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Critical role of a hydrogen bond in the interaction of phospholipase A_2 with transition-state and substrate analogues

(phospholipase A_2 inhibitor/pH effect/Triton X-100 mixed micelles/interfacial binding)

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ABSTRACT **The inhibition of phospholipase A_2 by an amide substrate analogue, 1-hexadecylthio-2-hexadecanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphocholine, and a phosphonate transition-state analogue, 1-hexadecylthio-1-deoxy-2-hexadecylphosphono-*sn*-glycero-3-phosphocholine, is dramatically influenced by pH. However, these two inhibitors show opposite pH dependencies. The amide analogue acts more potently under basic conditions, whereas the phosphonate acts more potently under acidic conditions. In both cases, ligand binding is perturbed by protonation of an enzyme functional group with an apparent pK_a of 6.1, which corresponds to that of a histidine residue. Thus, His-48, which has previously been implicated in catalysis, appears to be critically involved in the hydrogen bond interactions between the enzyme and these two inhibitors. The amide analogue binds most effectively to the enzyme when His-48 is deprotonated. Upon protonation of the histidine residue, the amide cannot form a critical hydrogen bond and loses its ability to interact effectively with the enzyme. In contrast, the phosphonate analogue binds much tighter to the protonated form of the enzyme than to the deprotonated form. The phosphonate analogue needs a bridging hydrogen between the oxygen on its phosphorus atom and the Nδ1 of His-48 to form a strong hydrogen bond. At optimal pH values for inhibitor binding, both the amide and the phosphonate analogues are potent competitive inhibitors of cobra (*Naja naja naja*) venom phospholipase A_2. The IC_{50} for the amide was 4.4 $\times 10^{-4}$ mol fraction and for the phosphonate was 1.6×10^{-5} mol fraction. Under the experimental conditions used, this corresponds to a bulk concentration of 2 μM and 70 nM, respectively.**

Recently, two types of reversible, tight-binding phospholipase A_2 inhibitors have been rationally designed and characterized. The first type is composed of amide analogues in which an amide functional group is used to replace the scissile ester bond at the *sn*-2 position of phospholipids (1, 2). The other is composed of phosphonate analogues in which a tetrahedral phosphonate group is used in place of the *sn*-2 ester bond of phospholipids to mimic the putative transition state of phospholipase A_2-catalyzed hydrolysis (3). Crystallographic studies of phospholipase A_2 complexed with these analogues have suggested that both inhibitors interact with the enzyme via a strong hydrogen bond (4–6). These studies indicate that the amide analogue forms a hydrogen bond through the amide hydrogen with the Nδ1 atom of His-48. The phosphonate analogue forms a hydrogen bond through one of the nonbridging oxygen atoms of the phosphonate group with the protonated Nδ1 atom of His-48. In this manuscript, we explore the ramifications of these findings on the binding of various inhibitors to the enzyme.

The concept of transition-state analogues has been successfully applied to develop potent inhibitors of a variety of enzymes (7). Such transition-state analogues have been designed based on the premise that an enzyme catalyzes a reaction by binding the transition state much more tightly than the ground state of the substrate (8–10). Therefore, a molecule that closely resembles the transition state would act as a tight enzyme inhibitor. We have now synthesized two inhibitors, an amide and a phosphonate analogue, both of which contain the same fatty acid chain length at the *sn*-2 position, a thioether function at the *sn*-1 position, and a phosphorylcholine head group (1); they differ only in the amide and phosphonate functional groups in the *sn*-2 position. We found that the phosphonate analogue functions as a 4-fold less potent inhibitor than the amide analogue at the pH most often used to study the enzyme (pH 8.5), although both compounds are effective in the micromolar concentration range and bind to the enzyme ≈3 orders of magnitude tighter than its natural substrate. It was surprising that the putative transition-state analogue binds to the enzyme less well than the amide substrate analogue. To investigate the unexpected behavior of the phosphonate analogue, we have explored the effect of pH on the interaction of phospholipase A_2 with the amide and phosphonate analogues. As will be shown, the inhibition of phospholipase A_2 by amide and phosphonate analogues depends strongly on the ionization of the catalytic histidine residue of the enzyme. Under optimal conditions, which may not be physiologic, the phosphonate analogue is indeed a very effective inhibitor, which binds to the enzyme ≈30-fold tighter than the amide analogue.

