The phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* can be activated by nonsubstrate interfaces such as phosphatidylcholine micelles or bilayers. This activation corresponds with partial insertion into the interface of two tryptophans, Trp-47 in helix B and Trp-242 in a loop, in the rim of the αβ-barrel. Both W47A and W242A have much weaker binding to interfaces and considerably lower kinetic interfacial activation. Tryptophan rescue mutagenesis, reinsertion of a tryptophan at a different place in helix B in the W47A mutant or in the loop (residues 232–244) of the W242A mutant, has been used to determine the importance and orientation of a tryptophan in these two structural features. Phosphotransferase and phosphodiesterase assays, and binding to phosphatidylcholine vesicles were used to assess both orientation and position of tryptophans needed for interfacial activation. Of the helix B double mutants, only one mutant, I43W/W47A, has tryptophan in the same orientation as Trp-47. I43W/W47A shows recovery of phosphatidylinositol-specific phospholipase C (PC) activation of d-myo-inositol 1,2-cyclic phosphate hydrolysis. However, the specific activity towards phosphatidylinositol is still lower than wild type enzyme and high activity with phosphatidylinositol solubilized in 30% isopropyl alcohol (a hallmark of the native enzyme) is lost. Reinserting a tryptophan at several positions in the loop composed of residues 232–244 partially recovers PC activation and affinity of the enzyme for lipid interfaces as well as activation by isopropyl alcohol. G238W/W242A shows an enhanced activation and affinity for PC interfaces above that of wild type. These results provide constraints on how this bacterial phosphatidylinositol-specific phospholipase C binds to activating PC interfaces.

Phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme with a critical role in membrane-associated signal transduction, carries out PI cleavage by an intramolecular phosphotransferase step that cleaves PI to cIP followed by a cyclic phosphodiesterase step that converts cIP to I-1-P (1, 2).

Bacterial PI-PLC enzymes, which are secreted by the bacterium, have a simpler structure than the complex multidomain mammalian enzymes. Whereas bacterial PI-PLC enzymes do not have a role in signaling in the bacterium, they can be critical for infection of other cells (3). The PI-PLC enzymes from *Bacillus cereus* and *Listeria monocytogenes* are composed of a distorted (βα)_{n} barrel that is structurally very similar to the catalytic domain of PLCε1 (4–7). Interfacial binding of both bacterial and mammalian enzymes to phospholipid interfaces, which was shown to be linked to the kinetic activity of PI-PLC in both steps in the reaction (8–15), was suggested to involve parts of the rim of the barrel. A good candidate for the interfacial binding site is provided by an unusual clustering of hydrophobic residues (and relative lack of charged residues) in the rim of the bacterial enzyme as well as in a ridge at the top of the barrel of PLCε1 (15).

The simpler structure of the bacterial enzyme makes it an excellent system for exploring the importance of hydrophobic rim residues for both interfacial binding and catalytic activity. Previously, we showed that two tryptophan residues, Trp-47 in helix B and Trp-242 in *Bacillus thuringiensis* PI-PLC (1–2) in a 13-member loop (232FTSLSGGTAWS244), play major roles in binding to PC surfaces and interfacial activation by that nonsubstrate phospholipid (16). The large hydrophobic side chain of tryptophan has the largest free energy for partitioning into PC membranes as measured by the Wimley and White hydrophobicity scale (17), and mutants that replace these bulky groups are predicted to have dramatically reduced affinities for surfaces. This was observed for W47A and W242A mutants binding to PC interfaces (16). In the present work, we have investigated the effects of orientation and position of tryptophan residues in helix B and the 232–244 loop on PI-PLC activity. If other factors as well as membrane insertion affect binding and interfacial activation of PI-PLC, replacement of different residues in the rim could alter PI-PLC kinetics and membrane binding.

