Determination of pK_a values of the histidine side chains of phosphatidylinositol-specific phospholipase C from *Bacillus cereus* by NMR spectroscopy and site-directed mutagenesis

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Abstract

Two active site histidine residues have been implicated in the catalysis of phosphatidylinositol-specific phospholipase C (PI-PLC). In this report, we present the first study of the pK_a values of histidines of a PI-PLC. All six histidines of *Bacillus cereus* PI-PLC were studied by 2D NMR spectroscopy and site-directed mutagenesis. The protein was selectively labeled with $^{13}\text{C}^{\epsilon 1}$ -histidine. A series of ^1H - ^{13}C HSQC NMR spectra were acquired over a pH range of 4.0–9.0. Five of the six histidines have been individually substituted with alanine to aid the resonance assignments in the NMR spectra. Overall, the remaining histidines in the mutants show little chemical shift changes in the ^1H - ^{13}C HSQC spectra, indicating that the alanine substitution has no effect on the tertiary structure of the protein. H32A and H82A mutants are inactive enzymes, while H92A and H61A are fully active, and H81A retains about 15% of the wild-type activity. The active site histidines, His32 and His82, display pK_a values of 7.6 and 6.9, respectively. His92 and His227 exhibit pK_a values of 5.4 and 6.9. His61 and His81 do not titrate over the pH range studied. These values are consistent with the crystal structure data, which shows that His92 and His227 are on the surface of the protein, whereas His61 and His81 are buried. The pK_a value of 6.9 corroborates the hypothesis of His82 acting as a general acid in the catalysis. His32 is essential to enzyme activity, but its putative role as the general base is in question due to its relatively high pK_a .

Keywords: $^{13}\text{C}^{\epsilon 1}$ -histidine; ^1H - ^{13}C HSQC; histidine pK_a ; NMR; phosphatidylinositol-specific phospholipase C; site-directed mutagenesis

Phosphatidylinositol-specific phospholipase C (PI-PLC) is the focus of considerable research because of its role in generating second messengers and modulating membrane traffic in eukaryotic cells (Berridge, 1993; Lee & Rhee, 1995; De Camilli et al., 1996). Moreover, the PI-PLCs from bacteria such as *Bacillus cereus*, *Bacillus thuringiensis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Costridium novyi* possess the activity of cleaving glycosylphosphatidylinositol (GPI), the lipid anchor that attaches many proteins to membranes (for reviews, see Turner, 1990; Englund,

1993). The bacterial PI-PLCs cleave phosphatidylinositol (PI) to form the lipid-soluble diacylglycerol (DAG) and water-soluble *myo*-inositol 1,2-cyclic phosphate [I(1:2cyc)P], which can be converted to acyclic 1-phosphate as shown in Figure 1 (for a review, see Bruzik & Tsai, 1994). The mammalian PI-PLCs hydrolyze phosphatidylinositol 4,5-bisphosphate to yield DAG and the corresponding polyphosphorylated inositol. With the recent completion of crystal structures of *B. cereus* PI-PLC (Heinz et al., 1995, 1996), and the mammalian PI-PLC δ 1 (Essen et al., 1996), more detailed studies of the molecular mechanisms of these enzymes are now possible. The catalytic domain of the mammalian PI-PLC has a similar folding topology to that of *B. cereus* PI-PLC, i.e., an irregular α/β -barrel, also named TIM barrel after the more regular structure of this type first observed in triose phosphate isomerase. There are two highly conserved histidines at the active sites of both bacterial and mammalian PI-PLCs. In the *B. cereus* isozyme, these two histidines are His32 and His82 (Fig. 2). Based on the presence of the stable cyclic product, I(1:2cyc)P, an in-line mechanism of general acid and base catalysis was postulated, analogous to that for ribonuclease A (Lin et al., 1990; Volwerk et al., 1990; Lewis et al., 1993).