EXPERIMENTAL PROCEDURES

Materials. Phospholipase A_2 was purified to homogeneity from lyophilized cobra venom (*Naja naja naja*) as described (11). 1-Hexadecylthio-1-deoxy-2-hexadecylphosphono-*sn*-glycero-3-phosphocholine and 1-hexadecylthio-2-hexadecanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphocholine were synthesized as described (unpublished data). 1,2-Bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine (thio-PC) was prepared as described (1, 12). 4,4′-Dithiodipyridine was purchased from Aldrich, and Triton X-100 was purchased from Sigma.

Enzyme Assay. Phospholipase A_2 hydrolysis of phosphatidylcholine in mixed micelles with Triton X-100 was determined spectrophotometrically at 324 nm using thio-PC as a substrate (12). Activity was followed at 30°C by monitoring the production of 4-thiopyridine, a product of the reaction of 4,4′-dithiodipyridine with the thiol released on phospholipid hydrolysis. The standard buffer contained 0.1 M KCl, 10 mM $CaCl_2$, 0.25 mM Triton X-100, and 25 mM buffer at a given pH. The buffers used were acetate (pH 5.5), 2-(*N*-morpho-

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Abbreviation: thio-PC, 1,2-bis(decanoylthio)-1,2-dideoxy-*sn*-glycero-3-phosphocholine.

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line)ethanesulfonic acid (pH 6.0–7.5), Tris·HCl (pH 8.0–9.0). As the extinction coefficient of 4-thiopyridine is pH dependent, enzyme activity was calculated by using the corresponding extinction coefficient at each pH as reported elsewhere (12).

Kinetic Studies. In this study, the thio-PC substrate is in the form of mixed micelles with Triton X-100, which has a critical micelle concentration of 0.24 mM. To define this micellar substrate, both its bulk and surface concentrations have to be given. The bulk concentration is expressed in terms of the molar concentration of the total substrate dissolved in the solution and the surface concentration in the mol fraction of the substrate in the micelle, which depends on the effective ratio (R_e) of detergent to substrate in aggregates (13). Since some of the detergent is still present as monomers in the mixed micellar solution, it has to be taken into account when one calculates the substrate surface concentration. In the present study, 0.24 mM Triton X-100 is included in the standard buffer to approximate the monomer concentration, so that the substrate surface concentration can be simply calculated based on the additional Triton X-100 added.

Phospholipase A_2 exhibits the phenomenon of "surface dilution kinetics" in that its activity depends not only on the bulk concentration of substrate, S, but also on its surface concentration, X (14–16). This is shown in the boxed scheme of Fig. 1, along with the pH effects and the effect of a competitive inhibitor that binds to the enzyme only in the second step. This scheme was developed from the kinetic model of Hendrickson and Dennis (16) with the assumption of rapid equilibrium kinetics. To deal with this surface dilution phenomenon, two sets of kinetic experiments were carried out, each of which simplifies the kinetics into a pseudo-single substrate system (16). In the first, substrate dependence curves were obtained by varying the bulk concentration of substrate while the surface concentration was held constant (case I). In the second, the surface concentration of substrate was varied while the bulk concentration was held constant (case II). In the present study, the bulk concentration of substrate was varied in case I from 0.02 to 0.5 mM at a surface concentration of 0.111 mol fraction. In case II, the experiments were carried out by varying the surface concentration of substrate from 0.02 to 0.2 mol fraction at a bulk concentration of 0.5 mM. A substrate dependence curve was obtained for both cases I and II at different pH values. The data from these substrate dependence curves were fitted by nonlinear regression analysis. The apparent maximal velocity, V_{app}, and substrate dissociate constant, K_{app}, at each pH were calculated by using the Michaelis–Menten equation for case I and the Hill equation for case II with a Hill coefficient of 1.66.

