The Catalytic Role of Aspartate in a Short Strong Hydrogen Bond of the Asp$_{274}$–His$_{32}$ Catalytic Dyad in Phosphatidylinositol-specific Phospholipase C Can Be Substituted by a Chloride Ion*

Li Zhao‡, Hua Liao§, and Ming-Daw Tsai‡§**††

From the Departments of ‡Chemistry and §Biochemistry and the §Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210 and the **Genomics Research Center, Academia Sinica, Taipei 115, Taiwan

Phosphatidylinositol-specific phospholipase C from Bacillus thuringiensis catalyzes the cleavage of the phosphorus–oxygen bond in phosphatidylinositol. The focus of this work is to dissect the roles of the carboxylate side chain of Asp$_{274}$ in the Asp$_{274}$–His$_{32}$ dyad, where a short strong hydrogen bond (SSHB) is shown to exist based on NMR criteria. A regular hydrogen bond (HB) was observed in D274N, and no low field proton resonance was detected for D274E and D274A. Comparison of the activity of wild type, D274N, and D274A suggested that the regular HB contributes significantly (~4 kcal/mol) to catalysis, whereas the SSHB contributes only an additional 2 kcal/mol. The mutant D274E displays high activity similar to wild type, suggesting that the negative charge is sufficient for the catalytic role of Asp$_{274}$. To further support this interpretation and rule out possible contribution of regular HB or SSHB in D274E, we showed that the activity of D274G can be rescued by exogenous chloride ions to a level comparable with that of D274E. Comparison between different anions suggested that the ability of an anion to rescue the activity is due to the size and the charge of the anion not the property as a HB acceptor. In conclusion, a major fraction of the functional role of Asp$_{274}$ in the Asp$_{274}$–His$_{32}$ dyad can be attributed to a negative charge (as in D274E and D274G–Cl$^-$), and the SSHB in the wild type enzyme provides minimal contribution to catalysis. These results represent novel insight for an Asp–His catalytic dyad and for the mechanism of phosphatidylinositol-specific phospholipase C.

Metal-independent bacterial phosphatidylinositol-specific phospholipase Cs (PI-PLCs; EC 3.1.4.10) catalyze the conversion of phosphatidylinositol (PI) to diacylglycerol and 1,2-cyclic inositol phosphate, as well as the subsequent hydrolysis of 1,2-cyclic inositol phosphate to inositol phosphate, a step that is 1000 times slower than the first one (1). Extensive mechanistic studies have led to the proposal of an elaborate mechanism involving a catalytic dyad Asp$_{274}$–His$_{32}$ and a novel catalytic triad His$_{58}$–Asp$_{33}$–Arg$_{69}$, as summarized in Fig. 1. In this mechanism, the three residues in the triad function together to activate the phosphate group and protonate the leaving group in a highly cooperative manner, as established by the extensive use of mutagenesis and the study of thio effects. Several other studies have suggested that the function of the dyad Asp$_{274}$–His$_{32}$ is to serve as the general base (2–4). The focus of this paper is to report our in-depth studies, which further dissect the roles of the dyad, as elaborated below:

The three-dimensional structure of Bacillus cereus PI-PLC complexed with myo-inositol, a substrate analog, has been determined by x-ray crystallography (2). It was found that the N$_{22}$ of His$_{32}$ is optimally positioned (2.8 Å) for proton abstraction from the C2-hydroxyl group of myo-inositol, whereas its N$_{51}$ forms a hydrogen bond (HB) (2.7 Å) with the O$_{2}$ of Asp$_{274}$ (Fig. 2, left panel). A recent NMR study showed that the H-bond between Asp$_{274}$ and His$_{32}$ in the free enzyme fits into the NMR criteria for a short strong hydrogen bond (SSHB): a highly deshielded proton resonance, a bond length of 2.64 Å, a D/H fractionation factor significantly lower than 1.0, and a protection factor of >100 (5). This type of H-bond has been postulated to play a crucial role in enzymatic reactions, particularly those involving the general acid/base catalytic mechanism, by providing substantial stabilization energy (10–20 kcal/mol) for the intermediate or transition state (6). However, we have shown that replacing Asp$_{274}$ with an asparagine residue has a relatively mild effect (40-fold decrease) on catalysis (4). In addition, x-ray structural analyses showed that the side chain of Asn$_{274}$ in D274N is virtually unchanged when compared with Asp$_{274}$ in WT, and Asn$_{274}$ still forms a hydrogen bonding interaction with N$_{51}$ of His$_{32}$ (3) (Fig. 2, right panel). These data led us to ask the following questions: (i) Does SSHB exist in mutants D274N and D274E and therefore account for the mild effect on catalysis? (ii) If the answer is no, what is the difference between a SSHB and a regular HB in their energetic contributions to catalysis? (iii) Can the role of Asp$_{274}$ in the catalytic dyad be further dissected?