With this in mind, we prepared the following mutant PI-PLCs: double mutants, I43W/W47A, G48W/W47A, G48W/W47A, M49W/W47A, S236W/W242A, G238W/W242A, and N243W/W242A, as well as a mutant with three tryptophan residues in the rim, G238W. These were characterized for kinetic activation of PI and cIP hydrolysis by dIC-PC (9, 13, 16) and the water-miscible organic solvent isopropyl alcohol (18, 19), the ability to bind to PC interfaces (comparing both intrinsically fluorescent presence in the PC micelles and a vesicle filtration assay), and any changes in secondary structure (analyzed by CD spectroscopy). Results indicate that the orientation of the tryptophan in helix B is very critical for interfacial binding and kinetic activation by both PC and iPrOH. There are fewer...
constraints on the position of tryptophan in the rim loop. In fact, replacing Gly-238 with tryptophan in W242A improves the vesicle binding ability of PI-PLC and enhances cleavage of PI solubilized in diC7-PC above that of native PI-PLC. Having three tryptophans in the rim does not dramatically enhance vesicle binding or the enzyme-specific activity indicating that only two tryptophans (one in helix B and another in the 232–244 loop) are inserting into PC membranes. The ability to uncouple PC activation from water-miscible solvent activation of the enzyme for many of these mutants strongly suggests that the two methods of activation occur by different pathways.

MATERIALS AND METHODS

Chemicals—POPC, diC7-PC, diC8-PC, and PI were purchased from Avanti; crude PI for preparing cIP was purchased from Sigma. cIP was prepared from PI as described previously (9). myo-inositol, Triton X-100, and iPrOH was purchased from Sigma. All other chemicals were reagent-grade.

Overexpression of Bacterial PI-PLC and Construction of Mutants—A plasmid containing the Bacillus thuringiensis PI-PLC gene obtained from Dr. Ming-Daw Tsai (Ohio State University) was transformed into E. coli BL21 cells (BL21-Codonplus (DE3)-RIL from Stratagene). Overexpression and purification of the recombinant protein and tryptophan mutants W47A and W242A have been described previously (16). Protein solutions were concentrated using Millipore Centraplus 10 filters; concentrations were estimated by both A280 (and the calculated extinction coefficient) and by Lowry assays. All of the mutations of the PI-PLC gene were carried out by QuikChange methodology (21, 22) using a site-directed mutagenesis kit from Stratagene. Two complementary mutagenic primers (all purchased from Operon) purified by high pressure liquid chromatography and containing the desired mutation were annealed to the same sequence on opposite strands of the plasmid. The primer CAAGTCTGGTCTCACCAGGGCAC and its complement were used for construction of G238W using the WT PI-PLC gene. Construction of the double mutants started with W47A or W242A single mutants (16). The primers for I343W were I343W-forward and I343W-reverse which were designed so that no more than 20% substrate hydrolysis occurred in 2 h. Assays to check for activation by PC typically used 5 mM cIP in the absence or presence of 50 mM diC7-PC to probe for PC activation. Samples were usually assayed in duplicate (and often in triplicate); errors in specific activities based on duplicate or triplicate samples were <10%.

PC Vesicle Binding Studies—SDS-PAGE (12% polyacrylamide) was used to quantitate free PI-PLC separated (via centrifugation/filtration) from PI-PLC bound to POPC vesicles. A stock of small unilamellar POPC vesicles (5 mM) with an average diameter ~300 Å (as estimated by light scattering using a Malvern particle sizer) was prepared by sonication in 10 mM Tris, pH 7.5. Vesicle samples were incubated with 0.03 mg/ml protein in 10 mM Tris, pH 7.5. The bulk POPC concentration was 0.01, 0.02, 0.05, 0.1, and 0.2 mM for WT PI-PLC and G238W and 0.0, 0.02, 0.05, 0.10, 0.15, and 0.2 mM for all double mutants. In the POPC binding assay with W47A/W242A, the bulk POPC concentration was increased to 2 mM. After incubation for 15 min, the samples were centrifuged to separate free protein from vesicle-bound protein (which was retained on the membrane). The eluant was collected and lyophilized overnight and analyzed by SDS-PAGE. Coomassie Blue-stained gels were imaged and the PI-PLC band intensities monitored. Comparison of band intensities to a sample carried through the filtration and centrifugation but without POPC vesicles was used to measure the fraction of free enzyme (E.F/O, where O is the total amount of enzyme). The fraction of enzyme bound (E.B/O), was then evaluated as (1–E.F/O).

