

A Low-barrier Hydrogen Bond Between Histidine of Secreted Phospholipase A₂ and a Transition State Analog Inhibitor

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This work describes in-depth NMR characterization of a unique low-barrier hydrogen bond (LBHB) between an active site residue from the enzyme and a bound inhibitor: the complex between secreted phospholipase A₂ (sPLA₂, from bee venom and bovine pancreas) and a transition-state analog inhibitor HK32. A downfield proton NMR resonance, at 17–18 ppm, was observed in the complex but not in the free enzyme. On the basis of site-specific mutagenesis and specific ¹⁵N-decoupling, this downfield resonance was assigned to the active site H48, which is part of the catalytic dyad D99-H48. These results led to a hypothesis that the downfield resonance represents the proton (H^{ε2} of H48) involved in the H-bonding between D99 and H48, in analogy with serine proteases. However, this was shown not to be the case by use of the bovine enzyme labeled with specific [¹⁵N^{ε2}]His. Instead, the downfield resonance arises from H^{δ1} of H48, which forms a hydrogen bond with a non-bridging phosphonate oxygen of the inhibitor. Further studies showed that this proton displays a fractionation factor of 0.62(±0.06), and an exchange rate protection factor of >100 at 285 K and >40 at 298 K, which are characteristic of a LBHB. The pK_a of the imidazole ring of H48 was shown to be shifted from 5.7 for the free enzyme to an apparent value of 9.0 in the presence of the inhibitor. These properties are very similar to those of the Asp...His LBHBs in serine proteases. Possible structural bases and functional consequences for the different locations of the LBHB between these two types of enzymes are discussed. The results also underscore the importance of using specific isotope labeling, rather than extrapolation of NMR results from other enzyme systems, to assign the downfield proton resonance to a specific hydrogen bond. Although our studies did not permit the strength of the LBHB to be accurately measured, the data do not provide support for an unusually strong hydrogen bond strength (i.e. >10 kcal/mol).

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Abbreviations used: d₆-DMSO, perdeuterated dimethylsulfoxide; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; GARP, globally optimized alternating-phase rectangular pulses; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; LBHB, low-barrier hydrogen bond; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement correlated spectroscopy; PI-PLC, phosphatidylinositol-specific phospholipase C; ppm, parts per million; sPLA₂, secreted phospholipase A₂; TSA, transition-state analog; WT, wild-type.

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Introduction

Secreted phospholipases A₂ (sPLA₂) are small (13–18 kDa) calcium-dependent lipolytic enzymes that belong to a family of enzymes that catalyze the hydrolysis of phospholipids at the *sn*-2 ester bond.¹ It was observed from crystal structures that the active site is composed of an Asp-His dyad, which is proposed to couple with the conserved catalytic water molecule to form a catalytic triad analogous to the well-studied serine proteases and lipases (Figure 1(a)). One notable exception is that the orientation of the imidazole ring in sPLA₂s and serine proteases/lipases is flipped with respect to each other. A second exception is that in serine proteases it is the *syn* lone pair of the Asp that accepts a H-bond from His, but sPLA₂s use the *anti* lone pair of Asp in forming a H-bond to His. In serine proteases, the catalytic triad consists of Asp, His, and Ser residues, and a proposed low-barrier hydrogen bond (LBHB) is formed between Asp and His in both free and inhibitor-bound states.^{2,3} Since sPLA₂s also employ the general base mechanism, it would be interesting to know whether such a “LBHB” exists and plays an equivalent role. Two of the sPLA₂ members, bovine pancreatic sPLA₂ and bee venom sPLA₂, serve as excellent models for investigating such a question, since they have been extensively studied by multifaceted approaches including site-directed mutagenesis, scooting-mode kinetics, X-ray crystallography, and NMR spectroscopy.^{1,4,5}

Hydrogen bonding is a ubiquitous feature in protein structures. Although weak or conventional H-bonds are common in biological systems, a short and stronger type often referred to as a LBHB has been postulated to play a crucial role in enzymatic reactions, particular those that involve a general acid-general base mechanism, by provid-

ing substantial stabilization energy (10–20 kcal/mol) for the intermediate or transition state.^{6–8} Most of this interest has stemmed from the role of a LBHB in the catalysis of serine proteases, proposed by Cleland & Kreevoy⁶ and Frey *et al.*² While the unusual role of LBHBs in enzymatic function has been under intensive debate,^{9–13} evidence in support of the existence of LBHB is accumulating.^{3,8} The most commonly used approach to characterize LBHBs in solution has been the observation, by NMR spectroscopy, of several physicochemical properties. The widely accepted features that are commonly associated with LBHBs are: (i) unusual downfield proton NMR resonance at 16–20 ppm, which is considered to be the most distinctive feature; (ii) ²H/¹H fractionation factor < 1.0; (iii) exchange rate protection factor > 10; and (iv) hydrogen bond strength > 7 kcal/mol.^{2,14,15}

Here, NMR spectroscopy has been employed to detect and characterize a possible LBHB in bee venom and bovine pancreatic sPLA₂s. Our results show that both sPLA₂s display a downfield proton resonance (17.6–18.0 ppm) in the presence of a phosphonate transition-state analog (TSA) (Figure 1(b)). For bovine pancreatic sPLA₂, combined use of site-directed mutagenesis and specific isotopic-labeling led to the convincing assignment of this resonance to the H^{δ1} of the catalytic H48. According to crystal structures,^{16–18} H^{δ1} of H48 is engaged in a H-bond with one of the non-bridging phosphonate oxygen atoms in the transition-state analog, rather than with D99 (Figure 1(c)). Further analyses demonstrated that this H-bond displays other physicochemical properties similar to Asp...His LBHB's of serine proteases. These results provide new insight to the structure–function relationship of both classes of enzymes.

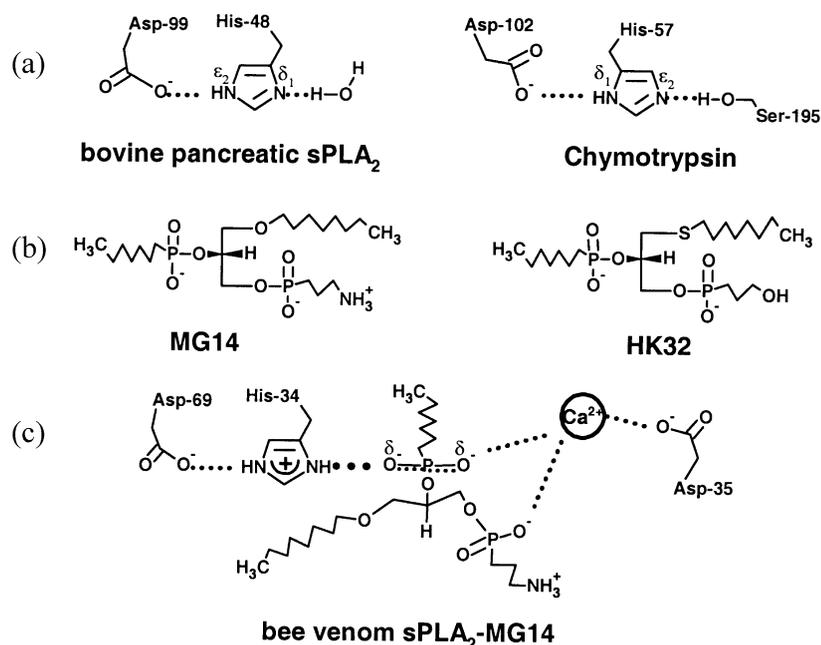


Figure 1. (a) Comparison of the Asp-His catalytic dyad in sPLA₂ and the Asp-His-Ser catalytic triad in chymotrypsin. (b) Structures of phosphonate transition-state analogs MG14 and HK32. (c) Active site interactions between bee venom sPLA₂ and MG14 as seen in the crystal structure.¹⁷