Herein, we report the use of site-directed mutagenesis in conjunction with $^1$H NMR spectroscopy and chemical rescue analyses to dissect the functional role of Asp$_{274}$ in the catalytic dyad (Asp$_{274}$–His$_{32}$) of PI-PLC. Asp$_{274}$ was substituted by asparagine, glutamate, alanine, and glycine. These mutant enzymes were analyzed by kinetic, NMR, and chemical rescue techniques. Our results suggest that the major functional role of Asp$_{274}$ can be achieved by forming a regular HB (as in D274N) or by providing a negative charge (as in D274E and Cl$^-$ rescued D274G) and that the SSHB in the wild type enzyme provides only a modest additional contribution to catalysis.

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Asp$^{274}$–His$^{32}$ Catalytic Dyad in PI-PLC

This work represents a novel insight for Asp–His catalytic dyads and for the mechanism of PI-PLC.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylinositol from bovine liver and 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DHPC) were purchased from Avanti Polar Lipids. L-α-Imidazol-2-β-[H]N-phosphatidylinositol was purchased from PerkinElmer Life Sciences. 3-5NCl (at 99% 35N) was from Isotech Inc. The oligonucleotides were purchased from Integrated DNA Technologies, Inc. The Escherichia coli strains XL1 Blue and BL21 (DE3) LysS used for gene manipulation and protein overexpression were from Stratagene. All DNA-modifying enzymes were from New England Biolabs. 99.9% atom D$_2$O was purchased from Cambridge Isotope Laboratories.

Construction and Purification of WT and Mutant PI-PLC—Mutations were introduced by the double-stranded, site-directed mutagenesis method (Stratagene). The mutagenic primers used are listed 5′ to 3′ with base substitutions underlined and codons of interest in italics. Only forward primer sequences are shown for each mutant oligonucleotide, and the mutation produced is in parentheses: 5′ GGATG GAC TAC ATA AAT GAA AAG TG-3′ (D274E), 5′ TGG GTA ATT CAA AAC GTA ATG GCT-3′ (D274N), and 5′ TGG GTA ATT CAA GCC TAC ATA AAT GAA AAG TG-3′ (D274Q).

All of the mutations were verified by sequencing. All of the proteins were purified as described previously (4,7).

NMR Methods—One-dimensional 1H NMR experiments were carried out on a Bruker DMX-600 spectrometer at 280 K using a jump-return sequence for solvent suppression (8). The NMR samples contained ~0.05 mM protein. Unless otherwise stated, lyophilized protein was prepared in 90% H$_2$O, 10% D$_2$O with 20 mM Tris/maleic buffer at pH 4.0–9.0. To determine the pK$_a$ of His$^{32}$ in both WT and mutant PI-PLC, one-dimensional 1H NMR experiments were conducted on PI-PLC samples upon pH titration (pH 5.0–9.0). Care was taken to minimize the loss of sample, and less than 10% volume changes were introduced.