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Abbreviations: DAG, diacylglycerol; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt; EDTA, ethylenediaminetetraacetate; GLN-INS, glucosaminyl(α 1 $\rightarrow$ 6)-D-*myo*-inositol; GPI, glycosylphosphatidylinositol; H-bond, hydrogen-bond; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; HSQC, heteronuclear single-quantum coherence; I(1)P, *myo*-inositol 1-phosphate; I(1:2cyc)P, D-*myo*-inositol 1,2-cyclic phosphate; NMR, nuclear magnetic resonance; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; Tris, tris(hydroxymethyl)aminomethane.

Fig. 1. The reactions catalyzed by *B. cereus* PI-PLC. The first reaction is fast and yields diacylglycerol (DAG) and *myo*-inositol 1,2-cyclic phosphate [I(1:2cyc)P]. The second reaction is much slower and converts I(1:2cyc)P to *myo*-inositol 1-phosphate [I(1)P].

To understand the roles of His32 and His82 in catalysis, we have initiated the investigation of the microenvironments of the histidines by NMR spectroscopy. There are a total of six histidine residues (Fig. 2) in *B. cereus* PI-PLC, a 298-residue protein with a molecular weight of 35 kDa. In this report, we present the pK_a determination for all six histidines by 2D NMR spectroscopy aided by site-directed mutagenesis. This is the first study of histidine pK_a values for a member of the PI-PLC family of enzymes.

Results

Resonance assignments for the histidines in the NMR spectra

A ^1H - ^{13}C correlated spectrum of $^{13}\text{C}^{\epsilon 1}$ -histidine-labeled *B. cereus* PI-PLC is shown in Figure 3a. All six histidine residues are present and resolved. Individual resonances in the spectrum were assigned from the NMR spectra of a set of mutants in which one histidine at a time was replaced by alanine (Fig. 3b–f). The impurity peak marked in the spectrum 3(e) is present in every sample but dominant in the H81A sample resulting from poor overexpression of the mutant protein and consequently low protein concentration

(<0.1 mM). The other two weak peaks above and below the His92 peak in the ^1H dimension are data truncation artifacts from the strong His92 resonance. The relatively low intensity for the His32 resonance is due to exchange broadening at the pH value (8.0) near its pK_a (7.6). The ^1H chemical shifts observed for the histidines that were not substituted in the mutants are summarized in Table 1. Overall, the remaining histidines in the mutants show little chemical shift changes in the ^1H - ^{13}C HSQC spectra except for His32 and His81 in the H82A mutant. The replacement of His81 has only a slight effect on the chemical shift of His82. The alanine substitution of His82 results in ~ 0.4 ppm downfield shift for His32 and ~ 0.2 ppm upfield shift for His81, respectively, in the ^1H dimension. Replacement of His32 with alanine causes only ~ 0.08 ppm downfield shift of His82 in ^1H and has no effect on the chemical shifts of the other histidines.

The ^1H and ^{13}C chemical shifts of the $\text{C}^{\epsilon 1}\text{H}$ s of His61 and His227 are identical at pH 7.6 (Fig. 4). As the pH decreases, one resonance shifts downfield, whereas the other one remains the same. To determine the continuity of the titration curves from the crossover point at pH 7.6, several ^1H - ^{13}C HSQC spectra were acquired for the H61A mutant between pH 6.8–8.2. The chemical

Fig. 2. Stereo ribbon diagram of the crystal structure of *B. cereus* PI-PLC showing the histidine side chains together with *myo*-inositol (INS) at the active site. The plot was generated using the program MOLSCRIPT (Kraulis, 1991).

Fig. 3. The ^1H - ^{13}C HSQC NMR spectra of $^{13}\text{C}^{\epsilon 1}$ -histidine-labeled *B. cereus* PI-PLC acquired at pH 8.0 and 25 °C. (A) Wild type; (B)–(F) mutants with one histidine replaced by alanine in each case. The arrows in the spectra (A)–(C) indicate data truncation artifacts from the strong His92 resonance. In (E), the peak marked with an asterisk (*) is due to an impurity, which is present in every sample but dominant in the H81A sample due to poor overexpression of the mutant protein and consequently low protein concentration.

shifts of the remaining resonance (His227) of the pair in these spectra are the same as in the wild type, so we were able to trace the curve for His227 in the absence of His61. Thus, the resonance that does not titrate over the range of pH 4–9 belongs to His61.