Results

Detection of a downfield proton NMR resonance characteristic of a LBHB in sPLA₂-inhibitor complexes

Our initial studies were performed with bee venom sPLA₂ and the transition state analog, MG14, a water-soluble phospholipid analog containing a tetrahedral phosphonate replacing the ester at the *sn*-2 position (Figure 1(b)).¹⁶ The crystal structure of the bee venom sPLA₂-MG14 complex has been solved, and the active site interactions are shown in Figure 1(c).¹⁷ In the 1D ¹H NMR experiments, a downfield peak at 17.6 ppm and another peak at 14.1 ppm were observed in the spectrum of the bee venom sPLA₂-MG14 complex but not in the free enzyme (data not shown). In all subsequent studies, the inhibitor HK32 was used instead of MG14 because of availability. HK32 is similar in structure to MG14 except that the *sn*-1 ether oxygen of MG14 is replaced by sulfur, and the ammonium portion of the polar head group of MG14 is replaced by a hydroxyl group in HK32 (Figure 1(b)). Downfield peaks were observed with identical chemical shifts for the bee venom sPLA₂-HK32 and bee venom sPLA₂-MG14 complexes (Figure 2). Selective ¹⁵N decoupling experiments revealed that the proton at 17.6 ppm is attached to a nitrogen at ~200 ppm and the one at 14.1 ppm is attached to a nitrogen at ~170 ppm (HSQC-type experiments proved futile, most likely due to exchange broadening and/or inherently small transverse relaxation time *T*₂ values, same as in the NMR work of bovine pancreatic sPLA₂). On the basis of previous studies of serine proteases,^{2,3,19} the NMR results with bee venom sPLA₂ are indicative of a very strong hydrogen bond, i.e. a LBHB.

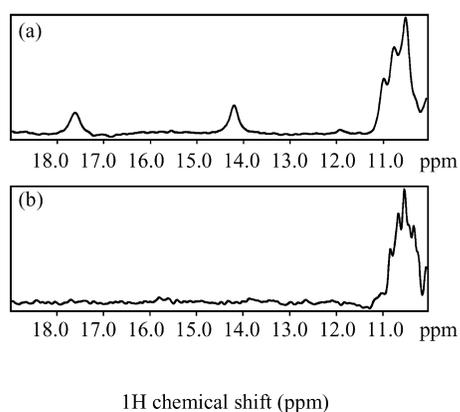


Figure 2. Downfield region of the NMR spectrum of bee venom sPLA₂ at 500 MHz using a jump-return sequence for solvent suppression. (a) ¹⁵N-labeled WT bee venom sPLA₂ in the presence of HK32 inhibitor, pH 4.8 at 305 K with broadband decoupling of ¹⁵N during acquisition using a GARP sequence. (b) Unlabeled WT bee venom sPLA₂, pH 4.8 at 298 K with no inhibitor present.

Since there are seven histidine residues in bee venom sPLA₂, it is difficult to verify that the above-mentioned downfield resonances are due to the active site histidine. An initial attempt to assign the resonance to hydrogen-bonded proton in the D64...H34 catalytic dyad led to ambiguous results since the resonance was still observable, though very broad, in the D64N-HK32 complex (data not shown). Also, due to severe loss of exchangeable proton signals at pH values above 5.0, and a strong tendency for the protein to precipitate with any change to the buffer system, neither pH titration, nor experiments at higher pH values could be performed. The lowest pH, at which the downfield peaks were investigated and observed, was 3.8, and the highest was 4.8. These problems made bee venom sPLA₂ unfavorable for further investigations and prompted us to switch to bovine pancreatic sPLA₂, whose solution structure has already been solved by NMR at neutral pH²⁰ and which contains only two histidine residues. The wild-type (WT) bovine pancreatic sPLA₂ has been shown to be stable against pH and chemical denaturation.²¹ Its two histidine residues are located at the active site (H48) and in the C terminus (H115), and the latter could be mutated without affecting the catalytic activity.²²

Like bee venom sPLA₂, an unusual downfield peak at 18.00 ppm and another peak at 12.67 ppm were observed with bovine pancreatic sPLA₂ upon addition of HK32 at neutral pH and at 285 K. The most downfield peak was absent in the free enzyme (Figure 3(a)) despite exhaustive search in the temperature range of 275–310 K and the pH range of 3–10. The peak at 12.67 ppm was also absent in the spectrum of the free enzyme, but another proton resonance appeared at a different chemical shift (13.02 ppm) under acidic conditions (Figure 3(l) and (m)), which will be addressed later. Since the inhibitor was dissolved in d₆-DMSO before mixing with the enzyme, a control experiment was also performed to ensure that the observed downfield peaks in the complex did not result from addition of the small amount of d₆-DMSO (Figure 3(b)). The two downfield peaks were both shown to arise from nitrogen-attached protons based on observation of a ¹⁵N NMR splitting in jump-return experiments carried out on a uniformly ¹⁵N-labeled WT-HK32 complex sample (Figure 3(c)). The ¹⁵N chemical shifts were determined to be 171(±2) ppm (Figure 3(d)) for the peak at 18.00 ppm and 195(±2) ppm for the peak at 12.67 ppm (Figure 3(e)) by performing a series of continuous wave decoupling experiments with step-by-step increase in ¹⁵N carrier frequency. These observations, like for bee venom sPLA₂, provide evidence for a LBHB involving a histidine residue in the bovine pancreatic sPLA₂-HK32 complex.

Specific assignment of the downfield proton resonances

Since the two downfield proton peaks originate from imidazole ring(s) of histidine residue(s), their