Activity Assay of PI-PLC with [15]H[PI] Substrate—The specific activities of mutants were measured according to the procedure reported earlier (9) with minor modifications. L-α-[1-15N]-Imidazol-2-β-[H]N-phosphatidylinositol was mixed with unlabeled PI from bovine liver to obtain an overall PI concentration of 5 mM and a specific activity of ~1.25 × 10$^{6}$ cpm/mol. In the pH dependence study, the reaction mixture contained 2 mM PI, 8 mM DHPC, 20 mM succinate (pK$_a$ = 4.2), 20 mM HEPES (pK$_a$ = 7.48), and 20 mM borate (pK$_a$ = 9.23), where the pH of the buffer was adjusted solely by NaOH to avoid introduction of chloride ions. Both pH adjustment and assays were performed at 25 °C. In the anion activation study, the reaction mixture contains 2 mM PI, 8 mM DHPC, 0–1 mM NaX, and 50 mM HEPES, pH 7.5, where the pH of the buffer was adjusted solely by NaOH to avoid introduction of chloride ions (X stands for different anions tested in this study: F$^−$, Cl$^−$, Br$^−$, N$_3^−$, COO$^−$, and Ac$^−$). The assays were performed at 37 °C. Because of the low solubility of NaF (4.22 g/100 ml of H$_2$O), 0–400 mM NaF was used in the F$^−$ activation study. An aliquot of 20 µl of PI-PLC solution was added to the reaction mixture and incubated for 10 min. The concentrations of enzymes were adjusted so that the substrate conversion does not exceed 10–30%. The reaction was stopped by the addition of 0.5 ml of CHCl$_3$–CH$_3$OH–HCl (66:33:1). The phases were separated by a brief centrifugation, and radioactivity of 50 µl of the aqueous phase was measured by scintillation counting (Beckman). Enzymatic activity was expressed in µmol × min$^{-1}$ × (µg of enzyme)$^{-1}$ or units/mg.

RESULTS AND DISCUSSION

Proton NMR Evidence for SSHB in WT and Normal HB in D274N Bacillus thuringiensis PI-PLC—A low field proton peak at 16.3 ppm at low pH (<6) that arose from H$_3^{31}$ of His$_{32}$ has been observed for the WT but not for the mutant D274A. B. cereus PI-PLC (5). Under the same conditions, we were able to observe the same characteristic low field chemical shift of the SSHB proton at 16.3 ppm for the WT B. thuringiensis PI-PLC (Fig. 3B). Note that the amino acid sequence of B. thuringiensis PI-PLC is identical to that of B. cereus PI-PLC, except at eight nonconservative positions (1). Therefore, we did not further acquire other experimental parameters (D/H fractionation and factor protection factor) used as NMR criteria for assigning the SSHB between N$_3^{31}$ of His$_{32}$ and O$_{41}$ of Asp$_{274}$. However, we did perform a decoupling experiment to confirm that this proton is attached to nitrogen. In short, when uniformly 15N-labeled WT enzyme was used, a low field (16.3 ppm) splitting proton peak was observed for WT (Fig. 3A). The J-coupling is estimated to be ~90 Hz, suggesting that this peak arises from the nitrogen-attached proton. The 15N chemical shift was determined to be 180 ± 10 ppm by a series of continuous wave decoupling experiments (spectra not shown). This 15N chemical shift value is close to the typical value for a = NH$^−$ in model compounds (~175 ppm) (10, 11), suggesting that His$_{32}$ is largely positively charged. The protonation state of His$_{32}$ at pH 5.0 is consistent with its pK$_a$ value described in the next section.

Under the same conditions, we found that this low field proton peak shifted to 13.6 ppm in D274N (Fig. 3C) and was missing in D274E (Fig. 3D). The crystal structures of WT and D274N have been determined at resolutions of 2.6 and 2.3 Å, respectively. The H-bond distance between N$_3^{31}$ of His$_{32}$ and O$_{41}$ of Asp$_{274}$ in the WT was estimated to be 2.7 Å, and the distance between N$_3^{31}$ of His$_{32}$ and O$_{41}$ of Asn$_{274}$ in the D274N mutant is estimated to be 2.5 Å (3). However, x-ray crystallographic results at a resolution of 2.3–2.6 Å are insufficient to distinguish between small changes in bond distances. On the basis of the NMR data, a SSHB exists between Asp$_{274}$ and His$_{32}$ in the WT enzyme, whereas a normal double-well H-bond forms between Asn$_{274}$ and His$_{32}$ in the D274N mutant. It should be noted that in the absence of structural information the negative result for D274E should not preclude a possible H-bond interaction (SSHb or regular HB) between Glu$^{274}$ and His$^{32}$. However, we will address this question through functional studies in the subsequent sections.

