and are likely to be displaced when the surface concentration of the anionic lipid decreases. These two additional lipids are held by polar contacts that are not specific to choline or phosphatidylcholine.

Do Both Choline Sites in N254Y/H258Y Bind PC?—A comparison of *S. aureus* N254Y/H258Y PI-PLC structures in the absence or presence of a 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. 6A). Interestingly, in the choline bound conformation, binding pocket 1 of the double mutant overlays well with the side chains of the *B. thuringiensis* PI-PLC structure (PDB

entry 1T6M) (Fig. 6B), whereas choline site 2 appears unique to the *S. aureus* PI-PLC mutant but resembles other choline binding sites that can use a Trp residue to form part of the cation- π box (supplemental Table S1). Although the secondary structure of the two proteins is quite different, the spatial arrangement of tyrosine residues in choline site 1 is very similar to how choline is bound in OpuBC (14). 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 μ s (22). 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 $2.35 \times 10^{-7} \text{ s}^2$ for N254Y/H258Y, and $1.48 \times 10^{-7} \text{ s}^2$ for *B. thuringiensis* PI-PLC, consistent with PC binding closer to the spin label site in *B. thuringiensis* 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, 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 *B. thuringiensis* 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. 7A).

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.

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 head-

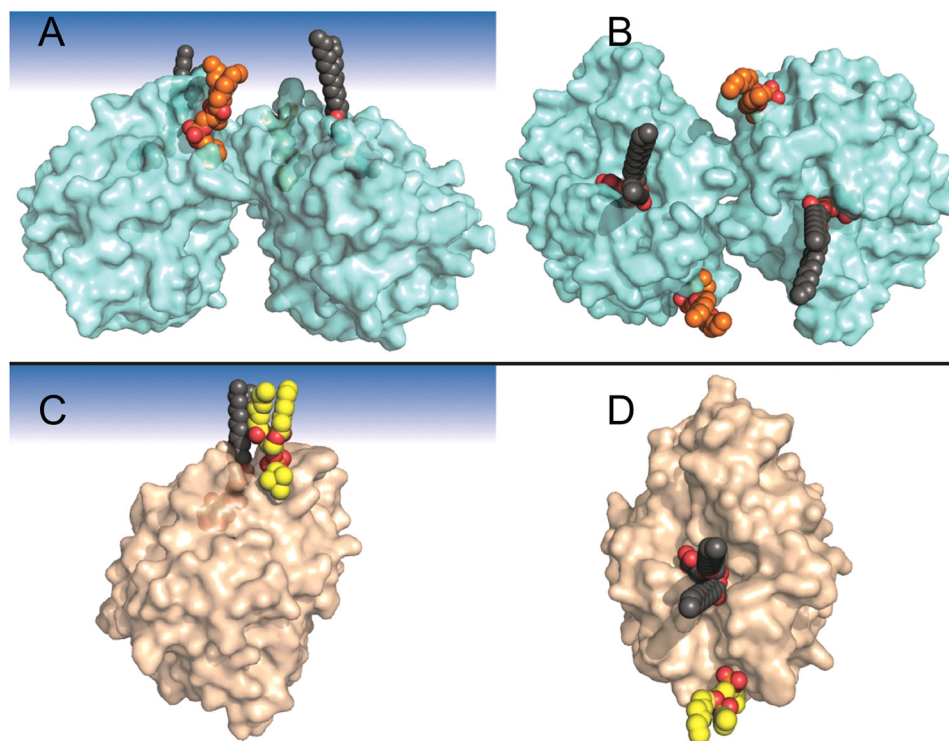


FIGURE 7. *In silico* models of PC and PI binding to discrete sites on *S. aureus* PI-PLC N254Y/H258Y and *B. thuringiensis* PI-PLC. The *S. aureus* mutant structure is shown as a dimer (mediated through helix B) with one molecules of diC₇-PC in site 2 (orange) and one molecule of PI (dark gray) in the active site in views (A) with the membrane interface at the top, and (B) looking down from the membrane. The *B. thuringiensis* monomer is shown with diC₇-PC modeled in site 1 (yellow) along with one molecule of PI (dark gray) in the active site in views (C) with the membrane at the top or (D) looking down from the membrane.

groups of zwitterionic phospholipids. *Bacillus* PI-PLC is activated by PC (10), in large part by enhancing vesicle binding (25). The G helix region of *Bacillus* PI-PLCs has several Tyr residues that could form a sandwich or cage around a choline group (22, 28), 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 *B. thuringiensis* 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 (site 1 between tyrosines on helix F and helix G and site 2 between helix G and a loop), only choline site 2 between helix G and a loop is occupied by a PC molecule in the *S. aureus* protein. *B. thuringiensis* 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 *B. thuringiensis* 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 (40). 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, $K_d(\text{no PC site})/K_d(\text{intact PC site})$, should be similar for both enzymes. Data for $X_{\text{PC}} = 0.8$ SUVs were chosen for the comparison,

because no K_d could be obtained for *S. aureus* WT binding to pure PC SUVs (23). For the *S. aureus* PI-PLC, $K_d(\text{WT})/K_d(\text{N254Y/H258Y}) = 48$. For *B. thuringiensis* PI-PLC, the Y251A mutant was selected as one where the site analogous to choline site 1 should be abolished (Tyr-251 is the equivalent of *S. aureus* H258Y). Binding data at the same mole fraction PC (41) yield $K_d(\text{Y251A})/K_d(\text{WT}) = 45$. 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 (40). 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 *S. aureus* PI-PLC N254Y/H258Y binds to pure PC bilayers with significantly weaker affinity than *B. thuringiensis* PI-PLC. Tallying up the tyrosine residues around the rim of the $\alpha\beta$ -barrel it is clear that the *B. thuringiensis* PI-PLC has far more aromatic residues that could either (i) insert into the bilayer or (ii) form transient cation- π complexes.

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.

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