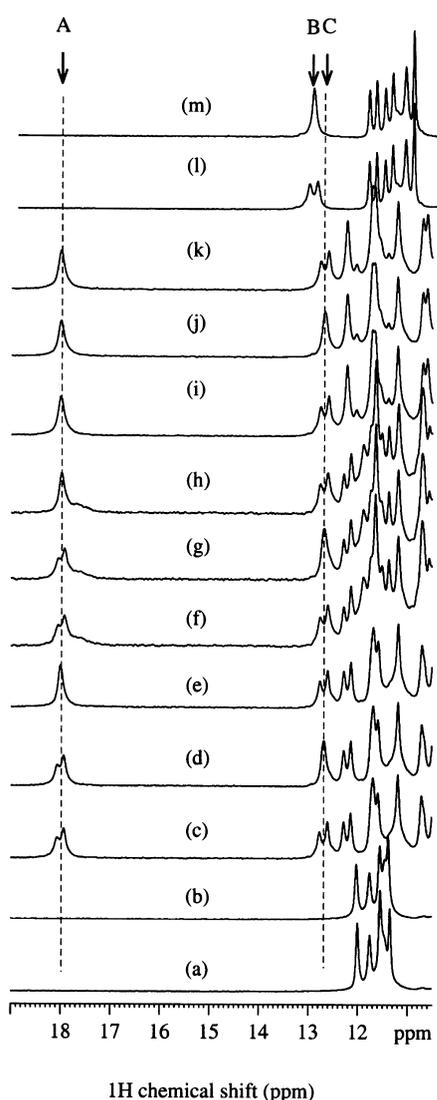


Figure 3. Downfield region of the ^1H NMR spectra at 600 MHz acquired on bovine pancreatic sPLA₂ with a jump-return pulse sequence at 285 K: (a) free WT, pH 6.9; (b) free WT + DMSO, pH 6.9; (c) ^{15}N -labeled WT + HK32, pH 7.1; (d) ^{15}N -labeled WT + HK32, pH 7.1, ^{15}N -decoupling at 171 ppm; (e) ^{15}N -labeled WT + HK32, pH 7.1, ^{15}N -decoupling at 194 ppm; (f) ^{15}N -labeled H115A + HK32, pH 7.5; (g) ^{15}N -labeled H115A + HK32, pH 7.5, ^{15}N -decoupling at 171 ppm; (h) ^{15}N -labeled H115A + HK32, pH 7.5, ^{15}N -decoupling at 194 ppm; (i) [$^{15}\text{N}^{\text{e}2}$]His labeled WT + HK32, pH 7.1; (j) [$^{15}\text{N}^{\text{e}2}$]His labeled WT + HK32, pH 7.1, ^{15}N -decoupling at 171 ppm; (k) [$^{15}\text{N}^{\text{e}2}$]His labeled WT + HK32 at pH 7.1, ^{15}N -decoupling at 194 ppm; (l) free ^{15}N -labeled WT, pH 5.1; and (m) free ^{15}N -labeled WT, pH 5.1, ^{15}N -decoupling at 168 ppm. The peaks designated by A, B and C are $\text{H}^{\text{e}1}$ /H48 in sPLA₂-HK32 complex, $\text{H}^{\text{e}2}$ /H48 in the imidazolium form of free PLA₂, and $\text{H}^{\text{e}2}$ /H48 in sPLA₂-HK32 complex, respectively.

assignments in bovine pancreatic sPLA₂ were simplified by constructing the H115A mutant that contains only the active site H48. As shown in Figure 3(f)–(h), both peaks were observed in this

mutant upon addition of the inhibitor with virtually identical chemical shifts and ^{15}N -decoupling behavior as for the WT-HK32 complex. Consequently, they were assigned to proton(s) attached to the imidazole ring of H48. For serine proteases (Asp-His-Ser catalytic triad) and a recently reported bacterial phosphatidylinositol-specific phospholipase C (PI-PLC; Asp-His catalytic dyad), the low-field ^1H NMR signal (16–20 ppm) has been attributed to the proton that is engaged in hydrogen bonding between the β -carboxylic group of Asp and the imidazole $\text{N}^{\text{e}1}$ of His at the active site (Figure 1(a)).^{2,23–25} It was thus tempting to assign the peak at 18.00 ppm to the $\text{H}^{\text{e}2}$, which bridges H48 and D99 in the case of bovine pancreatic sPLA₂, an assignment that appeared to be supported by the absence of this peak in the experiments performed with the D99N and H48Q mutants (spectra not shown). However, in order to establish the assignment, a [$^{15}\text{N}^{\text{e}2}$]histidine-labeled WT-sPLA₂ sample was prepared to distinguish $\text{H}^{\text{e}2}$ from $\text{H}^{\text{e}1}$. Surprisingly, it is the peak at 12.67 ppm, instead of 18.00 ppm, that shows a one-bond J -coupling with ^{15}N (Figure 3(i)–(k)). This observation conclusively shows that the peak at 18.00 ppm is $\text{H}^{\text{e}1}$ of H48 while the other at 12.67 ppm is $\text{H}^{\text{e}2}$ of H48.

The specific-labeling experiments were performed twice with two [$^{15}\text{N}^{\text{e}2}$]His samples obtained independently. We chose to repeat the experiments with the ^{15}N labeling at the same position, instead of at the other position, in order to exclude the possibility of switching or mislabeling samples. In the repeated experiment, the ^{15}N isotope position in the commercially obtained [$^{15}\text{N}^{\text{e}2}$]His sample was verified by chemical analyses before use. The J coupling constants and chemical shifts obtained in the NMR experiments were consistent with those previously published,²⁶ and the molecular ion peak $(\text{M} + \text{H})^+$ was observed at m/z 157.0 (>98% enrichment) by mass spectrometry.

Fractionation factor and exchange rate of the downfield proton resonance

The furthest downfield proton resonance exhibited the following additional physicochemical properties characteristic of a LBHB.^{15,27} (i) The $^2\text{H}/\text{H}$ fractionation factor was determined by integrating the downfield proton resonance as a function of the $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ solvent ratio. The results (Figure 4) show that the wild-type sPLA₂-HK32 complex displays a fractionation factor of $0.62(\pm 0.06)$, indicative of a relatively strong hydrogen bond.^{15,27–29} (ii) An Arrhenius plot of the temperature effect on the observed linewidth ($\Delta\nu_{1/2}$) of the downfield proton resonance in the wild-type sPLA₂-HK32 complex yielded a $k_{\text{ex}} \sim 83 \text{ s}^{-1}$ at 285 K and $\sim 237 \text{ s}^{-1}$ at 298 K. $k_{\text{intrinsic}}$ was estimated to be approximately 10^4 at pH 6.0, yielding an exchange rate protection factor of >100 at 285 K, and >40 at 298 K (Figure 5).

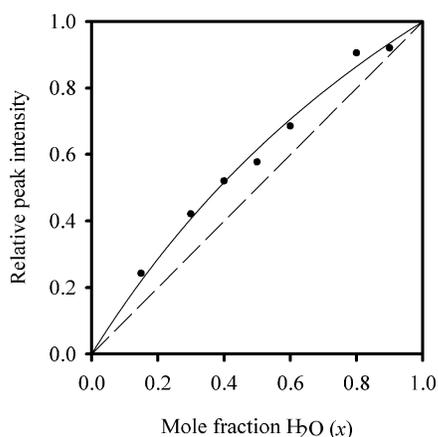


Figure 4. Determination of the fractionation factor ϕ^{H^1} of the most downfield proton resonance of the bovine pancreatic sPLA₂ complex with the transition-state analog (HK32). The value was found to be $0.62(\pm 0.06)$ by non-linear least squares fit to equation (1). The continuous curve and broken line correspond to the expected behavior when ϕ equals 0.62 and 1.0, respectively.

Determination of the pK_a change of H48

Formation of a LBHB is also characterized by a change in the pK_a of the donor and the acceptor.^{15,30} Here, the pK_a for the free enzyme was obtained by following the chemical shift change of [¹³C^{ε1}-¹H^{ε1}]His cross-peak from ¹H-¹³C HMQC experiments using a [¹³C^{ε1}]His labeled WT sample. It was noticed that during pH titration the peak of [¹³C^{ε1}-¹H^{ε1}]H48 disappeared around pH 6.0, likely broadened out due to an intermediate proton association/dissociation process. Furthermore, when pH is ≤ 3.5 , the cross-peak of

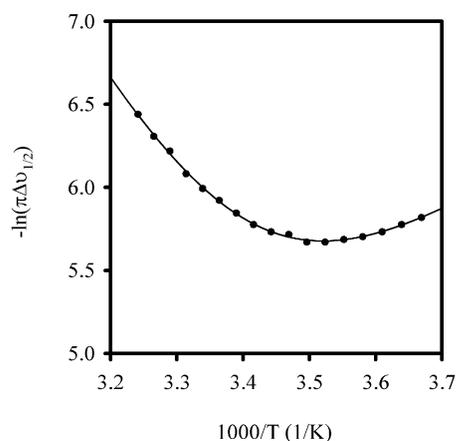


Figure 5. Determination of the exchange rate k_{ex} . The continuous curve through the experimental data is the Arrhenius plot of the temperature effect on the observed linewidth of the most downfield resonance in the WT sPLA₂-HK32 complex with the following parameters: $E_{\text{ex}} = 13.5(\pm 0.6)$ kcal/mol, $C_{\text{ex}} = 28.3(\pm 0.9)$, $E_{\text{d}} = -4.9(\pm 0.4)$ kcal/mol, and $C_{\text{d}} = -3.2(\pm 0.8)$.