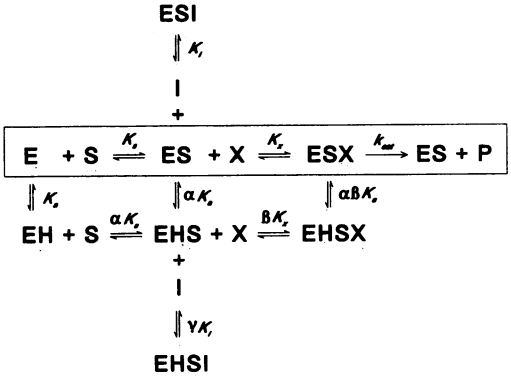

FIG. 1. Proposed kinetic scheme for phospholipase A_2 inhibition by the amide and the phosphonate analogues. All of the constants are dissociation constants.

The kinetic parameters and the pK_a were determined from the V_{app} and K_{app} values obtained above by fitting the data to Eqs. 1 and 2:

$$V_{app} = C_1/(1 + C_2 H/K_a) \quad [1]$$

$$K_{app} = (1 + C_3 H/K_a)/(C_4 + C_5 H/K_a), \quad [2]$$

which are derived from the scheme in Fig. 1 in the absence of an inhibitor. As shown in the scheme, the enzyme is assumed to exist in both protonated and deprotonated forms, and both forms are assumed to bind substrate but with different affinities. In Eqs. 1 and 2, H represents the proton concentration, K_a is the dissociation constant of the ionizable group, and the meaning of the remaining constants depends on how the experiments were carried out. They are listed in Table 1.

Inhibition Studies. The inhibition of phospholipase A_2 by phospholipid analogues was evaluated by measuring the half-maximum inhibition (IC_{50}) as described (1, 2). For the amide analogue, the enzymatic reaction was initiated by adding the enzyme to the mixture of the inhibitor and the substrate in Triton X-100 mixed micelles. For the phosphonate analogue, the assay was carried out by adding the substrate to the enzyme solution containing the desired amount of the inhibitor in mixed micelles to initiate the reaction. The linear portion of the reaction progress curve was used to calculate the initial velocity. The standard assay mixture consisted of 0.5 mM thio-PC and 4 mM Triton X-100 in the standard buffer at a given pH. IC_{50} values were calculated by fitting data to Eq. 3:

$$v = C/(IC_{50} + I), \quad [3]$$

where v is the velocity at the inhibitor surface concentration I, and C is equal to $V_{max}K_iX/K_x$. IC_{50} is determined by adding the inhibitor to the substrate solution containing constant bulk concentrations of thio-PC substrate and Triton X-100. Since the maximal inhibitor concentration used in this study is 0.05 mM, which is ≈1% of the total surfactant concentration, the dilution of the substrate surface concentration is negligible. At a constant surface concentration of substrate, C is also a constant. This equation is also derived from the scheme in Fig. 1 at a constant pH with the assumption of competitive inhibition. IC_{50} values are given in surface concentration units (mol fraction), assuming that all of the inhibitor is bound to the mixed micelle; for discussion purposes only, the corresponding bulk concentration is also given.

RESULTS

Dependence of Enzyme Activity on pH. To ascertain that the pH effect on enzyme catalysis and substrate binding was not caused by enzyme denaturation, the stability of cobra venom phospholipase A_2 at different pH values was determined. The enzyme was first incubated at a given pH for 5 min, and then its activity was measured at pH 8.5 using 0.5 mM thio-PC substrate. At pH values between 5.5 and 9.0, there was no