Because the $^1\text{H}^{\epsilon 1}$ chemical shifts of His32 and His82 are similar, one might raise the question whether the resonance assignments of

the two histidines were reversed. However, the $^{13}\text{C}^{\epsilon 1}$ chemical shifts of His32 and His82 are very different. The pK_a s determined from both ^1H and ^{13}C data are the same, and this rules out the possibility of confusion in the assignments. Furthermore, when a weak competitive inhibitor of the enzyme, glucosaminyl($\alpha 1 \rightarrow 6$)-D-myoinositol, is added to the protein sample, only the resonance assigned

Table 1. ^1H Chemical shifts (ppm) of the histidine $\text{C}^\epsilon\text{H}$ s in the histidine mutants

Mutants	pH	His32	His82	His61	His81	His92	His227
wt	8.0	8.03	7.94	7.48	7.01	7.52	7.39
H32A	8.1		8.02	7.45	7.00	7.52	7.37
H82A	8.1	8.43		7.45	6.83	7.54	7.37
H61A	8.0	8.06	7.93		7.02	7.52	7.39
H81A	8.1	7.99	7.90	7.46		7.49	7.39
H92A	8.0	8.01	7.92	7.47	7.00		7.39

to His32 is affected (Fig. 5). The resonance assigned to His82 is not shifted. This is consistent with the crystal structure data in that His32 is close to the 2OH group (2.9 Å) of the inositol ring, whereas His82 is over 5 Å away from the inhibitor molecule. Therefore, we conclude that the assignments for these two histidines are correct.

Determination of the pK_a values for the histidines

The chemical shift versus pH data for all six histidines of *B. cereus* PI-PLC are shown in Figure 4. The pK_a values derived from these titration curves are presented in Table 2. His32 and His82, located in the active site, display pK_a values of 7.6 and 6.9, respectively. His92 and His227 exhibit pK_a values of 5.4 and 6.9. Both His61 and His81 display abnormal titration behaviors with inflections at $\text{pH} > 8.5$ and < 4 , respectively, i.e., they did not change their protonation states in the pH range measured. Their pK_a values

were not determined due to protein precipitation at lower pH and limitation of the buffer system at higher pH.

Enzymatic activity of the histidine mutants of PI-PLC

The enzyme activity of the mutants as well as the wild-type PI-PLC were assayed using radiolabeled PI, and the results are shown in Table 3. H61A and H92A are essentially as active as the wild type, whereas H81A retains about 15% of the enzymatic activity of the wild type, presumably due to its effect on the active site residue His82. Less than 0.001% and 0.01% of wild-type enzymatic activity were detected for the H32A and H82A mutants, respectively, strongly suggesting that these two histidines are important for catalysis.

Discussion

pK_a values and the chemical environment of the histidines

B. cereus PI-PLC is a 35 kDa enzyme, and because of the size of the protein the 1D and 2D NMR spectra are too complex and unresolved to study the individual histidines. We therefore specifically labeled the protein with $^{13}\text{C}^\epsilon\text{H}$ -histidine and performed a ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) experiment. This greatly simplifies the analysis, because only the protons attached to the ^{13}C atoms are detected and, therefore, there is only one peak per histidine residue in the HSQC NMR spectrum. With this approach we successfully resolved all six histidines.

To assign each resonance in the spectrum, we utilized site-directed mutagenesis to generate histidine mutants. In each of the five mutants generated, one histidine was selectively replaced by

Fig. 4. The pH titration curves of the six histidines in *B. cereus* PI-PLC. The pK_a values were determined by non-linear regression fitting of the data points.

