Role of Tryptophan Residues in Interfacial Binding of Phosphatidylinositol-specific Phospholipase C*

Received for publication, January 29, 2002, and in revised form, March 18, 2002
Published, JBC Papers in Press, March 23, 2002, DOI 10.1074/jbc.M200938200

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The phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus thuringiensis exhibits several types of interfacial activation. In the crystal structure of the closely related Bacillus cereus PI-PLC, the rim of the active site is flanked by a short helix B and a loop that show an unusual clustering of hydrophobic amino acids. Two of the seven tryptophans in PI-PLC are among the exposed residues. To test the importance of these residues in substrate and activator binding, we prepared several mutants of Trp-47 (in helix B) and Trp-242 (in the loop). Two other tryptophans, Trp-178 and Trp-280, which are not near the rim, were mutated as controls. Kinetic (both phosphotransferase and cyclic phosphodiesterase activities), fluorescence, and vesicle binding analyses showed that both Trp-47 and Trp-242 residues are important for the enzyme to bind to interfaces, both activating zwitterionic and substrate anionic surfaces. Partitioning of the enzyme to vesicles is decreased more than 10-fold for either W47A or W242A, and removal of both tryptophans (W47A/W242A) yields enzyme with virtually no affinity for phospholipid surfaces. Replacement of either tryptophan with phenylalanine or isoleucine has moderate effects on enzyme affinity for surfaces but yields a fully active enzyme. These results are used to describe how the enzyme is activated by interfaces.

Phosphatidylinositol-specific phospholipase C (PI-PLC)

* This work was supported by National Institutes of Health Grant GM 60418 (to M. F. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PI-PLC, phosphatidylinositol-specific phospholipase C; cIP, D-inositol 1,2-cyclic-phosphate; I1P, D-inositol 1-phosphate; CMC, critical micelle concentration; PC, phosphatidylcholine; diC, PC, 1,2-diacylglycerolphosphatidylcholine with n carbons in each acyl chain; POPC, 1-palmitoyl-2-oleoylglycerol-3-phosphocholine; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; CD, circular dichroism; TX-100, Triton X-100; WT, wild type PI-PLC; PA, phosphatidic acid; PMe, phosphatidylmethanol; PG, phosphatidylglycerol; PrOH, isopropanol.
PI-PLC Tryptophan Mutants and Surface Binding

sites. Modification of this residue should play little role in substrate and activator binding.

Analyses of these mutant PI-PLCs show that the two tryptophan residues Trp-47 and Trp-242 are critical for the enzyme to bind to zwitterionic activating interfaces. Trp-242 is also the major fluorophore responding to micelle binding.

MATERIALS AND METHODS

Chemicals—POPC, diC6PC, diC7PC, and PI were purchased from Avanti; crude PI for preparing cIP was purchased from Sigma Chemical Co. diC6PA was prepared from the corresponding short-chain PCs using Streptomyces chromofuscus phospholipase D (13). cIP was prepared from PI as described previously (8). myo-Inositol was purchased from Sigma.

Overexpression of Bacterial PI-PLC and Construction of Mutants—A plasmid containing the B. thuringiensis PI-PLC gene obtained from Dr. Ming-Daw Tsai (Ohio State University) was transformed into Escherichia coli BL21 cells (BL21 CodonPlus(DE3)-RII, from Stratagene). Overexpression of the recombinant protein was induced by addition of isopropyl-thio-β-galactopyranoside (0.8 mM) to the E. coli grown at 37 °C to an A600 of 0.7 in LB medium, pH 7.0, containing ampicillin and chloramphenicol. Continued incubation until A600 reached 1.2 (2–3 h) yielded a reasonable amount of PI-PLC protein in the cytoplasm. After centrifugation, the cell pellet was lysed by sonication, and the solution was centrifuged again. The supernatant was subjected to two chromatographic steps, a Q-Sepharose fast flow column followed by a phenyl-Sepharose column, to purify the PI-PLC. Millipore Centraplus 10 filters were used to concentrate the protein.

All of the mutations of the PI-PLC gene were carried out by QuikChange methodology (15, 16) using a site-directed mutagenesis kit from Stratagene. Two complimentary mutagenic primers (all purchased from Operon) containing the desired mutation (codon indicated in bold) were used to anneal the sequence on opposite strands of the plasmid. The 33 base mutagenic primers CCGATTAAGCAAGTGCCCATCACTTAT (for W178A) and CTTCTGGTGCAATGGGATCTCTGGC (for W280A), respectively. All primers were purified by high performance liquid chromatography before mutagenesis.