[¹³C^{ε1}-¹H^{ε1}]H115 split into two peaks: one corresponds to the native protein (gradually disappeared at lower pH), and the other to a random coil (this observation is consistent with the previous suggestion that denaturation of this enzyme at acidic pH starts at the region close to the C terminus²¹). In spite of these problems, the NMR data were of sufficiently high quality to be fitted to a one-proton titration curve, and pK_a values of $5.7(\pm 0.1)$ and $6.2(\pm 0.1)$ were obtained for the imidazole rings of H48 (Figure 6(a)) and H115 (Figure 6(b)), respectively.

The pK_a of H48 in the WT-HK32 complex was measured by monitoring changes in the peak

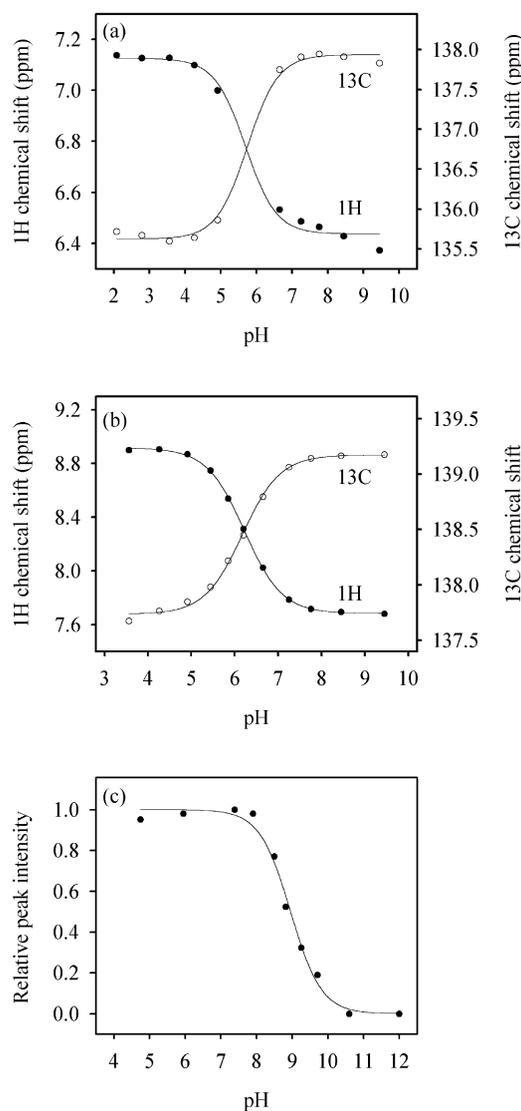


Figure 6. Determination of the pK_a of histidine residues in bovine pancreatic sPLA₂. (a) The pH titration curves of [¹³C^{ε1}]/[¹H^{ε1}]H48 and the fitting curves. Data were acquired by performing ¹H-¹³C HMQC experiments at 600 MHz on [¹³C^{ε1}]His specifically labeled WT sample. (b) The pK_a of H115 was obtained in the same experiments as (a). (c) The pK_a in the complex was obtained by following peak intensity changes of H^{ε1}/H48 upon pH titration. Data acquired by running 1D jump-return experiments were fitted to equation (5).

intensity rather than the chemical shift of the H^{δ1} resonance, because the free enzyme and enzyme–inhibitor complex are in slow exchange on the NMR timescale. The experimental data can be nicely fit to a one-proton titration curve, and the pK_a of H48 was found to be elevated to 9.0(±0.2) (Figure 6(c)). Therefore, in contrast to the free enzyme, H48 should be protonated at neutral pH when in the complex with inhibitor.

Active site histidine is partially positively charged in the sPLA₂-HK32 complex

It has been suggested that in chymotrypsin and subtilisin complexes with inhibitors the active site histidine is only partially charged (between 0.5 and 1.0) due to the depolarization of LBHB,^{2,3,25} as if the bridging proton is partially (about 20–30%) transferred from the donor (active site His) to the acceptor (active site Asp). However, the only experimental evidence presented is the ¹⁵N^{δ1} chemical shift (197.6 ppm) of the active site histidine of subtilisin, which is about 22.6 ppm downfield from a typical value (~175 ppm) for a =NH⁺ in model compounds.^{25,31} Here we present a more comprehensive analysis of both imidazole NH chemical shifts in comparisons with those in free enzyme as well as in model compounds, which strongly supports a partially positively charged H48 in the sPLA₂-HK32 complex.

In the previous NMR structural work of free bovine pancreatic sPLA₂ carried out at 310 K, neither H^{δ1}/H48 nor H^{ε2}/H48 could be observed.²⁰ In the present work, when the temperature was lowered to 285 K, a cross-peak was clearly detected at 11.46 ppm in the ¹H-dimension and at 167.6 ppm in the ¹⁵N-dimension in the 2D ¹H–¹⁵N HSQC spectrum recorded on a uniformly ¹⁵N-labeled sample at neutral pH. An identical cross-peak was observed when the [¹⁵N^{ε2}]His labeled sample was used (Figure 7(a)), resulting into two possible candidates for its assignment: [¹⁵N^{ε2}–¹H^{ε2}]H48 and [¹⁵N^{ε2}–¹H^{ε2}]H115. The peak was further assigned to [¹⁵N^{ε2}–¹H^{ε2}]H48 because of its NOE to H^{ε1}/H48 and H^{δ2}/H48 in the 2D NOESY spectrum (data not shown). The [¹⁵N^{ε2}–¹H^{ε2}]H48 peak was shifted to 13.02 ppm ¹H/172.2 ppm ¹⁵N when the sample was titrated to pH 5.1 (Figure 7(b)). The proton resonance at 13.02 ppm has been mentioned above (Figure 3(l) and (m)). Based on the results of pK_a determination, these cross-peaks are consequently assigned to ¹⁵N^{ε2}–¹H^{ε2} of H48 in the imidazole form (Figure 7(a)) and imidazolium form (Figure 7(b)) for spectra obtained at pH 7.1 and 5.1, respectively.