Fig. 5. The effect of the inhibitor glucosaminyl(α 1 $\rightarrow$ 6)-D-myo-inositol on the chemical shifts of the histidines. The ^1H - ^{13}C HSQC spectra for ^{13}C -histidine labeled PI-PLC at pH 7.4 and 25°C in the absence (**A**) and presence (**B**) of 7.5 mM racemic glucosaminyl(α 1 $\rightarrow$ 6)-D-myo-inositol (GLN-INS). Only the resonance of His32 is shifted upfield. The rest of the histidines are not affected.

alanine, and one specific peak in the NMR spectrum was absent, providing conclusive assignments of all six histidines (Fig. 3). The chemical shifts for the remaining histidines in the mutants show little change except for His32 and His81 in the H82A mutant. This indicates that individual alanine substitutions have little or no effect on the tertiary structure of the protein.

Table 2. The pK_a values of the histidines of *B. cereus* PI-PLC

Histidine	pK _a	Location ^a
H32	7.6	Active site
H82	6.9	Active site
H61	—	Buried
H81	—	Buried
H92	5.4	Protein surface
H227	6.9	Protein surface

^aThe locations are from the crystal structure (Heinz et al., 1995).

Table 3. Enzymatic activity of the histidine mutants assayed with radiolabeled PI

Protein	Specific activity (U/mg) ^a
wt PI-PLC	2162
H92A	2210
H61A	1830
H81A	344
H32A	<0.02 ^b
H82A	<0.14 ^c

^a1 Unit ($\mu\text{mol min}^{-1}$) is the amount of water-soluble hydrolysis products released from phosphatidylinositol under standard assay conditions at pH 7.0 and 37°C.

^bNo activity was detected at concentrations of 1.1×10^{-5} -fold of the wild-type PI-PLC.

^cNo activity was detected at concentrations of 1.5×10^{-4} -fold of the wild-type PI-PLC.

With the assignments complete, the pK_a for each histidine in the wild-type *B. cereus* PI-PLC was determined from the chemical shift data over a range of pH values (Fig. 4). The resulting pK_a values indicate that the histidines are in different chemical environments. The histidines can be divided into three groups, based on their locations in the three-dimensional structure. The first group comprises the important histidines, His32 and His82, which are located at the active site and required for activity. Based on its location at the active site, His82 has been proposed to act as an acid in a general acid-base catalytic mechanism (Heinz et al., 1995). For His82 the pK_a derived from the NMR data is 6.9. This pK_a value coincides with the pH value at the half maximum enzyme activity on the descending limb of the pH profile (Ryan, unpublished data), which suggests that this histidine can act as a general acid for catalysis. The other histidine residue at the active site, His32, exhibits a pK_a of 7.6, which is relatively high, considering that it is proposed to act as the base in the general acid-base mechanism of *B. cereus* PI-PLC catalysis. There is an aspartate residue, Asp274, adjacent to His32. The distance is 2.6 Å between the nearest oxygen of the carboxyl group of Asp274 and the N δ 1 of His32. Raised pK_a values of histidines due to interaction with an aspartate or glutamate residue have been observed in other proteins such as ribonuclease T₁ and T4 lysozyme (Inagaki et al., 1981; Anderson et al., 1990). The interaction between Asp274 and His32 is most likely to account for the pK_a shift of His32. In addition, this interaction may serve to stabilize the orientation of the imidazole ring of the histidine for catalysis.

The second group of histidine residues, His61 and His81, do not titrate over the range of pH 4 to 9. Both His61 and His81 are in the upfield region for the aromatic protons, probably due to the aromatic ring effects from the nearby tyrosine and tryptophan residues. His81 is more buried inside the molecule than His61. His61 is probably hydrogen bonded to the backbone O atom of Tyr57 (3.05 Å) and the carboxyl group of Glu278 (2.98 Å), based on the distances in the crystal structure. The third group of histidines, His92 and His227, exhibit normal titration behavior and pK_a values of 5.4 and 6.9, respectively. Both His92 and His227 are located on the surface of the enzyme. Thus, the NMR data for all six histidines are consistent with the crystal structure.