CD Spectroscopy—WT and mutant secondary structure and thermal stability as monitored by the thermal denaturation transition (Tm) were measured by CD spectroscopy using an AVIV 202 spectrophotometer. Proteins were dialyzed in 10 mM borate buffer, pH 5.0. For Tm measurements, protein (0.03–0.04 mg/ml) was incubated in a 1-cm cell, and the wavelength at 222 nm was monitored as the temperature was increased from 25 to 75 °C in 1 °C steps with an equilibration time of 1 or 2 min. For comparing secondary structure, wavelength scans (180–300 nm) were carried out at 25 °C with protein (0.2–0.3 mg/ml) in a 0.1-cm cell. Estimation of secondary structure content was done with CDNN using ellipticity in the 195- to 300-nm range (17–19).

Fluorescence Spectroscopy—PI-PLC intrinsic fluorescence spectra were obtained with a Shimadzu RF5000U spectrofluorometer. All fluorescence measurements were carried out at 25 °C with ~2 μM protein in 50 mM HEPES, pH 7.5, with 1 mM EDTA. The excitation wavelength was 290 nm, with both excitation and emission slit widths set at 5 nm. The wavelength for the maximum in PI-PLC fluorescence was the same, 337 nm, for WT and all the tryptophan mutants. Changes in the fluorescence intensity were expressed as (I - I0)/I0, where I0 is the intensity of protein alone, and I is the intensity in the presence of an additive.

Kinetic Analysis of PI-PLC Mutants—PI-PLC activity assay was carried by two methods. (i) For long-chain PI as substrate, 200-μl aliquots were removed from the reaction mixture at defined intervals and extracted with 300 μl of CHCl3 (this also stops the reaction). The content of cIP and 1P in the water-soluble phase was determined by 31P NMR (202.3 MHz) spectroscopy as described previously (8, 13) using a Varian INOVA spectrometer. (ii) For cIP, hydrolysis was monitored by 31P NMR spectroscopy by measuring the decrease of substrate and increase in product (1P) resonance intensities as a function of incubation time with the enzyme. The amount of protein added was adjusted so that no more than 20% substrate hydrolysis occurred within 2 h. Assays to check for activation by PC typically used 5 mM cIP in the absence or presence of 5 mM diC6:PC to probe for PC activation.

PC Vesicle Binding Studies—SDS-PAGE (12% polyacrylamide) was used to quantify free PI-PLC separated (via centrifugation/filtration) from PI-PLC bound to POPC vesicles. A similar method was used to quantify S. chromofuscus phospholipase D partitioning to phospholipid vesicles (20). A stock of small unilamellar POPC vesicles (5 mM) with an average diameter ~300 Å was prepared by sonication in 10 mM Tris, pH 7.5. Large unilamellar vesicles were prepared by multiple passages of an aqueous POPC solution through polycarbonate membranes (100-nm pore diameter). Samples for binding assays with SUVs were prepared with 0.03 mg/ml protein in 10 mM Tris, pH 7.5; the bulk POPC concentration was 0, 0.01, 0.02, 0.05, 0.1, and 0.2 mM for WT PI-PLC, W47A, W242A, W47F, and W242F, respectively. All primers were purified by high performance liquid chromatography before mutagenesis. Analyses of these mutant PI-PLCs show that the two tryptophan residues Trp-47 and Trp-242 are about 10 Å apart.

RESULTS

Secondary Structure and Thermal Stability of Wild Type and Mutant PI-PLCs—The crystal structure of bacterial PI-PLC enzyme shows a single distorted (βα)8-barrel domain with the active site located at the C-terminal side (as determined by strand orientation) of the β-barrel (Fig. 1). Replacement of tryptophan residues at the mouth of the barrel is unlikely to
have a dramatic effect on secondary structure as long as the mutant proteins fold correctly. To check this, CD spectra of WT and mutant PI-PLC proteins were acquired and used to check for overall structural elements. WT and mutant thermostabilities were also measured by monitoring the loss of secondary structure with temperature. As shown in Table I, estimates of WT secondary structure calculated from the CD wavelength spectra by CDNN (17–19) agreed moderately well with the secondary structure elements in the crystal structure (9). All the tryptophan mutants except W178A had essentially the same proportion of secondary structure elements. They also had very similar thermal denaturation temperatures (Table I). Thus, any changes in PC binding and kinetics are unlikely to be due to protein that has a significantly altered structure from WT.