RESULTS

Strategy for Tryptophan Rescue Mutants, Secondary Structure, and Thermostability—The active site of PI-PLC is located at the C-terminal side (as determined by strand orientation) of the distorted (βα)6-barrel. Helix B and loop 232–244 are at the mouth of the barrel (Fig. 1). The distance between Cα of Trp-47 to Ca of Trp-242 is 10.5 Å. These residues are both quite far from the active site, a measure of this is the distance between
CD spectra of WT and mutant PI-PLC proteins were acquired and used to check for overall structural elements. A large change might not be expected in either secondary structure or stability, since helix B and loop 232–244 had weaker density in the crystal structure and were assumed to be flexible (4). Estimates of WT secondary structure calculated from the CD wavelength spectra by CDNN (23, 24) agreed moderately well with the secondary structure elements in the crystal structure (4), and nearly all the single and double tryptophan mutants had essentially the same proportion (±1%) of secondary structure elements (16) and $T_m$ values within 1 °C of the $T_m$ for WT PI-PLC (54.4 °C). The one exception was M49W/W47A, which exhibited a 2 °C decrease in $T_m$. Residue 49 is close to the C-terminal end of helix B and interacts with other portions of the protein. Insertion of a bulky tryptophan side chain at this position could slightly destabilize the molecule. However, the lack of significant changes in secondary structure in all these PI-PLC mutants indicates that any changes in PC binding and kinetics are not due to protein that has a significantly altered structure.

**Catalytic Properties of Helix B Mutants**—By using W47A activities (low compared with native protein (16)) as a point of reference, cIP hydrolysis and PI cleavage activities of the helix B mutants were obtained. Of all the helix B double mutants constructed, only I43W/W47A had significantly increased specific activity toward PI in the presence of diC$_7$PC compared with W47A (Fig. 3A). All the other mutants in this region of the protein had PI cleavage activity reduced compared with the parent W47A as follows: 53, 40, and 20% for G48W/W47A, M49W/W47A, and Q45W/W47A, respectively. Phosphodiesterase activities (cIP hydrolysis) of these mutants were consistent with the trends in PI hydrolysis. With 5 μM cIP, native PI-PLC activity was enhanced 8–9-fold with diC$_7$PC added (16). W47A shows −60% of the diC$_7$PC activity of WT (16). Hence, an improved double mutant would have a specific activity toward cIP in the presence of diC$_7$PC higher than W47A. As shown in Fig. 3A, the specific activity of I43W/W47A toward cIP in the presence of PC micelles was basically the same as WT. The other helix B mutants exhibited cIP hydrolysis rates with diC$_7$PC present that were less than W47A, indicating that PC activation of this step was not restored (and impaired even more than the removal of Trp-47 for some of the mutants).

An alternate means of activating PI-PLC is the addition of moderate percentages of water-miscible organic solvents (18). 30% iPrOH has been postulated to activate PI-PLC by changing the local polarity of the active site and also possibly altering the protein conformation slightly. This amount of water-miscible solvent solubilizes PI and can be used as an assay system for PI-PLC (19). In the presence of isopropyl alcohol, all helix B mutants, including I43W/W47A, which could be activated by diC$_7$PC, showed reduced activity compared with WT protein that was comparable with that of W47A. A tryptophan at any position but residue 47 produces an enzyme that cannot be activated by iPrOH. These results indicate that not only orientation (face of the helix) but position of tryptophan in helix B is critical for optimized phosphotransferase and phosphodiesterase activities. Tryptophan (or a large hydrophobic group) located at residue 47 is absolutely necessary for PI cleavage to be enhanced by iPrOH.

**Catalytic Properties of Loop 232–244 Mutants**—The specific activity of W242A toward PI solubilized in diC$_7$PC was 3-fold less than WT. This mutant also showed impaired PC activation of cIP hydrolysis (16). Reinsertion of tryptophan at three different positions in the loop (residues 236, 238, and 243) was much more successful at increasing PI cleavage rates above that for W242A (Fig. 3B). For S236W/W242A and N243W/

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**Fig. 2.** A, orientation of Trp at different positions in helix B mutants. B, orientation of Trp at different positions in the 232–244 loop. These place the Trp in the orientation of the side chain that was replaced and do not represent energy-minimized conformations since most of these two structural features are fully exposed to solvent and hence very flexible in the absence of surfaces.