Table 1. Parameters in Eqs. 1 and 2

	Case I	Case II
C_1	$V_{max}X/(K_x + X)$	V_{max}
C_2	$[K_x/\alpha + X/(\alpha\beta)]/(K_x + X)$	$1/(\alpha\beta)$
C_3	1	$(K_sK_x + K_xS/\alpha)/(K_sK_x + K_xS)$
C_4	$(K_x + X)/(K_sK_x)$	$S/(K_sK_x + K_xS)$
C_5	$[K_x/\alpha + X/(\alpha\beta)]/K_sK_x$	$S/[(\alpha\beta)(K_sK_x + K_xS)]$

V_{max} is the product of k_{cat} and E_0 (total enzyme). All other constants are defined in Fig. 1.

significant change in enzymatic activity within experimental error. This result indicates that the enzyme is stable over the pH range used in the present studies.

A series of substrate-dependence experiments were carried out at each pH, and V_{app} and K_{app} values were determined. The effects of pH on the apparent maximal velocity V_{app} for both case I and case II are shown in Fig. 2. The phospholipase A_2 activity increases with pH, reaching a plateau at pH 7 for both cases, consistent with the titration of a single base (Fig. 2). The effects of pH on the apparent binding constants, K_{app} values for case I and case II, are shown in Fig. 3. It should be noted that the units in apparent binding constants K_{app} for case I and case II are different; mol fraction is used for the latter and micromolar is used for the former. The pH profiles are identical. Substrate bindings increase with increasing pH, plateauing above pH 7. The pK_a determined was 6.1 ± 0.4, which is consistent with the pK_a value of 5.9 previously determined via calcium binding studies (17). The kinetic parameters were also determined. V_{max} is 620 μmol·min^{-1}·mg^{-1}. K_s is 0.233 mM and K_x is 0.0425 mol fraction. α and β equal 1.3 and 2.6, respectively.

The kinetic data collected under case II should follow simple Michaelis–Menten kinetics according to the working model proposed in Fig. 1. However, the experimental data obtained between pH 5.5 and 9.0 all fit best to a Hill equation with the Hill coefficient of 1.66, indicating that substrate binds to the enzyme in a cooperative manner at the interface. Previous studies have suggested that the enzyme has two binding sites (a catalytic and an activator site) and phosphatidylcholine substrates bind to both (18–20). Whether an additional binding step should be considered for the hydrolysis of thio-PC is not clear at this time.

Effect of pH on the Interaction Between Phospholipase A_2 and Its Inhibitors. The analysis of phospholipase A_2 inhibition was carried out by using Eq. 4:

$$IC_{50} = [C_1 + C_2(H/K_a)]/[1 + H/(\alpha\gamma K_a)], \quad [4]$$

which is derived from a rapid equilibrium mechanism with assumption of the competitive inhibition of phospholipase A_2 by substrate analogues. As shown in Fig. 1, the enzyme first binds to the lipid–water interface through as yet undefined interactions (probably electrostatic interactions) with zwitterionic PC substrate and substrate analogue in the interface. After being bound to the interface, the enzyme then binds either a substrate or an inhibitor in the catalytic site. Since the inhibitor concentration is always <1/100th of the substrate concentration, its effect on the binding of the enzyme to the lipid–water interface is negligible. Thus, the interaction of the enzyme and its potent inhibitor is only shown after the enzyme binds to the interface. This kinetic scheme does not distinguish between ordered and random additions of substrate and inhibitor or between monomers and dimers as the active form of the enzyme.

In Eq. 4, C_1 is equal to $(K_s/S + 1 + X/K_x)K_i$ and C_2 is equal to $[K_s/S + 1/\alpha + X/(\alpha\beta K_x)]K_i$. As discussed in *Experimental Procedures*, the effect of the addition of the inhibitor to the assay solution on the substrate surface concentration is negligible. Therefore, the substrate surface concentration is considered to be a constant. Since the IC_{50} values were determined under conditions where both the bulk and surface concentrations of substrate were held constant, both C_1 and C_2 are constants. The assumption that these inhibitors are competitive was made because the crystallographic studies indicate that a single amide or phosphonate analogue binds to the enzyme in the putative catalytic site (4–6).