Upon addition of HK32 at pH 7.1, the cross-peak due to [¹⁵N^{ε2}–¹H^{ε2}]H48 was shifted to a location very close to that of Figure 7(b) (12.67 ppm ¹H/170.6 ppm ¹⁵N as shown in Figure 7(c)), accompanied by the appearance of the most downfield proton resonance at 18.00 ppm. The peak in Figure 7(c) can be assigned to the protonated form

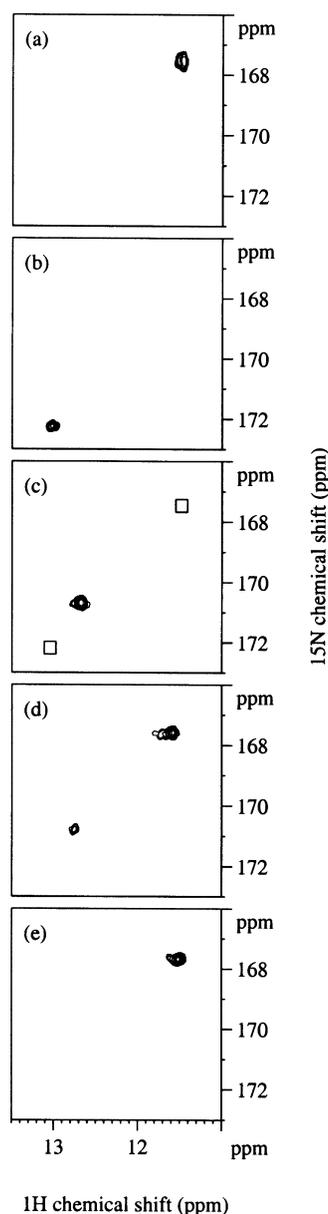


Figure 7. 2D ¹H–¹⁵N HSQC spectra at 600 MHz showing the positions of the [¹⁵N^{ε2}]/[¹H^{ε2}]H48 cross-peak in WT bovine pancreatic sPLA₂ under different conditions: (a) free WT at pH 7.1; (b) free WT at pH 5.1; (c) WT + HK32 at pH 7.1, the two boxes indicate the positions of cross-peaks in (a) and (b) for comparisons; (d) WT + HK32 at pH 8.5; and (e) WT + HK32 at pH 9.9.

of the sPLA₂-HK32 complex since the pH dependence studies described above indicate that the complex is protonated at pH 7.1. Upon titration toward the basic direction, the peak decreases in intensity and a new peak at the position identical to that of the peak in Figure 7(a) appears (Figure 7(d) and (e)). These observations suggest that the protonated complex is in slow exchange with the deprotonated free enzyme, although it cannot be ruled out that the inhibitor remains bound to the deprotonated enzyme and the chemical shift of H^{ε2}/H48 in the complex and the

enzyme are coincidentally the same. As shown in Figure 7(c), the [¹⁵N^{ε2}-¹H^{ε2}]H48 cross-peak in the complex sits almost on the diagonal line that links the unprotonated and protonated peaks of the free enzyme, suggesting that H48, though protonated, bears only a partial positive charge in the complex. When the chemical shifts of H^{ε2}/H48 in three different states (Figure 7(a)–(c)) are used for charge estimation, a positive charge of 0.78 is obtained for H48 in the complex.

It has been established from model compounds that the average chemical shift of the two nitrogen atoms of the imidazole ring indicates the ionization state of the ring; typically the ¹⁵N chemical shift is 176.5 ppm for an imidazolium ion and 207.5 ppm for the neutral species.³¹ In the sPLA₂-HK32 complex, the corresponding average ¹⁵N shift was found to be 182.8(±1) ppm for H48, which suggests a charge on the imidazole ring of 0.81. It should be noted that it is the [¹⁵N^{δ1}]H48 nitrogen resonance rather than the [¹⁵N^{ε2}]H48 resonance that significantly deviates from the typical value of ~176 ppm for an imidazolium ion. This is consistent with the proposed LBHB formed between N^{δ1}/H48 and the phosphonate oxygen of the inhibitor.

Discussion

Existence of a LBHB between H48 and inhibitor

Here, the use of single ¹⁵N-labeled histidine led to the unequivocal assignment of the downfield proton to H^{δ1}/H48, the proton that is bridging the active site His side-chain and a non-bridging phosphonate oxygen of the inhibitor. To the best of our knowledge, the only other example in which specific [¹⁵N]His has been used in the assignment of the downfield resonance (16–20 ppm) in protein NMR is that of α-lytic protease.²³ The results of the present study raise concern about assignment of downfield NMR peaks solely based on site-directed mutagenesis experiments or by simple comparison with serine proteases, which is commonly adopted by others.^{24,25,32,33} Even among serine proteases, it has been reported that the prolyl oligopeptidase family exhibits important dissimilarities in the active site compared to the pancreatic and subtilisin classes of serine proteases.³⁴ In the case of the prolyl oligopeptidase, non-catalytic histidine imidazole ring-attached protons are engaged in relatively strong hydrogen bonds based on the observation that their downfield chemical shifts are virtually independent of pH up to pH 9.5.³⁴ Furthermore, while in most situations a LBHB involves a carboxyl group from Asp and Glu residues,³⁵ our results indicate that this is not always the case. Conclusive assignment of the downfield proton resonance, to the specific His residue as well as to the specific ring nitrogen in our case, is important for the interpretation of experimental results.

The results reported here strongly suggest that in the bovine pancreatic sPLA₂-HK32 complex the H^{δ1} of His48 is involved in a LBHB, whereas H^{ε2} of His48 is engaged in a normal hydrogen bond. Both NMR studies²⁰ and the recent 0.97 Å ultra-high resolution crystal structure³⁶ of free bovine pancreatic sPLA₂ show that H48 and D99 form a H-bond involving the N^{ε2} of the imidazole ring. Taken together, we conclude that the LBHB is formed between H48 and the inhibitor. Another possibility to explain our NMR results is that the imidazole ring of H48 rotates 180 degree around the C^γ–C^β bond upon addition of the inhibitor,³⁷ such that N^{δ1} forms a H-bond with D99 in the complex. However, such a possibility is unlikely for the following reasons. (i) X-ray structures of several sPLA₂-inhibitor complexes do not reveal imidazole ring flipping.^{16–18,38,39} (ii) H48 ring flipping would require significant backbone conformational adjustment to bring N^{δ1}/H48 to within hydrogen bonding distance of O^{δ1}/D99. A 180-degree rotation around the C^β and C^γ of H48 would interchange N^{δ1} and C^{δ2}, and the new distance would be 3.84 Å for the atomic pair O^{δ1}/D99–N^{δ1}/H48,³⁶ which is too far apart for a H-bond. Thus, the N^{δ1} atom of H48 likely forms a hydrogen bond only with the transition-state analog. Although the presence of a LBHB between a catalytic residue(s) and substrate/inhibitor has also been suggested in other systems,^{33,40–42} our results are the first to indicate its existence in an Asp-His system and the first to be demonstrated convincingly by NMR. Furthermore, our results suggest that one should pursue the possible existence of a LBHB with binding of a transition state analog, even if it is clearly absent in the free enzyme.

The crystal structures of sPLA₂-MG14 complexes show that one of the non-bridging phosphonate oxygen atoms forms a hydrogen bond with the N^{δ1} atom of the active site histidine as shown in Figure 1(c).^{16–18} While such bond lengths were found to be 2.62 Å and 2.81 Å, respectively, for the crystal structures of two bovine pancreatic sPLA₂-TSA complexes and bovine pancreatic sPLA₂-inhibitor complex,^{18,39} it is possible that the bond length falls into the LBHB category, that is 2.5 < *d* < 2.6 Å,^{14,15} considering that the *R*-factors are 0.184 (resolution 1.89 Å) and 0.180 (resolution 1.96 Å), respectively.

Strength of the LBHB in the sPLA₂-TSA complex

Since the first proposal of LBHB in enzyme catalysis, two key issues have been under intensive debate. The first is whether the spectroscopic evidence, as described here and in many other papers, is sufficient for concluding the existence of LBHB. The second issue is the bond energy of the LBHB and how much it contributes to the catalysis of each specific enzyme. To date, the spectroscopic evidence has been largely accepted. However, experimental and theoretical data bearing on the

contribution of LBHB to enzyme catalysis varies greatly. The energy of LBHB has been suggested to be as large as 10–20 kcal/mol, and rate enhancements as much as five orders of magnitude have been proposed.^{2,3,6} On the other hand, Warshel *et al.*^{10,11} and Guthrie¹² have argued that evidence for short bond lengths of LBHBs in enzymes does not imply an unusually strong bond nor does the presence of a LBHB mean it has an important role in catalysis.