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The Ca atoms of the two rim tryptophans and the Ca of Trp–178, a residue at the bottom of the active site that forms a hydrogen bond with a residue critical for substrate binding (Asp–198). Trp–47 is 22.9 Å and Trp–242 is 28.8 Å from the Trp–178 Ca. Because W47A and W242A exhibited impaired interfacial activation by PC and reduced affinities for PC surfaces, a series of tryptophan rescue double mutants was prepared to explore where a tryptophan could be reintroduced to restore enzymatic activity and activation by PC surfaces. Helix B has only two turns with Trp–47 at the C-terminal end of this short helix (Fig. 2A). Gln–45 is on the same turn as Trp–47 but with an orientation on the opposite face of the helix. Ile–43 is in the first turn of helix B and has the same orientation as Trp–47 but is displaced along the helix. Gly–48 and Met–49 residues follow Trp–47 but are not part of the short helix. Starting with W47A, each of these residues was replaced by tryptophan to test the importance of tryptophan placement in helix B for optimal catalysis and PC binding.

The 232–244 loop appears to be very flexible in the crystal structure, although replacement of Trp–242 by alanine profoundly effects PI cleavage and binding to PC vesicles (16). Because replacement of the tryptophan by phenylalanine or isoleucine rescued PI cleavage rates, insertion of a tryptophan at many positions in the loop could lead to WT behavior. With this in mind, we chose Ser–236, Gly–238, and Asn–243 as target positions to introduce tryptophan into the W242A protein (Fig. 2B). Asparagine and serine are uncharged polar residues; Asn–243 is adjacent to Trp–242, and Ser–236 is 6 residues away. Gly–238 is in the middle of the loop and might contribute significantly to loop flexibility. Therefore, mutation of glycine to tryptophan should alter the loop flexibility as well as significantly increase protein hydrophobicity, both properties of the local structure that could be relevant to PI-PLC interfacial activity.
W242A the increases in PI cleavage rates (1.88- and 1.25-fold above W242A, respectively) were moderate, and specific activities were still less than that exhibited by WT enzyme. However, G238W/W242 was able to cleave PI with a rate 4.8-fold higher than W242A and 1.6-fold higher than WT in the presence of a PC interface. The movement of tryptophan from residue 242 in the loop to position 238 produces an enzyme with an enhanced capacity to cleave PI. With PI dispersed in water-miscible organic solvent (PI in 30% iPrOH), all three loop double mutants had higher activity than W242A, in fact N243W/W242A and G238W/W242A had activity comparable with WT enzyme. If loop 232–244 is a flexible structure, then one might expect insertion of tryptophan almost anywhere would enhance enzymatic activity, and indeed this occurs for PI cleavage. Enhanced specific activities toward cIP in the presence of diC7PC (compared with W242A) were also observed for G238W/W242A and N243W/W242A but not for S236W/W242A. Because G238W/W242A had high activity with PI in micellar diC7PC and in organic solvent, construction of the mutant G238W was undertaken. Addition of another tryptophan to this loop would be predicted to enhance binding of the protein to PC vesicles (assuming all rim tryptophans partition into the membrane) and might also enhance catalytic activity assuming surface binding and catalysis are coupled. Indeed, both G238W and G238W/W242A had similar activities toward PI (1.6-fold higher than WT) and similar rates toward cIP with diC7PC added (Fig. 3B). Placement of a tryptophan at residue 238 enhances interfacial activation compared with Trp-242. However, having two tryptophan residues in the 232–244 loop does not significantly increase activity any further.

**Binding of Mutant PI-PLC Enzymes to PC Vesicles**—Moving tryptophan in helix B or loop 232–244 could alter the affinity of these mutants for activating interfaces (e.g. PC). This possibility was investigated by measuring the partitioning of all enzymes to PC SUVs using a centrifugation/filtration assay (16, 27). The data were fit with a hyperbolic curve to generate an apparent dissociation constant, $K_d$. The results, shown in Fig. 4, provide a way of comparing affinities of the different PI-PLCs for a PC surface. The apparent $K_d$ of WT PI-PLC for PC SUVs was 88 μM with the $K_d$ of W47A and W242A had similar activities toward PI (1.6-fold higher than WT) and similar rates toward cIP with diC7PC added (Fig. 3B). Placement of a tryptophan at residue 238 enhances interfacial activation compared with Trp-242. However, having two tryptophan residues in the 232–244 loop does not significantly increase activity any further.