Fig. 6. Comparison of the mechanisms of ribonucleases and PI-PLC. The first step is a phosphotransferase (transesterification) reaction to produce a cyclic phosphate. The second step is a cyclic phosphodiesterase activity, which occurs rapidly in the ribonucleases but very slowly in *B. cereus* PI-PLC. The general base (Enz-B:) is His12 in RNase A, and Glu 58 in RNase T₁. The general acid (Enz-BH⁺) is His-119 in RNase A, and His92 in RNase T₁. For *B. cereus* PI-PLC, the general acid is His82 and the base is probably His32 or Asp33. R, R', and R'' are abbreviations for portions of the RNA and PI. Pu and Py represent a purine or pyrimidine, respectively.

Comparison of the catalytic residues of *B. cereus* PI-PLC and ribonucleases

Although hydrolyzing different substrates, PI-PLCs and ribonucleases cleave the same chemical bond, a phosphodiester bond, and yield similar relatively stable intermediates, cyclic phosphates (Fig. 6). The ribonuclease catalytic mechanism has been extensively studied, and involves a general acid-base catalysis (Richards & Wyckoff, 1971; Thompson & Raines, 1994). By analogy, PI-PLC is thought to cleave PI by a similar in-line mechanism (Fig. 6). A base abstracts the proton from the 2-OH group, which in turn carries out a nucleophilic attack on phosphorous while a general acid donates a proton to the leaving group (Lin et al., 1990; Volwerk et al., 1990; Lewis et al., 1993; Heinz et al., 1995). In pancreatic ribonuclease A both the acid and base are histidines. Moreover, as shown in Figure 7a, the distances between the im-

idazole rings of the two active site histidine pairs are quite similar between ribonuclease A and PI-PLC, in spite of the large differences in the overall structures (Heinz et al., 1995). Ribonuclease T₁, an acidic enzyme from the fungus *Aspergillus oryzae*, also has two histidines at the active site. The general acid for catalysis is one of these histidines. However, the base is a glutamate, assisted by the second histidine. Again, the two histidines of ribonuclease T₁ superimpose well with His32 and His82 of PI-PLC, as shown in Figure 7b.

Despite the distance similarity, the pK_a values for the active site histidines of these enzymes are different. In the case of bovine pancreatic ribonuclease A, the two catalytic histidines, His12 and His119, exhibit pK_a values of 5.8 and 6.2 (Markley, 1975), which fit their roles as general base and acid, respectively. In contrast, ribonuclease T₁ has a high average pK_a value of 7.6 for its three histidines (Inagaki et al., 1981). The two active site histidines,

Fig. 7. Superpositions of the active site histidines of ribonucleases and *B. cereus* PI-PLC. (A) His12 and His119 of ribonuclease A are aligned with the corresponding catalytic residues His32 and His82 of PI-PLC. (B) His40 and His92 of ribonuclease T₁ are superimposed with His32 and His82 of PI-PLC. With this alignment, Asp33 of PI-PLC is in the same orientation as Glu58 of ribonuclease T₁, the general base for catalysis. This suggests a role for Asp33 in the enzymatic action of PI-PLC. The bolded residues are from *B. cereus* PI-PLC.

His40 and His92, display pK_a values of 7.9 and 7.8, respectively. His92 is the general acid for catalysis (Heinemann & Saenger, 1982). In the crystal structure, His40 forms a hydrogen bond with Glu58 in the free enzyme (Martinez-Oyanedel et al., 1991), which is responsible for the high pK_a value of the histidine (McNutt et al., 1990; Steyaert et al., 1990). Similarly, in the *B. cereus* PI-PLC structure, there is a second aspartate, Asp33, close to His32 (3.4 Å) in addition to Asp274. Remarkably, when the side chains of His32 and His82 from PI-PLC are superimposed onto the corresponding side chains of His40 and His92 from ribonuclease T₁, not only are the histidines aligned well, but also Asp33 is in the same orientation as Glu58 in ribonuclease T₁ (Fig. 7b). Furthermore, His32 of PI-PLC and His40 of ribonuclease T₁ have similar pK_a values. Thus, it is possible that Asp33 could act as a base in the catalytic mechanism of *B. cereus* PI-PLC, or it is the cooperative act of Asp33 and His32 that is important for catalysis as in the case of ribonuclease T₁. Additional site-directed mutants of active site residues should clarify the roles of Asp33 and His32, and this work is in progress.