Extensive studies and debates have appeared in the literature since the idea of low barrier hydrogen bond (LBHB or SSHB) was first proposed (12–15). Two of the key questions are the strength of the LBHB and its contribution to the rate enhancement of the reaction catalyzed by the enzyme. To address these issues for the SSHB in PI-PLC, we performed pH dependence studies on the hydrogen bonding and the catalytic activity of both WT and D274N. The different properties of the two mutants provide insight to these issues.

pH Dependence of the Low Field Proton Resonance of D274N and WT PI-PLC—We examined the effects of pH on these low field proton peaks. The results from the pH titration of WT and D274N are shown in Fig. 4. For WT, the low field proton peak shifted from 16.3 ppm at pH 6.0 to 13.8 ppm at pH 9.0, and the calculated pK$_a$ is 7.4 at 7°C (Fig. 5A). For D274N, the low field proton peak shifted from 13.5 ppm at pH 5.0 to 11.5 ppm at pH 8.0, and the calculated pK$_a$ is 6.4 at 7°C. This result shows that the D274N mutation leads to a 1-pH unit decrease in the pK$_a$ of His$_{32}$, which can be explained by the perception that the negative

$^a$The pK$_a$ value of His$_{32}$ in B. cereus PI-PLC derived from 1H NMR studies by Ryan et al. (5) is 8.0 at 6°C. The difference between the pK$_a$ values could be due to various causes including the experimental conditions. Because the pK$_a$ difference between WT and D274N is our major focus, this small discrepancy was not further emphasized.
atively charged carboxyl oxygen can better stabilize the positively charged imidazole ring. Similarly, the LBHB exists in the protonated dyad His$^{57}$–Asp$^{102}$ of the free chymotrypsin, and the $pK_a$ of His$^{57}$ was reported to be 7.5 at 3°C (16) as shown in Fig. 5B. However, $pK_a$ of His$^{57}$ of chymotrypsin increases by 5 units when complexed with transition state analogs (Fig. 5C), which indicates that the LBHB strengthens in the transition state complex (17). Although no transition state analog for PI-PLC catalysis is available to test this property, it is likely that the SSHB in the protonated dyad His$^{32}$–Asp$^{274}$ further strengthens in the transition state complex. The strength of the SSHB in the transition state of PI-PLC is not known. However, its contribution to catalysis can be estimated by comparing the activities of WT and D274N as described in the next section.

The SSHB of PI-PLC Contributes Only Modestly to Catalysis— Kinetic data obtained previously (4), show that the activity of D274N (21.0 units/mg) is only modestly reduced relative to that of WT PI-PLC (1300 units/mg). However, previous studies were performed at a fixed pH of 7.5. Because WT and the mutant showed different pH dependence in hydrogen bonding as described in the preceding section, it is likely that their activities have different pH optima. Here, we constructed the pH activity profiles for WT, D274N, and D274E PI-PLC (Fig. 6). The apparent $pK_a$ values of the general base obtained from the equation in the Fig. 6 legend are 4.91 (WT), 3.98 (D274N), and 4.67 (D274E). Thus the D274N mutation lowers the apparent $pK_a$ value by 1 pH unit, whereas the D274E mutation does not affect the apparent $pK_a$ value (within the error range). Although the trend seems to be consistent with that from the NMR study, the exact values do not match. The apparent $pK_a$ values derived from pH activity profiles may not be a true reflection of the $pK_a$ values of His$^{32}$ (in the enzyme-substrate complex), possibly because of the complexity of the ionizable catalytic residues around the active site as well as the stability of the protein as a function of pH.