**FIG. 3.** Specific activities of WT PI-PLC and tryptophan rescue double mutants toward cIP (5 mM) in the presence of 5 mM diC7PC (black bars) and toward PI (8 mM) dispersed in 32 mM diC7PC (hatched bars), or 30% iPrOH (white bars). In A are shown helix B mutations, and in B are shown the rim loop mutations.

**FIG. 4.** Comparison of apparent $K_d$ values measured for PC vesicles (black bars) with the $K_d$ values estimated for each double mutant (hatched bars) assuming that the new tryptophan partitions into the bilayer and that the changes in free energy for the protein side chains can be derived from the experimental hydrophobicity scale of Wimley and White (17). In A are shown helix B mutations, and in B are shown the rim loop mutations.
Apparent $K_D$ values and specific activity toward PI were loosely correlated for the helix B double mutants. Mutants with enhanced PI cleavage exhibited $K_D$ values lower than that for W47A. For example, the $K_D$ for I43W/W47A was 0.85 mM, 4-fold lower than W47A but still 10-fold higher than WT. Q45W/W47A had a similar $K_D$ value to W47A. The other two mutants, G48W/W47A and M49W/W47A, exhibited slightly improved binding affinity for POPC SUVs ($K_D$ of 0.45 and 1.02 mM, respectively). For each of the mutants one can predict the change in $K_D$ for a mutant compared with WT using the Wimley-White hydrophobicity scale (17) to estimate the change in $\Delta G$ caused by the replacement of a given residue by tryptophan and assuming that the residues so modified insert into a PC bilayer. As shown in Fig. 4A, inserting a tryptophan in place of Ile-43 or Gly-48 in helix B is compatible with partial (but not complete) insertion of the tryptophan into PC vesicles. However, only I43W/W47A was able to regain diC$_7$PC activation of PI cleavage and cIP hydrolysis.

In contrast to the helix B double mutants, apparent $K_D$ values for double mutants of the 232–244 loop were all significantly decreased (10–100-fold) compared with W242A (Fig. 4B). N243W/W242A had the largest discrepancy between experimental and predicted $K_D$ values suggesting that a tryptophan in this position is not effectively partitioning the protein to the membrane surface. The affinity of G238W/W242A for the PC surface was increased above that for WT protein, and the affinity of G238W with three tryptophan residues in the rim region was not dramatically enhanced over G238W/W242A.

Fluorescence Sensitivity of Helix B and Loop 232–244 Mutants to Ligand Binding—Previous studies (11, 13, 16, 25) have shown that the PI-PLC binding to activating PC micelles or vesicles causes an increase in PI-PLC intrinsic fluorescence intensity. In contrast, the water-soluble substrate analogue myo-inositol binds to the active site as a competitive inhibitor and causes a decrease in fluorescence (16). DiC$_7$PC forms monomers below 1 mM (its CMC is 1.5 mM (28)) and aggregates to form rod-shaped micelles above 1.5 mM (29). The intrinsic fluorescence of W47A, and to a much greater extent W242A and W47A/W242A, showed reduced increases in the presence of PC micelles compared with WT PI-PLC (16). Helix B double mutants, I43W/W47A, Q45W/W47A, G48W/W47A and M49W/W47A, were examined for the effect of diC$_7$PC micelle on their intrinsic fluorescence. The emission maximum, 337 nm, was the same for all unliganded proteins and not affected by the addition of PC micelles (up to 4.0 mM diC$_7$PC). As shown in Fig. 5A, the fluorescence intensity of native PI-PLC at 337 nm increased dramatically once micelles were formed. For all the helix B tryptophan rescue mutants, the intrinsic fluorescence also increased with micellar diC$_7$PC with an extent greater than W47A. However, since Trp-242 was shown to be responsible for most of the fluorescence change upon binding to PC surfaces (16), a near normal fluorescence profile would be expected for these proteins (as long as they bind to the PC micelles).