W178A was the least stable mutant ($T_m$ decreased from 54 °C for the WT to 41.5 °C), and it also showed a significant drop in β-sheet and increase in random coil. The side chain of Trp-178, at the bottom of active site, is critical for hydrogen bonding and hydrophobic interactions that stabilize the barrel and active site. Loss of these interactions would be expected to modify secondary structure, destabilize PI-PLC, and possibly reduce the mutant enzyme activity. The only other mutant with a significantly reduced stability was W280A. Trp-280 is positioned at a relatively unstructured region near the C terminus of the protein. Given its position, replacement of the aromatic side chain might not be expected to alter secondary structure elements as reflected in Table I. However, the stability of this mutant is decreased compared with WT, perhaps suggesting that packing of this tryptophan residue does contribute to stabilization of the structure.

Replacement of either tryptophan at the barrel rim had minor effects on the protein stability. The $T_m$ of W47A was within the error of the $T_m$ for WT PI-PLC. A large change might not be expected, because Trp-47 is at the middle of helix B. However, the $T_m$ of W242A was increased to 56.2 °C. Replacement of the bulky side chain of the tryptophan in the loop at the opening of the barrel slightly stabilized the protein. This might correlate with a small increase in α-helix (at the expense of random loop structure). It might also reflect a reduced hydrophobicity of the loop that affects its tertiary structure and dynamics; it is this change that leads to a small stabilization of the protein.

Effect of diC6PC and diC7PC on the Intrinsic Fluorescence of Mutant PI-PLC Enzymes—PI-PLC has seven tryptophan residues that contribute to its fluorescence emission spectrum. Previous studies have shown that the binding of PC activator micelles (12, 21) or vesicles (6, 12) causes an increase in PI-PLC intrinsic fluorescence intensity, whereas molecules that bind to the active site (PA, PME, PG) cause a decrease in fluorescence (12, 13). Mutants where a specific tryptophan has been replaced by alanine should lose some or all of this sensitivity if, indeed, that particular tryptophan were responsible for much of the spectral change upon interfacial binding. W47A, W242A, W178A, W280A, and W47A/W242A were examined as a function of added diC6PC and diC7PC and compared with WT PI-PLC. The emission maximum, 337 nm, was the same for all unliganded proteins and unshifted by the addition of PC micelles (up to 35 mM diC6PC or 4.0 mM diC7PC). As shown in Fig. 2A, the change in fluorescence intensity of WT at 337 nm showed a small increase below the diC6PC CMC (14 mM), then increased dramatically once micelles were formed and bound to the WT protein. Three of the tryptophan mutants, W47A, W178A, and W280A, exhibited similar behavior (Fig. 2). However, W242A showed significantly reduced sensitivity to diC6PC micelle binding. The fluorescence intensity still increased slightly with micelle formation but the enhancement of fluorescence was roughly one-third that of WT. The double mutant, missing the tryptophan in helix B and the 237–243

### Table I

| Enzyme           | α-Helix | β-Sheet | β-Turn | Random coil |
|------------------|---------|---------|--------|-------------|
| WT               | 22.1    | 39.8    | 19.8   | 18.3        |
| W47A             | 21.9    | 40.2    | 19.9   | 18          |
| W242A            | 23.3    | 39.4    | 19.5   | 17.8        |
| W47A/W242A       | 21.8    | 40.2    | 19.8   | 18.2        |
| W178A            | 22.6    | 37.2    | 19.9   | 20.3        |
| W280A            | 22.8    | 39.1    | 19.6   | 15.5        |
| W47A/W242A       | 21.8    | 40.2    | 19.8   | 18.2        |

$^a$ Estimated using the program CDNN and data for the wavelength range 195–300 nm.

$^b$ The $T_m$ was extrapolated from the loss of negative ellipticity at 222 nm with temperature; $T_m$ is the maximum in the derivative of the temperature dependence of this signal. The error in each $T_m$ is less than 0.4 °C.
loop, showed no change in intrinsic fluorescence upon micelle binding (Fig. 2B).