Under the optimal pH conditions, the $V_{max}$ values are 1345 ± 86 units/mg (WT), 33.3 ± 2.1 units/mg (D274N), and 446 ± 85 units/mg (D274E). Previously, WT and a series of mutant PI-PLC enzymes were shown to display the same $R_p$ thio effect ($k_v/k_R$) using 1,2-dipalmitoyl-sn-glycero-3-thiophospho-1-myoinositol as the substrate and DHPC as the detergent, suggest-
ing that the chemistry step is rate-limiting under the assay conditions (9, 18). If the rate is limited by micelle exchanges, the thio effect should increase as the chemical step slows in the mutant. Thus the 40-fold decrease in activity caused by the D274N mutation corresponds to 2.0 kcal/mol energy loss in transition state stabilization ($\Delta G^\ddagger_{\text{WT}} - \Delta G^\ddagger_{\text{D274N}} = RT \ln (k_{\text{D274N}}/k_{\text{WT}}) = 2.0 \text{ kcal/mol}$). This indicates that the contribution of the SSHB (relative to the normal HB) to catalysis is relatively small in comparison with that of serine proteases.

The results of D274E and D274A are more difficult to interpret because their structures are not available. Although the D274A mutant retains only 0.005% of activity relative to that of WT (4), D274E retains nearly 50% of the WT activity as indicated above. The significant loss of activity in D274A can be attributed to the loss of the SSHB and the negative charge. However, it is unclear whether the nearly complete retention of activity for D274E is contributed by the negative charge alone or by the regular HB and/or SSHB. As stated earlier, the absence of low field proton resonance does not necessarily mean the absence of a hydrogen bond. This issue is addressed in the next section.

Dissecting the Roles of the H-bond and the Negative Charge of Asp274

To further dissect the roles of the H-bond and the negative charge, we performed anion rescue experiments. The rationale is to introduce anions that can provide negative charge but not hydrogen bonding. Asp274 in PI-PLC was first changed to alanine and glycine by the site-directed mutagenesis. The activities of the two cavity mutants (D274A and D274G) were tested in the presence of different anions (data not shown). The activity levels of D274A and D274G in the absence of rescuing anions at pH 7.5 were 0.033 and 0.19 units/mg, respectively. D274G, but not D274A, showed enhanced activity in the presence of exogenous anions. Further analyses demonstrated that the enhanced activity of D274G by these anions obeys saturation kinetics with respect to the anion concentration (Fig. 7). The magnitude of D274G activation by different anions is summarized in Table I. The maximal velocities of D274G in the presence of NaF, NaCl, NaBr, NaN₃, NaCOO, and NaAc at saturating substrate concentrations give a 13-, 67-, 54-, 71-, 24-, and 4-fold activation, respectively. Although the high $K_{\text{app}}$ values indicate the low binding affinity of D274G toward those exogenous anions, this is the first example in which a mutated Asp/His catalytic dyad is rescued by halide ions. The identical activation effect from NaCl and KCl suggests that counter cations have no effect on the rescued activity.

![Fig. 5. Comparison of the Asp-His catalytic dyad in PI-PLC and Asp-His-Ser catalytic triad in chymotrypsin. A, the free PI-PLC in which the Asp274-His32 dyad is protonated. B, the free chymotrypsin in which Asp102-His57 is protonated. C, the peptidyl adducts formed between Ser195 and trifluoro ketone (N-acetyl-L-leucyl-L-phenylalanyl trifluoromethyl ketone) in which the dyad is protonated. The chemical shifts for the SSHBs and information about the $pK_a$ values for the His residues from this and previous studies (17) are included.](image)

![Fig. 6. pH dependence of $V_{\text{max}}$ for WT (●, right coordinate), D274E (●, right coordinate), and D274N (●, left coordinate) PI-PLC. The pH dependences of the reaction were measured in 20 mM Borate-Succinate-HEPES buffer at 25 °C. Each specific activity represents the average value determined from two parallel experiments. The curves were obtained by fitting the data to the equation $V_{\text{max}} = k_{\text{cat}}/K_{\text{M}} [S] + k_{\text{cat}}/K_{\text{M}}$ with SigmaPlot. The data above the optimal pH were not fitted.](image)
Anion Concentration, mM

![Graph showing anion concentration vs. specific activity](image)

**Fig. 7.** Activation of the D274G PI-PLC by different anions: N\(^-\) (\(\gamma\), Na\(\gamma\)), Cl\(^-\) (\(\delta\), NaCl), Cl\(^-\) (\(\epsilon\), KCl), Br\(^-\) (\(\upgamma\), NaBr), COO\(^-\) (\(\mu\), NaCOO\(\gamma\)), Ac\(^-\) (\(\kappa\), NaAc), and F\(^-\) (\(\lambda\), NaF). The assay conditions are described under “Experimental Procedures.” Each specific activity represents the average value determined from two parallel experiments, and the error bars show the average deviations.