The bond strengths of LBHB have been estimated from the change in pK_a of the donor (increase) or acceptor (decrease), relative to the pK_a values for these species under conditions in which they are not engaged in a LBHB.^{15,30,43} This is illustrated with the aid of Figure 8. As shown, H48 can be in the protonated or non-protonated form, and enzyme in either protonation state can be bound to TSA (HK32). Under the assumption that the only difference in energetics between the complex of neutral H48 enzyme with TSA and the complex of protonated H48 enzyme with TSA is that the latter involves a LBHB between protonated H48 and phosphonate oxygen whereas the former does not contain a hydrogen bond between neutral H48 and phosphonate oxygen, the free energy change for the formation of the LBHB is given by $\Delta G_{\text{formation}} = 2.303RT[p^{\text{E}}K_a - p^{\text{EI}}K_a]$, where the equilibrium constants are defined in Figure 8. Note from Figure 8 that in the absence of bound TSA, H48, protonated or not, forms a hydrogen bond with water and this water is displaced into bulk solution when TSA binds to the active site.

Thus, the above equation also assumes that the hydrogen bond between a water molecule and neutral H48 has the same strength as the hydrogen bond between protonated H48 and water. Although this assumption may not be true, the difference in H48–water hydrogen bond strengths is presumably small compared to the strength of the LBHB, if in fact the LBHB is unusually strong. Here we have shown that in the absence of TSA, $p^{\text{E}}K_a = 5.7$. In the presence of HK32, we suspect that the observed pK_a is an apparent value because the NMR evidence suggests that deprotonation of H48 is accompanied by dissociation of HK32 from the enzyme as described in Results. In principle we could determine whether the pK_a of H48 remains invariant with increasing concentrations of HK32; however, this is not feasible because the critical micelle concentration of this inhibitor is in the micromolar range. If sPLA₂ with neutral H48 is fully bound to HK32, one obtains $\Delta G_{\text{formation}} = 4.5$ kcal/mol on the basis of $p^{\text{E}}K_a = 5.7$ and $p^{\text{EI}}K_a = 9.0$. This is a lower limit estimate for the strength of the LBHB. As described in Results, we know from the pH titration of enzyme in the presence of inhibitor that half of the downfield NMR signal from the LBHB remains when the pH is increased to 9.0 (i.e. 50% of the total enzyme is in the form of the complex between HK32 and H48-protonated enzyme at pH 9.0). Using the known values of the proton concentration (at pH 9.0), the total concentration of protein, and the total concentration of inhibitor, $^{\text{EI}}K_a$ can be calculated by solving the appropriate simultaneously equilibrium equations

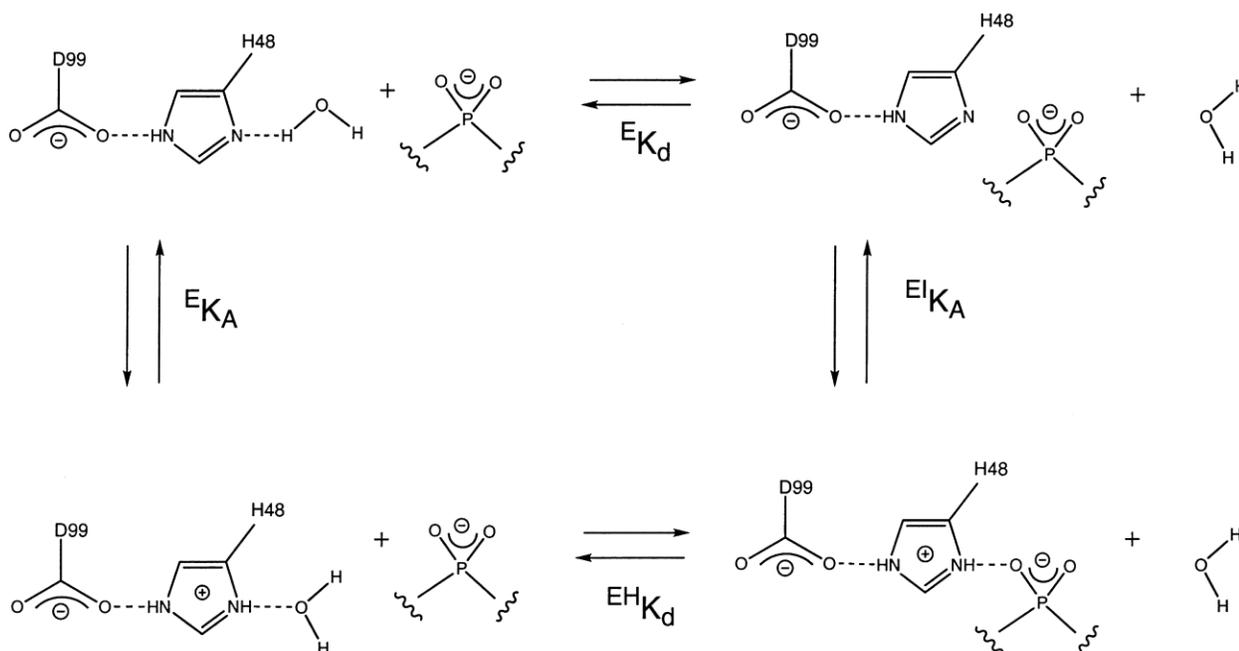


Figure 8. Thermodynamic scheme for the interaction of TSA with sPLA₂. $^{\text{E}}K_d$ is the equilibrium constant for the dissociation of TSA from enzyme containing H48 in the imidazole form, and $^{\text{EH}}K_d$ is the corresponding value when H48 is in the imidazolium form. $^{\text{E}}K_A$ is the equilibrium constant for the dissociation of the proton from the imidazolium enzyme, and $^{\text{EI}}K_A$ is the corresponding value when TSA is bound to the active site of the sPLA₂. For this closed thermodynamic box, $^{\text{EI}}K_A \cdot ^{\text{E}}K_d = ^{\text{E}}K_A \cdot ^{\text{EH}}K_d$.

as a function of ${}^E K_d$. Thus when ${}^E K_d$ is chosen to be well below the concentration of enzyme and HK32, enzyme remains bound to HK32 during the deprotonation of H48, and the limiting value of $\Delta G_{\text{formation}} = 4.5$ kcal/mol is obtained as described above. Since the data suggest that HK32 dissociates from enzyme when H48 deprotonates, ${}^E K_d$ is greater than the ~ 1 mM concentration of enzyme and HK32 in the NMR sample. For ${}^E K_d = 5$ mM, $\Delta G_{\text{formation}} = 5.4$ kcal/mol, for ${}^E K_d = 50$ mM, $\Delta G_{\text{formation}} = 6.6$ kcal/mol, and for ${}^E K_d = 500$ mM, $\Delta G_{\text{formation}} = 7.90$ kcal/mol. Thus, it would appear that 6–8 kcal/mol is a reasonable upper limit estimate for $\Delta G_{\text{formation}}$.

Due to the relatively weak strength, the “LBHB” referred to in this work should really be considered just as a “strong hydrogen bond” instead of a LBHB.