For all three of the loop tryptophan rescue mutants, intrinsic fluorescence as a function of added diC$_7$PC resembled that of W242F or W242I (16), i.e. only small increases in fluorescence occurred upon diC$_7$PC micellization (Fig. 6A), and the major change occurred at $>$2 mM diC$_7$PC (possibly indicating interactions of fluorophores with the rod-shaped PC micelles that increase in average length as the diC$_7$PC concentration increases (29)). Moving tryptophan away from residue 242 appears to uncouple it from responding to diC$_7$PC binding (or its response is buried in the fluorescence of the other six tryptophan in the molecule). G238W, which has two tryptophans in the loop (including Trp-242 as the major sensor of PC binding), shows a large increase in fluorescence upon diC$_7$PC micellization that occurs below 1 mM. The modified protein appears to lower the effective CMC of diC$_7$PC. Clearly, Thr-242 is the major fluorophore responding to micelle binding, and its position in the loop is key to its ability to detect any change in environment with added micelles. The observation that tryptophan fluorescence at other positions in the loop does not increase upon PC binding might suggest that its placement in these other positions puts it in a hydrophobic environment prior to surface binding that does not change significantly upon binding to surfaces.

In contrast to activating molecules such as diC$_7$PC, myo-inositol bound to the PI-PLC active site caused a decrease in the fluorescence intensity of WT PI-PLC. W47A, W242A, and W47A/W242A mutants required more myo-inositol added to decrease fluorescence, suggesting that altering key rim hydrophobic residues is coupled to substrate/analogue binding at the active site (16). The effect of myo-inositol on the fluorescence of I43W/W47A, Q45W/W47A, and M49W/W47A mutants was
very similar to that of WT PI-PLC (Fig. 5B). G48W/W47A fluorescence was less sensitive (i.e. exhibited the smallest decrease in intensity) to myo-inositol binding. Interestingly, this helix B double mutant had the tightest binding to PC membranes. The requirement for more inositol to elicit a decrease in intrinsic fluorescence might suggest that the active site conformation or accessibility to the active site has been affected in G48W/W47A. Similarly, all loop tryptophan rescue mutants (Fig. 6B) showed decreased fluorescence upon myo-inositol binding of this water-soluble inhibitor to the PI-PLC active site, although S236W/W242A and G238W/W242A intensity decreased the least over the inositol concentration range examined (possibly indicating that the added fluorophore has a larger contribution to PI-PLC fluorescence when it is at residue 236 or 238). Overall, these fluorescence results with myo-inositol binding strongly imply that in most cases moving a tryptophan to different positions of helix B or the 232–244 loop does not dramatically disrupt accessibility of the active site.

**DISCUSSION**

Enzymes (e.g. phospholipase C, D, and A$_2$) that show interfacial activation cleave phospholipid ester bonds with greater efficiency when the substrate is localized at an interface than when it is monomeric. Several of these enzymes are also activated by nonsubstrate interfaces (9, 30–32), in some cases suggesting that binding to the interface shifts the enzyme conformation to an activated form. In this way, the interface acts as a cofactor or positive effector of the reaction. Obtaining a molecular picture of the interfacial site and how this may affect enzyme conformation and catalysis is difficult. Binding of an enzyme to an interface likely has both hydrophobic and electrostatic components (32, 33). For many peripheral proteins, tryptophan penetration into the membrane is an important component of interfacial binding to PC surfaces (34). Furthermore, crystal structures of integral membrane proteins show that the bulky side chains of tryptophan tend to partition to PC activation surfaces (34).

The interfacial binding surface of B. cereus PI-PLC was suggested to be a hydrophobic ridge composed of a short $\alpha$-helix B and a loop between strand III and helix G composed of residues 232–244 (4). Both regions had reduced density in the crystal structure suggestive of multiple conformations or mobility. Flexibility in this area of the protein may be important for contacting an interface. Residues suggested to interact with an interface were Pro-42, Ile-43, Val-46, Trp-47, Ala-241, Trp-242, and Pro-245 (1, 4). In addition to these hydrophobic residues, there are three lysines (Lys-44, Lys-122, and Lys-201) that could interact with the negatively charged phosphate in phospholipids. We have shown previously (16) that PI-PLC requires two tryptophans, Trp-47 and Trp-242, in the barrel rim for tight binding to PC interfaces and for optimal PI cleavage activity. Removal of either reduces both phosphotransferase and phosphodiesterase activities as well as membrane binding. An unanswered question is whether the tryptophans are just necessary to anchor the protein to an activating interface or whether that interaction with the membrane alters the conformation of the protein to enhance its catalytic ability.