**Table I**

Kinetic parameters of D274G PI-PLC by different anions

| Anions tested | \(V_{\text{max}}\) \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) | \(K_{\text{app}}\) mM | Molecular volume \(A^3\) |
|---------------|------------------|------------------|------------------|
| None | 0.19 ± 0.03 | 2.39 ± 0.34 | 0.34 ± 0.03 |
| Cl\(^-\) (as in KCl) | 12.7 ± 0.3 | 66.2 ± 7.1 | 34.24 |
| Cl\(^-\) (as in NaCl) | 12.9 ± 0.3 | 66.4 ± 5.2 | 34.24 |
| Br\(^-\) | 10.2 ± 0.3 | 115 ± 13 | 35.62 |
| N\(_3\) | 13.5 ± 0.3 | 52.8 ± 4.7 | 57.82 |
| HCOO\(^-\) | 4.92 ± 0.27 | 434 ± 83 | 74.99 |
| CH\(_3\)COO\(^-\) | 0.73 ± 0.41 | 93.18 |

\(^{a}\) Solvent-excluded molecular volumes were calculated based on the Monte Carlo method, using Gaussian.

(\(-0.0394 ± 0.0060 \ A^3\) \(\times V\) (molecular volume \(A^3\)) + (3.45 ± 0.46)). This indicates that the steric factor is very important in determining rate constants for the anion rescued activity. Upon input of the log \(V_{\text{max}}\) values of Cl\(^-\) and Br\(^-\) activation, we found that the experimental values were below theoretical predications. In addition, the \(K_{\text{app}}\) values for Cl\(^-\) and Br\(^-\) activation are larger than that for N\(_3\) activation. These data hint that the bound form of Cl\(^-\) and Br\(^-\) might be hydrated. Although F\(^-\) is the smallest halide ion, it activates D274G to the least extent with a \(K_{\text{app}}\) value of 575 ± 146 mm. A possible explanation is that F\(^-\) forms big anionic clusters in aqueous solution because of the strong binding energy (23.3 kcal/mol) in the F\(^-\)·H\(_2\)O complex (19).

To explain at the chemical level the activation of D274G, but not D274A, by the exogenous anions, there are several mechanisms envisaged. (i) The cavity generated by D274A is small and hydrophobic, thus excluding water or anions from binding. In contrast, the cavity generated by D274G is large and hydrophilic, and water and anions can bind to this cavity. This bound water may account for the 6-fold higher activity demonstrated by D274G in the absence of rescuing anions. (ii) Cl\(^-\) (\(pK_a = -9\)) is very unlikely to be able to operate efficiently as a proton acceptor at pH 7.5, whereas N\(_3\) (\(pK_a = 4.72\)) might. Here, we showed that Cl\(^-\), like N\(_3\), is capable of rescuing the activity of D274G by ~70-fold. Therefore, it seems probable that a small anion, by implication the side chain of Asp\(^{274}\), offers a fixed negative charge in the active site. On the other hand, if the rescue by N\(_3\) is attributed to its ability in accepting a proton, we would expect a higher activation by N\(_3\) compared with that of Cl\(^-\) at lower pH. This possibility is examined in the next section.