The effect of diC7PC micelles on PI-PLC intrinsic fluorescence was similar to that for diC6PC (increase in fluorescence around the diC7PC CMC of 1.5 mM) with the exception that now W47A as well as W242A also showed a significantly lower fractional increase in fluorescence (Fig. 3). However, the fractional increase in fluorescence upon micelle binding was still the least for W242A. Again, the double mutant displayed no sensitivity to PC micelle binding.

Effect of diC6PA and myo-Inositol on the Intrinsic Fluorescence of Mutant PI-PLC Enzymes—Micellar diC6PA inhibits PI-PLC hydrolysis of cIP whereas monomeric diC6PA can partially activate the enzyme (13). Binding of monomeric diC6PA caused a decrease in WT PI-PLC fluorescence that correlates with binding of this molecule to the active site; as PA micelles formed, the intrinsic fluorescence increased. As shown in Fig. 4, low concentrations of diC6PA decreased PI-PLC fluorescence for all the PI-PLC mutants indicating active site binding of this molecule is not abolished by the tryptophan substitutions. The decrease in fluorescence was the least for W242A and the double mutant. Upon micelle formation of diC6PA (the CMC depends on the ionic strength and pH of the medium and is likely to be 5–7 mM under these buffer conditions (22), all proteins except W242A and the double mutant showed large increases in fluorescence consistent with micelle binding as well as active site binding of the PA molecule. This could suggest that Trp-242 not only senses micelle binding but contributes to the decrease in fluorescence as lipids bind to the active site.

myo-Inositol is a poor competitive inhibitor of PI-PLC (23). Because it is not amphiphilic it has no tendency to form interfaces. Like other molecules that bind to the PI-PLC active site, myo-inositol caused a decrease in the fluorescence intensity of WT PI-PLC (Fig. 5). Tryptophan mutants that required more myo-inositol added to exhibit a decreased fluorescence also exhibited a reduced sensitivity to short-chain PC micelles. That the fractional decrease in fluorescence for W47A, W242A, and the double mutant was less than that observed with WT PI-PLC, might suggest that the interfacial site is coupled to substrate binding. Interestingly, the double mutant showed a more pronounced decrease in fluorescence with increasing myo-inositol than both W47A and W242A. If the decreased fluorescence is correlated with binding, this would indicate that removal of both tryptophan residues may actually enhance the binding of small water-soluble inositol compounds at the active site. However, the altered changes in fluorescence with myo-inositol could also reflect changes in the disposition of the fluorophores or removal of ones that are the major reporters of this small molecule binding to the active site.

Catalytic Properties of Alanine-substituted PI-PLCs: Effect of PC on cIP and PI Cleavage—To investigate the role, if any, of the different tryptophans in the interfacial activation of PI-PLC, cIP hydrolysis (phosphodiesterase) and PI cleavage (phosphotransferase) were examined for WT and mutant proteins with and without PC activator (diC7PC). As summarized in Table II, removing either Trp-47 or Trp-242, which reside at the rim of the barrel, reduced the PI-PLC specific activity toward cIP to 60 and 82% of WT, respectively. Both single mutants exhibited kinetic activation by micellar diC7PC (5 mM), but not to the same extent as WT (Table II). The reduction in the extent of PC activation is real was provided by measuring diC7PC activation of W280A, which was essentially the same as WT. Trp-280 is near the C-terminal end of the

![Fig. 3. Fluorescence intensities of B. thuringiensis PI-PLC proteins at 337 nm as a function of added diC7PC. A, WT (○), W47A (■), and W242A (▲); B, W47A/W242A (◇), W178A (□), and W280A (○). The arrow indicates the CMC of pure diC7PC.](http://www.jbc.org/)

![Fig. 4. Fluorescence intensities of B. thuringiensis PI-PLC proteins at 337 nm as a function of added diC6PA. WT (○), W47A (■), W242A (▲), W47A/W242A (◇), W178A (□), and W280A (○).](http://www.jbc.org/)
The lack of change in the intrinsic fluorescence when diC7PC was titrated into the double mutant. There was no fluorescence decrease observed with diC7PC added, this mutant showed only a 1.5-fold increase. The lack of kinetic activation of W47A/W242A explains that both Trp-47 and Trp-242 have been removed, toward cIP was just changed the interfacial binding character, not the active site inositol binding region.