Possible functional roles of the LBHB in the catalytic mechanism of sPLA₂

The mechanism for sPLA₂ catalysis involving an Asp...His...H₂O catalytic triad (or Asp...His dyad) was first proposed in 1980 based on structural comparison of free enzymes with serine proteases.⁴⁴ Further evidence for their mechanism comes from the X-ray structures of sPLA₂ complexes with transition-state and substrate analogs for three of the four sPLA₂ classes.^{16,17,38,45} In this mechanism, the conserved catalytic water, which is found in all high-resolution crystal structures of free enzymes and sits adjacent to the active site histidine residue, is deprotonated by the histidine and attacks the carbonyl carbon of the *sn*-2 ester of the phospholipid substrate. The phosphonate transition-state analog was designed to mimic the tetrahedral intermediate formed during esterolysis, and the non-bridging phosphonate oxygen involved in the LBHB formation mimics the attacking hydroxyl group, the catalytic nucleophile. The fact that during the catalysis the catalytic histidine as well as the attacking nucleophile is buried in the enzyme's interior and devoid of hydrogen bonds to solvent⁴⁵ provides a favorable condition for potential LBHB formation between these two groups. Since the downfield proton was not observed in free sPLA₂ but arises upon addition of the transition-state analog, stabilization energy released from the formation of a LBHB may stabilize a reaction intermediate or the transition state, thus lowering the activation barrier of the esterolysis reaction. On the other hand, it is important to keep in mind that even though the HK32 here is a good transition state analog, the pK_a of phosphonate is different from that of the actual transition state structure, which is proposed to be a calcium-bound gem-diolate $(\text{HO}-\text{C}-\text{O}\cdots\text{Ca})^{+1}$. Since formation of LBHB depends critically on the pK_a of the hydrogen bond donor and the pK_a of the conjugate acid of the hydrogen bond acceptor, the conclusion derived from the use of phospho-

nate analogs could differ from that of the actual reaction involving natural substrates.

The importance of a H-bond between H48 and the substrate in the enzyme mechanism has also been implicated in inhibition studies of other sPLA₂.^{38,46} It was reported that the substrate analog with an amide functional group in place of the *sn*-2 ester displays stronger binding affinity for sPLA₂ from cobra venom at basic pH, whereas a phosphonate transition-state analog binds most effectively at acidic pH.⁴⁶ These results are consistent with X-ray structural studies showing that the amide inhibitor donates its amide NH to the non-protonated N^{δ1} of the active site histidine,³⁸ whereas the phosphonate oxygen accepts a H-bond from the protonated N^{δ1}.¹⁶

Comparison with serine proteases

Although the location of the LBHB is clearly different between the two types of enzymes, the spectroscopic properties for the LBHB of sPLA₂ reported above are largely the same as those of the LBHB of serine proteases. One notable difference is that the pK_a of the active site histidine changes from 5.7 in free sPLA₂ to 9.0 when it is bound to the TSA, which is somewhat smaller than the corresponding changes in serine proteases (from 5.8 to 11–12).^{30,47} Qualitatively, these results suggest that the strength of the LBHB in sPLA₂ is weaker than that in serine proteases by ca 2–3 kcal/mol.

In the case of serine proteases, the contribution of LBHB to catalysis is often estimated from the decrease in k_{cat}/K_m (by a factor of ca 10^5) when the active site Asp is mutated to Asn or Ala.^{14,15} It is important to note that this loss cannot be attributed entirely to the loss of LBHB. In the case of trypsin, it has been demonstrated that in the crystal structure of the D102N mutant the tautomeric form of the His residue is reversed.⁴⁸ Thus a substantial fraction of the loss of activity is likely to be caused by the change in the tautomeric form of the His residue. Consistent with this interpretation, a recent report has concluded that the LBHB is not an inherent requirement for substantial rate enhancement for subtilisin BPN', since mutation of the active site Asp32 to Cys leads to a modest eightfold decrease in k_{cat}/K_m compared to that of the WT enzyme.⁴⁹

Interestingly, the crystal structure of the D99N mutant of bovine pancreatic sPLA₂ shows that the carbonyl group of the N99 side-chain remains H-bonded to N^{ε2} of His48 as in WT sPLA₂, therefore maintaining the tautomeric form of H48,⁵⁰ which is in contrast to the observation in the corresponding Asp to Asn mutation in trypsin.⁴⁸ Thus, the active site structure is likely little perturbed in the D99N mutant of sPLA₂. This is in agreement with the observation that the LBHB could still be observed in the D64N mutant of bee venom sPLA₂. It is not clear why the 18 ppm peak was not detectable for the D99N mutant of bovine

sPLA₂ in complex with the transition state analog (data not shown); however, the negative result may not necessarily suggest the absence of LBHB. These results together can explain that various functional properties of the Asp-to-Asn mutant of PLA₂ are only modestly perturbed. For example, substitution of D99 with asparagine only resulted in a 20-fold decrease in k_{cat} relative to WT bovine pancreatic sPLA₂,⁵⁰ and the corresponding decrease for bee venom sPLA₂ is 50-fold.⁵

One might wonder why a LBHB forms between the Asp and His of serine proteases but not between the corresponding pair of residues in sPLA₂. A possible reason is the difference in structural arrangement of the Asp-His pair in these two classes of enzymes. In serine proteases, the H^{δ1} of the active site His donates a H-bond to the *syn* lone pair of the Asp, whereas sPLA₂s use the *anti* lone pair on the Asp to accept a H-bond from the H^{ε2}/His. Gandour has provided strong evidence that the *syn* lone pair of a carboxylate is more basic than the *anti* lone pair.⁵¹ In serine proteases, the use of the *syn* lone pair (Figure 1(a)) may allow more transfer of the H^{δ1} His proton toward the carboxylate oxygen, since the pK_a of the conjugate acid of the *syn* lone pair protonated Asp is closer in magnitude to the pK_a of the imidazolium. This may contribute to the observed LBHB formation. In sPLA₂, the pK_a of the *anti* lone pair protonated Asp may be far enough below the pK_a of the imidazolium (the sPLA₂ Asp pK_a is not known) such that the proton is much closer to the His N^{ε2} than to the carboxylate oxygen, resulting in a normal H-bond.

Conclusion

The two classes of hydrolases, sPLA₂s and serine proteases, utilize similar active site architecture (though with notable differences) and employ a general-base mechanism. Both contain a LBHB in their active sites; however, the site of formation of the LBHB is different. While the LBHB is formed between the catalytic Asp and His residues in serine proteases for both free and inhibitor-complexed forms, it is between the catalytic His residue and a non-bridging phosphonate oxygen of a bound transition-state analog in the case of sPLA₂. Our data do not provide support for an unusually strong hydrogen bond strength (i.e. >10 kcal/mol) for this LBHB. The LBHB is not observed in free sPLA₂. The normal H-bond between the active site Asp and His in sPLA₂ may serve to fix the orientation of the histidine imidazole ring.^{5,20}

Materials and Methods

Materials

Bovine pancreatic sPLA₂ was over-expressed in *Escherichia coli* strain BL21(DE3)[pLysS] carrying the

pET25b(m)-prosPLA₂ plasmid and purified as described.⁵² H115A was constructed by site-directed mutagenesis using the Quickchange method (Stratagene) with pET25(m)-prosPLA₂ as template. Oligonucleotides used were ordered from IDT Inc. (Coralville, IA), and the sequence (complementary set) was as follows: 5'-CCT TAT AAC AAA GAA GCC AAG AAT CTT GAT AAA-3'. The H115A mutant was found to be relatively unstable and only low yields, in comparison with WT, were obtained. The preparation of isotope-labeled samples was achieved essentially as described⁵² except that different media were used to grow cells. For ¹⁵N uniform labeling, the cells were grown in M9 minimal medium with (¹⁵NH₄)₂SO₄ as the sole nitrogen source. For selective labeling, the cells were grown in a synthetic rich medium with isotopically enriched amino acids ([¹⁵N^{ε2}]His or [¹³C^{ε1}]His). All of the labeling materials were from Cambridge Isotope Laboratories (Andover, MA). The [¹⁵N^{ε2}]His labeling materials were ordered twice, and the second sample was further characterized with NMR and mass spectrometry.