**pH Activity Dependence of the Rescue Effects of D274G by Cl\(^-\) and N\(_3\)** Although the D274G can be successfully activated by exogenous anions, the maximal activity at the assay condition (pH 7.5) is still 3-fold lower than that of D274N and 60-fold lower than that of D274E. It is possible that the pH optimum of D274G has changed, thus maximal activities were not observed. To test this possibility and the role of N\(_3\) (stated above), we examined the dependence of activity on the pH. As shown in Fig. 9, the pH activity profiles of D274G in the absence and presence of rescuing ions are both bell-shaped curves. The pH optimum changes from pH 5.0 to 6.0 in the presence of Cl\(^-\) (or...
to pH 5.6 in the presence of N\textsubscript{3}\textsuperscript{–}). This suggests that the general base in the Michaelis complex functions more efficiently at a higher pH in the presence of rescuing anions. It is striking that the activity of D274G at optimal pH increases from 1.75 to 100 units/mg in the presence of Cl\textsuperscript{–} (98 units/mg in the presence of N\textsubscript{3}\textsuperscript{–}). Note that this rescued activity of D274G (100 units/mg) is 3-fold higher than that of D274N (35 units/mg). As controls, the pH dependence of D274N, D274E, and WT in the presence of 1 M Cl\textsuperscript{–} were examined. Within experimental errors, the activities of D274N and D274E are not affected by Cl\textsuperscript{–}, and the pH profiles remain the same. For WT, the activities generally increased by −2−3-fold and the pH optimum became 7–8 instead of 6−9 in the presence of 1 M Cl\textsuperscript{–}.

Taken together, these findings suggest that anions with small radii can bind D274G by occupying the cavity created by the aspartate to glycine mutation and consequently stabilize the positive charge on the general base (His\textsuperscript{32}) through electrostatic interaction (lacking directionality) and activate D274G. These findings also infer that Asp\textsuperscript{274} may play a trivial role in the catalysis by PI-PLC. Thus our results provide another perspective of the role of Asp/His dyad. This pattern suggests strongly that the loss of LBHB, for the tautomeric form of the His residue is reversible in the D102N mutant of trypsin (24). A substantial fraction of the loss of activity is likely caused by the change in the tautomeric form of the histidine residue. Consistent with this interpretation, a recent study has shown that the LBHB (Asp\textsuperscript{274}–His\textsuperscript{32}) of subtilisin Bacillus protease N\textsuperscript{–} can be partially (2%) replaced by a normal strength hydrogen bond (Cys\textsuperscript{32}, His\textsuperscript{461} of mutant enzyme D29C (25).

For bovine pancreatic secreted phospholipase A\textsubscript{2} (sPLA\textsubscript{2}), the Asp\textsuperscript{99}–His\textsuperscript{48} dyad functions similarly to the Asp\textsuperscript{274}–His\textsuperscript{32} dyad of PI-PLC. A significant difference resides in the tautomeric form of the imidazole ring (in sPLA\textsubscript{2}, Asp\textsuperscript{99} H-bonds to the N\textsuperscript{2} of His\textsuperscript{48}, whereas in PI-PLC, Asp\textsuperscript{274} H-bonds to the N\textsuperscript{6} of His\textsuperscript{32} (26). Unlike PI-PLC and serine proteases, the SSHB in PLA\textsubscript{2} exists between the K\textsubscript{51} of His\textsuperscript{48} and one of the nonbridging phosphate oxygen atoms in the transition state analog, whereas a normal H-bond is formed between the N\textsuperscript{2} of His\textsuperscript{48} and the O\textsubscript{61} of Asp\textsuperscript{99} at low pH or in the presence of the transition state analog (HK32). Similar to that has been observed for the mutant D274N PI-PLC, both the x-ray crystal structure and NMR experimental results suggest that a regular HB exists between Asn\textsuperscript{99} and protonated His\textsuperscript{48} in the D99N mutant of sPLA\textsubscript{2}, and D99N still preserves 5% of the catalytic activity compared with that of WT (27, 28).

Conclusion—The detailed catalytic roles of the Asp–His catalytic dyad (or Asp–His–Ser triad) and the SSHB in this motif are subjects of active research and intense debate. Combination of detailed structural and enzymological analyses has allowed us to address this issue in depth for sPLA\textsubscript{2} previously and for PI-PLC in this work. Although there may be multiple functional roles for a particular structural element, our results with PI-PLC suggest that a glycine residue along with a chloride ion, in the absence of a regular HB or a SSHB, suffice nearly the entire role of the aspartate residue in the Asp\textsuperscript{274}–His\textsuperscript{32} dyad where a SSHB exists. The WT enzyme still does better overall in catalyzing the reaction and likely also in other factors, such as protein stability and substrate binding. However, dissection of the functional roles described here provides new insight into the structure-function relationship of enzymes.

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