An alternate means of activating PI-PLC is to have moderate percentages of water-miscible organic solvents present (24). It has been proposed that a co-solvent (e.g. 30% iPrOH) activates PI-PLC by changing the local polarity of the active site. 30% iPrOH activated WT and all the mutants examined (W47A, W242A, and W47A/W242A) for hydrolysis of cIP. The extent of iPrOH activation (comparing the specific activity with 30% iPrOH to cIP hydrolysis by that mutant in its absence) was ~4.5 for the single mutants versus 5.5 for WT. However, removal of both Trp-47 and Trp-242 generated PI-PLC that could only be activated 2.6-fold by that organic solvent (Table II).

Similar phenomena were observed comparing cleavage of 8 mM PI in 16 mM TX-100 or 32 mM diC7PC matrices. PI dispersed in diC7PC micelles is a better substrate than in TX-100 micelles (7). Interpretation of the kinetics is more complex, because both the activating phospholipid and the substrate have alyl chains. However, one gets a sense of whether tryptophan removal affects PI cleavage as well as PC activation of the first step of PI-PLC action. As shown in Fig. 6, removal of either rim tryptophan led to a 3-fold decrease in PI-PLC specific activity toward PI in both detergent matrices. Even though these mutants could be activated for cIP hydrolysis, cleavage of PI to cIP was reduced to the extent that activated rates for cIP hydrolysis and PI cleavage were closer. Removal of both tryptophans enabled enzyme to further reduce activity toward PI and was not activated by 30% iPrOH. In fact, the activity for PI dispersed in 30% iPrOH was considerably lower than PI in TX-100 suggesting that iPrOH was inhibitor for the double mutant. Therefore, the two rim tryptophan residues are critical for optimal PI cleavage as well as cIP hydrolysis.
This is consistent with the following scenario. The inositol headgroup of PI binds at the active site, while the acyl chains are oriented with hydrophobic side chains of the rim to enhance the PI binding with protein.

Phenylalanine and Isoleucine Substitutions at Trp-47 and Trp-242—Replacement of the bulky tryptophan side chain with a methyl group is a rather drastic change. It is possible that the roles of these residues in interfacial activation and amphiphilic substrate binding are not specific for tryptophan but just require a hydrophobic side chain. Isoleucine and phenylalanine residues are hydrophobic and much bigger than alanine. When compared with tryptophan using the Wimley-White hydrophobicity scale (25), phenylalanine is closer to tryptophan in partitioning whereas isoleucine is significantly less hydrophobic. Construction of W47I, W47F, W242I, and W242F was carried out, and fluorescence and kinetic analyses were done to address this point. The specific activities of W47I, W47F, W242I, and W242F toward PI dispersed in diC6PC were comparable to WT enzyme (Table III), although W57I was about 25% lower and the two Trp-242 mutants were 25% higher. With the alanine substitutions, PI specific activity was decreased substantially when the rim tryptophans were removed. Comparable PI cleavage is consistent with intact structural features for interfacial activation. That both isoleucine and phenylalanine mutants behave like WT is consistent with relatively nonspecific hydrophobic interactions contributing to interfacial activation.

Because these mutants exhibit PI specific activity comparable to WT, they can be used as a better assessment of the role of each tryptophan in contributing to the intrinsic fluorescence response to micelle binding. As shown in Fig. 7A, W47I and W47F intrinsic fluorescence show a dependence on diC6PC concentration similar in shape to WT but with about half the fluorescence increase; W242F and, in particular, W242I have greatly reduced changes upon diC6PC micelle binding. The same trends are observed when the enzymes were titrated with diC6PC micelles. With the larger diC7PC micelles, there were essentially no significant increases in the intrinsic fluorescence of W242I and W242F.

Binding of Mutant PI-PLC to PC Vesicles—The loss of diC6PC activation by the W47A/W242A double mutant strongly suggests that removal of both tryptophan residues altered the affinity of this mutant for activating surfaces. This possibility was investigated by measuring the partitioning of WT and mutant PI-PLC enzymes to PC SUVs using a centrifugation/filtration assay. SUVs were initially used because WT PI-PLC showed much higher activity toward small versus large vesicles (7, 21). A typical gel showing the loss of free W47F PI-PLC as the bulk concentration of PC increased is shown in Fig. 8A. The variation in the amount of PI-PLC partitioned to PC SUVs was fit with a hyperbolic curve to generate an apparent dissociation constant, $K_D$ (Table IV). This is not a true dissociation constant but an apparent $K_D$ that provides a way of comparing affinities of the different PI-PLCs for PC surfaces.