In summary, the NMR studies reported here provide the first pK_a values for the histidines of PI-PLC. The pK_a values corroborate the implication of His82 as the general acid for catalysis, while our results do not allow the clarification of the role of His32.

Materials and methods

Materials

Radiolabeled PI (³H-PI) was from NEN (Boston, MA) and bovine liver PI from Avanti Polar-Lipids, Inc. (Alabaster, AL). All amino acids and salts for the bacterial growth medium were from Sigma (St. Louis, MO). ¹³C^{ε1}-histidine, Tris-d₁₁, and maleic acid-d₂ were purchased from Cambridge Isotope Laboratories (Andover, MA). Enzyme concentrations were determined from the absorbance at 280 nm using a calculated E_{1%} of 18.4 (molar extinction coefficient of 6.4 × 10⁴ M⁻¹ cm⁻¹). Coordinates for ribonucleases A (ID code 1mc) and T₁ (ID code 9mt) were obtained from Protein Data Bank, Brookhaven National Laboratory.

Site-directed mutagenesis

Alanine substitutions of the histidines were carried out using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the PI-PLC protein expression vector pHS475, following the procedures in the manual provided by the manufacturer. This construct pHS475 is identical to pIC (Koke et al., 1991) except that 3 kbp of non-coding *B. cereus* sequence have been deleted. The selection primer was purchased from Stratagene and the mutagenic primers from Cruachem Inc. (Dulles, VA). The selection primer, 5'-CATCATTGGAAACGCTCTTCGGG GCG3', was used to remove the Xmn I site in the Amp^r gene. The mutagenic primers for the five histidines were: 5'-GTCCCACT ATCGGCTGTTCTTGG3' for His32A; 5'-GATATAATGGCCCA GCATGAAGAAC3' for H82A; 5'-GCGAGCTCCAGCGTCCATT TGATAGCG3' for H61A; 5'-GGCCCATGAGCAAGAAGTATCG3' for H81A; and 5'-CATTTATGAATTCAGCCAGTGTACG3' for H92A. The selection primer and the H82A mutagenic primer were chemically phosphorylated during the syntheses of the primers at the 5' ends and purified by gel electrophoresis. The rest of the primers were phosphorylated with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA) and purified using SELECT-D

G-25 spin columns (5 Prime → 3 Prime, Inc., Boulder, CO). Regions of the plasmids that contained the mutation were sequenced using the Sequenase Version 2.0 DNA sequencing kit (Amersham, Cleveland, OH).

Enzyme activity assays

Enzyme activities of the wild type and the mutants were assayed with ³H-PI, essentially as described previously (Volwerk et al., 1989, 1994). Typically, a 10 mM suspension of radiolabeled PI was prepared by rehydrating a mixture of unlabeled and radiolabeled PI after organic solvent evaporation under vacuum. The specific radioactivity of the PI was about 80,000 cpm/μmol. For the assay, 20 μL of the ³H-PI suspension, 20 μL of 0.8% sodium deoxycholate and 40 μL of 100 mM HEPES, 1 mM EDTA, pH 7.0 were combined and vortexed to form mixed micelles. The reaction was initiated by the addition of *B. cereus* PI-PLC in 20 μL of 0.1% bovine serum albumin, 20 mM HEPES, 1 mM EDTA, pH 7.0 to give a final volume of 100 μL. The reaction was vortexed briefly, incubated at 37 °C for 10 to 40 min, and terminated by the addition of 0.5 mL of chloroform/methanol/HCl (66:33:1, by volume) with vortex mixing. The phases were separated by brief centrifugation in a microfuge at 4 °C and water-soluble products were determined by scintillation counting of an aliquot of the upper aqueous phase. The amount of water-soluble product formed was linear with time for at least 40 min in the presence of about 1.8 ng/mL (54 pM) PI-PLC. Up to 10⁵-fold higher protein concentrations were used to detect residual activities in mutant PI-PLC enzymes.