The only helix B tryptophan rescue mutant of W47A that showed recovery of PI binding affinity and enhanced specific activity toward PI solubilized in diC$_7$PC was I43W/W47A. Ile-43 has the same orientation on the helix as Trp-47 but is displaced by one turn to the N-terminal end of the helix (Fig. 2A). The enhanced PC binding by I43W/W47A (compared with W47A) indicates that a tryptophan at this position can partially penetrate into the interface. However, more than just membrane penetration must be involved since other helix B double mutants (e.g. G48W/W47A) exhibit even tighter PC binding but do not recover the enzymatic activation by PC interfaces that is lost by the removal of Trp-47. Clearly, the orientation of this bulky side chain on the short helix is coupled to PC activation of the enzyme. The requirement of a tryptophan for interaction of PI with PI-PLC is even more constrained. None of the helix B mutants shows high rates of PI cleavage when it is monomeric. Several of these enzymes are also activated by nonsubstrate interfaces (9, 30–32), in some cases suggesting that binding to the interface shifts the enzyme conformation to an activated form. In this way, the interface acts as a cofactor or positive effector of the reaction. Obtaining a molecular picture of the interfacial site and how this may affect enzyme conformation and catalysis is difficult. Binding of an enzyme to an interface likely has both hydrophobic and electrostatic components (32, 33). For many peripheral proteins, tryptophan penetration into the membrane is an important component of interfacial binding to PC surfaces (34). Furthermore, crystal structures of integral membrane proteins show that the bulky side chains of tryptophan tend to partition to PC activation surfaces (34).

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The interfacial binding surface of B. cereus PI-PLC was suggested to be a hydrophobic ridge composed of a short $\alpha$-helix B and a loop between strand III and helix G composed of residues 232–244 (4). Both regions had reduced density in the crystal structure suggestive of multiple conformations or mobility. Flexibility in this area of the protein may be important for contacting an interface. Residues suggested to interact with an interface were Pro-42, Ile-43, Val-46, Trp-47, Ala-241, Trp-242, and Pro-245 (1, 4). In addition to these hydrophobic residues, there are three lysines (Lys-44, Lys-122, and Lys-201) that could interact with the negatively charged phosphate in phospholipids. We have shown previously (16) that PI-PLC requires two tryptophans, Trp-47 and Trp-242, in the barrel rim for tight binding to PC interfaces and for optimal PI cleavage activity. Removal of either reduces both phosphotransferase and phosphodiesterase activities as well as membrane binding. An unanswered question is whether the tryptophans are just necessary to anchor the protein to an activating interface or whether that interaction with the membrane alters the conformation of the protein to enhance its catalytic ability.