The apparent $K_D$ of WT PI-PLC for PC SUVs was 88 µM. For comparison, binding of PI-PLC to PC LUVs was considerably weaker (apparent $K_D > 1$ mM). Therefore, the effect of removing individual tryptophan residues on binding to PC vesicles was measured with SUVs. Apparent $K_D$ values extrapolated for W47A (3.2 mM) and W242A (8.6 mM) were much higher than for WT protein. The apparent $K_D$ values of W47I and W242F for PC SUVs were higher than WT, but both were lower than the alanine mutants. W47F (binding data shown in Fig. 7B) had an apparent $K_D$ comparable to the WT protein (86 µM). No significant binding of W47A/W242A to POPC SUVs could be detected with this assay even when the concentration of PC was increased to 2 mM. This puts a lower limit on the apparent $K_D$ of the double mutant of >20 mM for PC surfaces. Both Trp-242 and Trp-47 have a huge role in interfacial binding of protein. Conversion of these two residues to alanine removes the interfacial binding of PI-PLC. Replacement of tryptophan with the aromatic phenylalanine side chain preserved the affinity of the enzyme for PC surfaces. The binding studies are consistent with the kinetic results: Hydrophobic interactions of residues Trp-47 and Trp-242 are critical for interfacial activation.
the bilayer as well as in stabilizing transmembrane helices (26). For many peripheral proteins, tryptophan residues are also critical for interactions of the protein with the bilayer. In the case of lipoprotein lase, a tryptophan cluster in a C-terminal loop contributes to binding of the lipase to lipid/water interfaces (27). Similarly, Naja naja atra phospholipase $A_2$ has two tryptophans (Trp-19 and Trp-61) located in the membrane-water interface that are important for this phospholipase binding to phospholipid interfaces (11). These residues appear to penetrate into the membrane during the interfacial catalysis of PLA$_2$. However, for PLA$_2$ enzymes, electrostatic interactions as well as tryptophan insertion are critical for membrane binding and interfacial activation (28).

Considerably less is known about what drives the interfacial activation of PI-PLC enzymes. B. thuringiensis PI-PLC has seven tryptophan residues, two of them at the hydrophobic rim of the active site in helix B and a loop (residues 237–242), another at the bottom of the active site (Trp-178), two near the C-terminal end of the protein (Trp-270 and Trp-280) and two close to the N-terminal portion of the protein (Trp-10 and Trp-13). The two near the mouth of the $\beta$-barrel are of particular interest, because they would be near the path for substrates to enter the active site (Fig. 1). Replacement of either or both of these tryptophans with alanine (Trp-47, Trp-242, and W47A/W242A) or both (W47A/W242A) had only modest effects on activity (at most a 2-fold decrease in specific activity) of PI-PLC toward its monomeric substrate cIP. However, there was a much more significant reduction in PC activation for two of these mutations, W47A and W242A, that was accentuated with the W47A/W242A double mutant. The loss of PC activation by the double mutant did not correlate with altered secondary structure of the protein. A plausible explanation is that both aromatic side chains are directly involved in binding to a phospholipid interface.

Binding studies of the mutants to PC vesicles showed that replacement of either tryptophan with alanine decreased the affinity of the enzyme for an activating PC bilayer 37- and 98-fold (W47A and W242A, respectively). The Wimley-White hydrophobicity scale (25) can be used to predict changes in membrane partitioning of a series of related peptides. For a mutant where a single membrane interacting tryptophan is replaced by alanine ($\Delta G = 2.02 \text{ kcal/mol}$), the ratio of the mutant $K_D$ to that of WT would be about 30. Removal of both tryptophans would increase $K_D$ ~900-fold. The relative experimental $K_D$ values for Trp-47 and Trp-242 mutants compared with WT show the same trends as these predictions (Table IV).