Bee venom sPLA₂ was produced by refolding *E. coli* inclusion body protein as described.⁵³ The phosphonate transition-state analogs, MG14 and HK32, were synthesized as described.^{16,54}

NMR experiments on bee venom sPLA₂

1D ¹H NMR experiments were carried out on a Bruker DRX-500 spectrometer at 298 K using a jump-return sequence for solvent suppression.⁵⁵ The NMR samples contained 0.1–0.4 mM protein, prepared in 95% H₂O/5% ²H₂O with 25 mM NaCl, 4 mM CaCl₂, 0.02% (w/v) NaN₃, and 10–20 mM d₃-HOAc, at pH* 4.5–4.8 (uncorrected). The phosphonate transition-state analogs MG14 and HK32 were typically dissolved in a pH 4.5 stock of d₃-HOAc to a concentration of 20 mM, and added to the NMR sample to give a [inhibitor]/[protein] mole ratio of 2–5.

NMR experiments on bovine pancreatic sPLA₂

NMR samples of bovine pancreatic sPLA₂ typically were 0.4–0.7 mM protein, except 0.15 mM for H115A, which aggregates at higher concentration, and 0.2 mM for D99N, due to low yield. Unless stated otherwise, lyophilized protein was prepared in 90% H₂O/10% ²H₂O, with 200 mM NaCl and 50 mM CaCl₂, at pH* 6.0–7.5 (uncorrected). The phosphonate transition-state analog HK32 inhibitor was dissolved in d₆-DMSO to make a stock solution of 25–50 mM, and the [inhibitor]/[protein] mol ratio was 1–2. Since narrower linewidths were obtained around 285 K, which in turn improved the signal-to-noise ratio, NMR experiments were carried out at 285 K on a Bruker DMX-600 spectrometer, unless otherwise stated. The jump-return method⁵⁵ was used to suppress the water signal with the carrier frequency set on the water signal and the delay between the pulses optimized to achieve maximum excitation at 15 ppm. Selective ¹⁵N decoupling was achieved by applying low power (25 dB) on the ¹⁵N channel with the ¹⁵N carrier frequency set to a desired value. Data were processed using the XWINNMR software (Bruker). Typically zero-filled and window multiplication were applied prior to Fourier transformation. ¹H chemical shifts were calibrated against the external standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and ¹⁵N chemical shifts were referenced to liquid NH₃.

Fractionation factor determination on bovine pancreatic sPLA₂

The fractionation factor for the downfield peak was determined by conducting jump-return experiments on regular WT samples dissolved in H₂O/²H₂O solvent mixtures of seven different ratios. These samples contained 0.7 mM protein, 200 mM NaCl and 50 mM CaCl₂ at pH* 6.0 (uncorrected) and ²H₂O volume percentages of 10%, 20%, 40%, 50%, 60%, 70%, and 85%. An equal amount of HK32 stock solution was added to each sample to give a [inhibitor]/[protein] mol ratio of ~1.5. Samples were then incubated for at least 12 hours at room temperature before NMR experiments. Experiments were repeated after three months of incubation to ensure complete equilibrium of labile protons in the downfield region. The fractionation factor was deduced from the quantitative measurement of the downfield peak intensity as a function of deuterium content by non-linear regression analysis to the following equation:²⁸

$$I = \frac{I_{\max}(x)}{\phi(1-x) + x} \quad (1)$$

where x is the mol fraction of H₂O in the H₂O/²H₂O mixture, ϕ is the fractionation factor of the measured proton, I is the relative peak intensity normalized to an upfield methyl resonance (an internal reference) at a given x , and I_{\max} is the maximal intensity when x equals 1.0.

Determination of the proton exchange rate and protection factor in bovine pancreatic sPLA₂

The proton exchange rate of the downfield peak with solvent was determined based on the temperature dependence of the linewidth of this resonance in 1D ¹H NMR spectra. The regular WT sample was equilibrated in the probe for at least 15 minutes before acquisition at each experimental temperature (272.5–308.5 K), which has been calibrated with neat methanol as described elsewhere.¹⁵ Duplicate experiments were performed to ensure consistency of the data. The data were fitted to the following equation¹⁵ with the use of SigmaPlot software (SPSS Inc.):

$$\ln\left(\frac{1}{\pi\Delta\nu_{1/2}}\right) = \ln\left[\exp\left(-\frac{E_{\text{ex}}}{RT} + C_{\text{ex}}\right) + \exp\left(-\frac{E_{\text{d}}}{RT} + C_{\text{d}}\right)\right] \quad (2)$$

where $\Delta\nu_{1/2}$ is the linewidth at a given temperature; E_{ex} and C_{ex} are the activation energy and Arrhenius coefficient, respectively, for the proton exchange process, and E_{d} and C_{d} are the corresponding set associated with the dipolar contribution. The pseudo-first-order exchange rate at temperature T was calculated as follows:

$$\ln(k_{\text{ex}}) = -\frac{E_{\text{ex}}}{RT} + C_{\text{ex}} \quad (3)$$

The protection factor was calculated as $k_{\text{intrinsic}}$ over k_{ex} , in which $k_{\text{intrinsic}}$, the pseudo-first-order exchange rate in the absence of hydrogen bonding, can be estimated from the experimental conditions as described by Mildvan *et al.*¹⁵

Determination of the pK_a of H48

Two-dimensional ¹H-¹³C HMQC spectra were recorded on a [¹³C^{e1}]His specifically labeled WT bovine

pancreatic sPLA₂ over the pH range 2.1–9.5. The pK_a of H48 in the free form was determined by following the chemical shift changes of the [¹³C^{e1}]/[¹H^{e1}]H48 cross-peak upon pH titration. The experimental data were non-linear least-squares fitted to the following one-proton titration equation (SigmaPlot, SPSS Inc.):

$$\delta_{\text{obs}} = \delta_{\text{A}} + (\delta_{\text{HA}} - \delta_{\text{A}}) \times [10^{-\text{pK}_a} / (10^{-\text{pK}_a} + 10^{-\text{pH}})] \quad (4)$$

in which δ_{obs} is the observed peak chemical shift (¹H or ¹³C) at a given pH, and δ_{A} and δ_{HA} are the corresponding chemical shifts in the unprotonated and protonated imidazoles, respectively.

One-dimensional ¹H jump-return experiments were conducted on a regular WT sample in the presence of HK32 upon pH titration (pH 4.8–12.0).³⁰ Care was exercised to minimize the loss of sample, and small volume changes were taken into account for correction. The pK_a of H48 in the complex was obtained by fitting the titration data to the following equation:

$$I_{\text{obs}} = I_0 \times [10^{-\text{pK}_a} / (10^{-\text{pK}_a} + 10^{-\text{pH}})] \quad (5)$$

where I_{obs} is the relative peak intensity of the downfield peak at a given pH, and I_0 is the maximum relative peak intensity at low pH (normalized to 1.0).

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