The only helix B tryptophan rescue mutant of W47A that showed recovery of PI binding affinity and enhanced specific activity toward PI solubilized in diC$_7$PC was I43W/W47A. Ile-43 has the same orientation on the helix as Trp-47 but is displaced by one turn to the N-terminal end of the helix (Fig. 2A). The enhanced PC binding by I43W/W47A (compared with W47A) indicates that a tryptophan at this position can partially penetrate into the interface. However, more than just membrane penetration must be involved since other helix B double mutants (e.g. G48W/W47A) exhibit even tighter PC binding but do not recover the enzymatic activation by PC interfaces that is lost by the removal of Trp-47. Clearly, the orientation of this bulky side chain on the short helix is coupled to PC activation of the enzyme. The requirement of a tryptophan for interaction of PI with PI-PLC is even more constrained. None of the helix B mutants shows high rates of PI cleavage when it is monomeric. Several of these enzymes are also activated by nonsubstrate interfaces (9, 30–32), in some cases suggesting that binding to the interface shifts the enzyme conformation to an activated form. In this way, the interface acts as a cofactor or positive effector of the reaction. Obtaining a molecular picture of the interfacial site and how this may affect enzyme conformation and catalysis is difficult. Binding of an enzyme to an interface likely has both hydrophobic and electrostatic components (32, 33). For many peripheral proteins, tryptophan penetration into the membrane is an important component of interfacial binding to PC surfaces (34). Furthermore, crystal structures of integral membrane proteins show that the bulky side chains of tryptophan tend to partition to PC activation surfaces (34).
idues would cover a wide range of orientations if the loop adopts the same conformation as in the crystal structure (Fig. 2B). However, if this loop is reasonably flexible, insertion of a tryptophan at all of these positions might restore PI cleavage in the W242A mutant to WT values. In fact, all the loop mutants examined showed increased PI cleavage (compared with W242A) with both PI dispersed in dC,PC as well as 30% iPrOH compared with W242A. iPr,PC activation of dIP hydrolysis compared with W242A was also enhanced in most loop mutants. Because Trp-47 is present, as long as there is a tryptophan in the loop, activities in 30% iPrOH are higher than for W242A. The observation that activity toward PI in iPrOH was higher for all these loop mutants compared with helix B mutants confirms that the position and orientation of Trp-47 are very important for water-miscible solvent activation of PI-PLC. These differences in iPrOH activation imply that water-miscible solvent induces a conformational change of the protein and that Trp-47 is vital for this change. As long as the rim loop has a tryptophan, the enzyme shows high activity toward PI dispersed in iPrOH. PC activation requires a tryptophan with the right orientation on helix B (i.e. in position 43 or 47), whereas several positions in the rim loop can contribute to PC activation toward both dIP and PI cleavage. A tryptophan at residue 238 is particularly advantageous for PC activation of the enzyme. The phosphotransferase activities of both G238W and G238W/W242A toward PI dispersed in dC,PC were 60% higher than that of WT PI-PLC. These mutants have $K_D$ values for PC slightly lower than WT. G238W does not bind to PC as tightly as would be expected if both loop tryptophans were independently inserted into the surface. There are several possible explanations for this result: (i) a single tryptophan in the 232–244 loop is involved in interfacial PI-PLC activity and binding, but several regions of the loop can supply the single tryptophan; (ii) the lower $K_D$ in G238W maybe due to partial insertion of both loop tryptophan side chains, whereas in G238W/W242A the tryptophan at position 238 is fully inserted; or (iii) insertion of both tryptophan side chains occurs, but the change in free energy of binding is reduced because of ring stacking between Trp-238 and Trp-242 if the structural mimicry is present (12). The rim of the mammalian barrel, like the bacterial enzyme, has a hydrophobic ridge with residues Leu-320, Tyr-358, and Phe-360 in the comparable loop region and Trp-555 in Tβ7 analogous to Trp-47 in the bacterial enzyme. Katan and co-workers (15) have already shown that W555A exhibits reduced catalytic activity. If the structural similarity to B. thuringiensis PI-PLC holds, that mutant may also have impaired PC vesicle binding and PC (or phosphatidylinositol 4,5-bisphosphate) activation of dIP hydrolysis similar to the B. thuringiensis mutant.

Predictions for Other PI-PLC Enzymes—The interfacial binding interactions found in B. thuringiensis PI-PLC may also occur in other bacterial as well as mammalian enzymes. Two systems, in particular, may exhibit a similar link between tryptophan orientation and optimal kinetics. There is significant topological similarity of L. monocytogenes PI-PLC to the B. thuringiensis protein (35). However, the Listeria protein is much more basic and would be predicted to have a much higher affinity for anionic phospholipid surfaces. The L. monocytogenes PI-PLC, with only three tryptophans in the mature protein, has residues Trp-56 and Lys-60 in helix B and Phe-242 in a rim loop occupying positions analogous to Trp-47, Lys-44, and Trp-242 in the B. thuringiensis enzyme. The rim loop in L. monocytogenes PI-PLC is three residues shorter than that in B. thuringiensis PI-PLC. This may constrain Phe-244 and may enforce stricter positional requirements for an aromatic side chain in the rim loop that responds to PC and iPrOH activation. Assuming binding to a PC surface is similar for both bacterial proteins, one would predict the following: (i) this enzyme is activated by interfaces in a fashion similar to the B. thuringiensis enzyme; (ii) the orientation and position of

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