Interestingly, substitutions at Trp-47 had smaller effects than at Trp-242. There could be several explanations for this. Trp-47 is in a short helix and has a defined structure, whereas Trp-242 is in a more flexible loop. The Wimley-White scale is based on short peptides that have no structure either free in solution or partitioned to a PC membrane. Differences in mutants at the two positions could reflect differences for a residue in a helix versus a less structured loop partitioning into the surface. Alternatively, Trp-47 may not completely partition into the PC surface while Trp-242 is completely imbedded. The observation that Trp-242 contributes the most to the increase in fluorescence upon PI-PLC binding to PC surfaces is consistent with this explanation. Yet a third possibility is that the rim loop with Trp-242 undergoes a conformational or dynamical change upon insertion into the PC surface whose extent depends on the identity of the hydrophobic side chain. Regardless of the detailed explanation, both helix B and loop tryptophans play critical roles in B. thuringiensis PI-PLC binding to PC interfaces.

Differences in binding affinity of the WT and mutant PI-PLC

\begin{table}[h]
\centering
\caption{Apparent dissociation constants for PI-PLC binding to POPC SUVs}
\begin{tabular}{lccc}
\hline
Enzyme & $K_D$ & $K_D$ (mutant)/$K_D$ (WT) \\
\hline
WT & 0.088 & 1.0 & 1.0 \\
W47A & 3.23 & 3.0 & 13 \\
W47F & 0.084 & 1.0 & 3.3 \\
W242A & 8.60 & 98 & 30 \\
W242I & 0.540 & 6.1 & 13 \\
W47A/W242A & 0.540 & 6.1 & 13 \\
\hline
\end{tabular}
\flushleft
\footnotesize{\textsuperscript{a} This is the total concentration of PC present for half saturation of binding ($1/K_D$ is equivalent to the partition coefficient for PI-PLC on PC surfaces). Errors in estimating apparent $K_D$ values (determined from the hyperbolic fit to the data) were typically $<20\%$.} \\
\footnotesize{\textsuperscript{b} The ratio of $K_D$ for PI-PLC mutants binding to PC compared to $K_D$ for WT protein was estimated using the changes in free energy for the side chains derived in the experimental scale of Wimley and White (25).}
\end{table}
are reflected in catalytic activity of PI-PLC toward cIP. Under normal assay conditions micellar diC\textsubscript{6}PC is used as the activator, and the partitioning of the enzyme for this interface may be characterized by slightly different apparent \( K_d \) values. However, PC micelle binding trends should be similar to those exhibited with PC SUVs. With 5 mM diC\textsubscript{6}PC there should still be sufficient PC binding for a good deal of the kinetic activation but not as much as WT, which is indeed what is observed. The double mutant showed no partitioning to PC vesicles and only a very small increase in specific activity toward cIP when an activating interface was present. Thus, the binding of PI-PLC to an activating PC interface is mainly controlled by both Trp-47 and Trp-242 residues. However, the specificity for tryptophan at those positions is not absolute. Other hydrophobic groups (Ile or Phe) can substitute for tryptophan at these positions with the aromatic side chain generating protein whose activity is essentially equivalent to WT.

**Tryptophans and Interfacial Binding in cIP and PI Cleavage**—Both Trp-47 and Trp-242 clearly have roles in PC activation of PI-PLC hydrolysis of water-soluble cIP. Because they also affect PI cleavage to cIP, they are important for orienting the enzyme at any interface, either activating interfaces such as PC or a substrate interface (PI). Removal of both rim tryptophans produces an enzyme that is less sensitive to both activating and substrate interfaces. Curiously, the double mutant cleavage of PI was inhibited by 30% iPrOH. Under conditions where all the single mutants showed substantial activity toward PI in this cosolvent, the double mutant was inhibited by iPrOH. This is different from what was observed for cIP, where iPrOH enhanced hydrolysis by W47A/W242A 2.5-fold compared with the 4.5-fold for the single mutants (Table I). The observed inhibition could reflect dramatically altered thermostability in the presence of iPrOH, although the \( T_m \) for the double mutant was the same as that of WT. Reduced sensitivity to iPrOH paralleling reduced PC activation might suggest that, when the cosolvent changes the local polarity of the active site and rim, there is a change in the orientation of one or both tryptophans that is critical for optimal substrate binding and processing.