Selective labeling of the wild type and the mutants of PI-PLC with ¹³C^{ε1}-histidine and protein purification

A histidine auxotroph *E. coli* strain, GM31, was obtained from the laboratory of Dr. Franklin W. Stahl at the Institute of Molecular Biology, University of Oregon. To obtain selectively labeled protein, the bacteria were grown in a synthetic medium consisting of all 20 amino acids and other necessary nutrients as described previously (Muchmore et al., 1989). Seventy milligrams to 80 mg of ¹³C^{ε1}-histidine HCl was used for 1 L of medium. A single colony of GM31 carrying an appropriate plasmid was grown overnight at 37 °C in 25 mL of the synthetic medium with 100 μg/mL of ampicillin added. This preculture was subsequently used to inoculate 1 L of the synthetic medium. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM when OD₆₀₀ of the culture reached 1.0 and the culture was then incubated at 30 °C for 15–16 h before it was harvested.

Mutant and wild-type PI-PLCs were purified from the periplasmic space of recombinant *E. coli* as described previously (Koke et al., 1991) with the following modification: lysozyme was used in combination with mild osmotic shock as described by Witholt et al. (1976). The purified proteins were exchanged into 20 mM Tris-d₁₁-maleate-d₂ buffer for NMR experiments.

NMR data collection and processing

All NMR spectra were acquired at 25 °C on a General Electric Omega 500 MHz spectrometer equipped with an 8 mm triple-resonance ¹H/¹³C/¹⁵N probe with gradient (Nalorac, Martinez, CA). Both ¹H and ¹³C chemical shifts were referenced to an external standard, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm, following the suggestions by Wishart et al. (1995). The protein sample concen-

trations were typically 0.2–0.3 mM in 20 mM Tris- d_{11} -maleate- d_2 with 10% D_2O . A series of 1H - ^{13}C HSQC (Wider & Wüthrich, 1993) spectra were acquired over a pH range of 4.0–9.0 for pK_a determinations. In the HSQC experiments, 1.14 ms was used as the nominal $1/4J_{HC}$. Proton excitation was applied at water proton frequency of 4.75 ppm and ^{13}C excitation was applied at 146.5 ppm. Water signal was saturated with application of gradients. For each spectrum, a total of 90 real t_1 (^{13}C) and 1024 complex t_2 (1H) points were used with spectral widths of 5,000 and 6,666.7 Hz, respectively. Ninety-six scans were accumulated for each t_1 point. Acquisition times were 154 ms and 18 ms in the 1H and ^{13}C dimensions, respectively.

Data were processed using FELIX software (version 2.30, Biosym Technologies, San Diego, CA). Skewed sinebell 30° shift was applied along both dimensions and zero-filling was employed to generate 2D matrices of 512×1024 real data points each.

pH Titration and pK_a determination for the histidines

The pH titration was carried out at $25^\circ C$ over a pH range of 4.0–9.0. The pH was varied by adding aliquots of 20 mM Tris- d_{11} /10% D_2O or 20 mM maleic acid- d_2 /10% D_2O to the protein sample in 20 mM Tris- d_{11} -maleate- d_2 buffer with 10% D_2O . The pH values of the samples were measured at $25^\circ C$ and not corrected for isotope effect. A 1H - ^{13}C HSQC spectrum was acquired at each pH value. The 1H and ^{13}C chemical shifts of the histidine $C^{\epsilon 1}H$ s were plotted against pH values. The pK_a values of the histidines were determined from the one-proton titration curve (Equation 1)

$$\delta_{obs} = \frac{\delta_{HA} + \delta_A - 10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \quad (1)$$

where δ_{HA} is the chemical shift in the acidic pH limit and δ_A represents the chemical shift in the basic pH limit. The titration data were fitted by non-linear regression using the program GraFit Vs. 3.0 (Leatherbarrow, 1992).

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