**Intrinsic Fluorescence of PI-PLC: Do Trp-47 or Trp-242 Penetrate Interfaces?**—The insertion of tryptophan residues into interfacial regions of membranes can often be detected by changes in the fluorescence. For example, E. coli \( \alpha \)-hemolysin reversibly adsorbs to bilayers then inserts into the bilayer to form a tightly associated complex. Insertion not the reversible adsorption is accompanied by an increase in protein intrinsic fluorescence (29). Furthermore, chemical modification of any of the solvent-exposed tryptophans abolishes lytic activity of the \( \alpha \)-hemolysin (29). A number of other toxins show tryptophan insertion into membranes as key steps in their action (e.g. diphtheria toxin (30) and perfringolysin O (31)).

PI-PLC has seven tryptophan residues that dominate its fluorescence emission at 337 nm. Removal of Trp-242 generated PI-PLC that was the least sensitive to the presence of PC micelles, both diC\textsubscript{6}PC and diC\textsubscript{7}PC, suggesting that in WT this residue is responsible for most of the increase in fluorescence upon micelle binding. Removal of Trp-47 also generated an enzyme that had a somewhat reduced sensitivity to binding to diC\textsubscript{6}PC interfaces (small changes for W47A and more substantial for W47F and W47I). All of these tryptophan single mutants exhibited further reduced fluorescence upon binding to diC\textsubscript{6}PC compared with diC\textsubscript{7}PC micelles. diC\textsubscript{6}PC micelles are small and nearly spherical (32), whereas diC\textsubscript{7}PC forms much larger rod-like micelles (33). The different sensitivity of Trp-242 and Trp-47 to PC interfaces may arise from different orientation of the residues on the membrane surface. It is possible that the side chain of Trp-47 is disposed in a more water-accessible region of the bilayer, while Trp-242 penetrates further into the lipid layer (possibly because of the flexibility of loop region). Such a difference in orientation could be envisioned with a small highly curved micelle oriented in a somewhat asymmetrical fashion at the barrel rim. The larger diC\textsubscript{7}PC micelles would then provide a slightly altered environment for Trp-47 that contributes along with Trp-242 to the increase in fluorescence upon micelle binding. In either case, binding of W242A to a PC bilayer was considerably weaker than W47A consistent with a stronger interaction of Trp-242 with PC surfaces.

Trp-242 also contributes to the protein intrinsic fluorescence changes when myo-inositol or monomeric PA bind to the protein, because its removal leaves a protein that is much less sensitive to active site binding as well as activator micelle binding. However, W178A and W47A also exhibited a reduced change in fluorescence upon inositol binding at the active site. Thus, the fluorescence response to a molecule binding at the active site has contributions from at least three of the tryptophans.

**Comparison of Tryptophans in B. thuringiensis PI-PLC to Other PI-PLC Enzymes**—The crystal structure of PI-PLC from Listeria monocytogenes shows a close topological similarity to PI-PLC from B. thuringiensis, despite a low level of sequence homology (~24% sequence identity) (34). The catalytic site and hydrophobic rim are very similar in both crystal structures. There is one tryptophan residue near the N terminus of helix B in L. monocytogenes PI-PLC (Trp-47 is located at the C terminus of helix B in B. thuringiensis PI-PLC) and a phenylalanine residue in a rim loop that appears to correspond to Trp-242 in B. thuringiensis. Because the B. thuringiensis W242F mutant is active, one might expect the same mode of PC interfacial activation for this bacterial enzyme.

The crystal structures of PI-PLC31 (35–37) show the active site to be in a \( \beta \)-barrel similar to B. thuringiensis PI-PLC. The rim of this barrel, like the bacterial enzyme, has a number of hydrophobic residues (Trp, Leu, and Phe). This hydrophobic ridge surrounds one end of the active site opening and is thought to penetrate the membrane during catalysis. With this protein, the PH domain contributes substantially to membrane binding energy and is linked with substrate processivity. Nonetheless, replacement of the hydrophobic moieties in the ridge do affect enzyme activity. For example, mutagenesis of Trp-555 to alanine decreased the activity from 1080 mol min\(^{-1}\) to ~300 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\). Perhaps, the simpler bacterial PI-PLC may provide a model to quantify how changes in membrane binding (via helix B and the rim loop residues) are linked to catalytic activity: both phosphotransferase and cyclic phosphodiesterase steps.

**Acknowledgment**—We thank Dr. Ming-Daw Tsai, Ohio State University, for the plasmid containing the B. thuringiensis PI-PLC gene.

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J. Biol. Chem. 2002, 277:19867-19875. doi: 10.1074/jbc.M200938200 originally published online March 23, 2002

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