The inhibition of phospholipase A_2 by the amide substrate analogue is pH dependent. The affinity of the amide for the enzyme is enhanced with increasing pH as shown in Fig. 4. The data were fit to Eq. 4. Utilizing the pK_a and α values determined from kinetic studies, the difference in the dissociation constant for the binding of the amide to the active site of the deprotonated enzyme (K_i) and to the active site of the protonated enzyme (γK_i) is calculated to be ≈17-fold. Thus, the amide substrate analogue binds much tighter to the deprotonated enzyme than to the protonated enzyme.

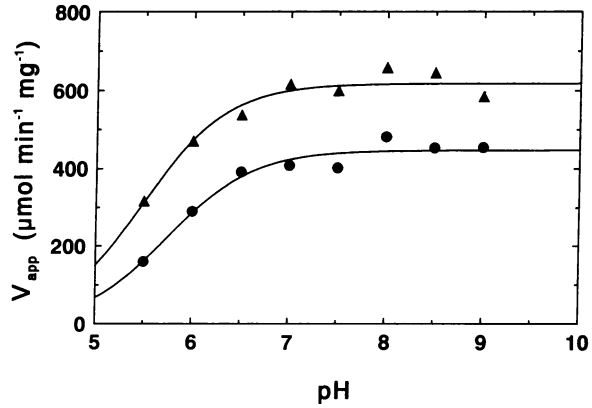

FIG. 2. Phospholipase A_2 activity (V_{app}) as a function of pH. Standard assay conditions are described in *Experimental Procedures*. Solid lines are drawn from a nonlinear least-squares fit to Eq. 1. ●, Case I; ▲, case II.

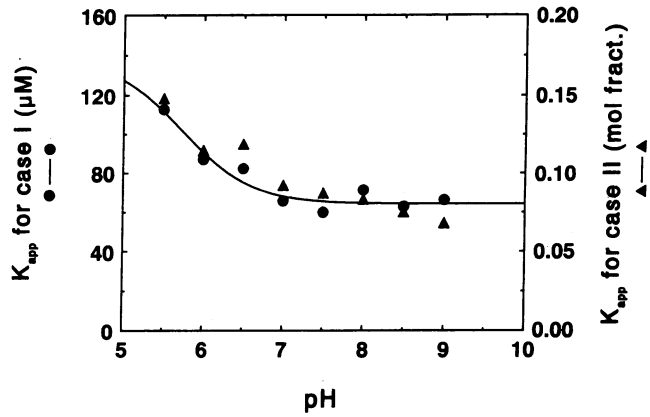

FIG. 3. Plot of apparent substrate binding constants as a function of pH. ●, K_{app} values for case I determined by fitting to the Michaelis–Menten equation; ▲, K_{app} values for case II determined by fitting to the Hill equation with a Hill coefficient of 1.66.

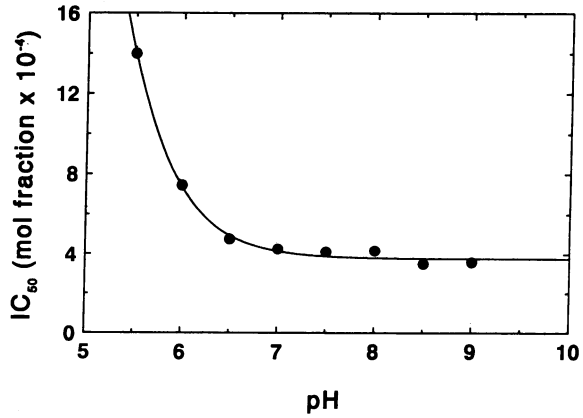

FIG. 4. Effect of pH on phospholipase A_2 inhibition by amide substrate analogue. IC_{50} values were determined by using substrate consisting of 0.5 mM thio-PC in Triton X-100 mixed micelles with an R_e of 8:1 (Triton X-100/phospholipid). Solid line was drawn by fitting the data to Eq. 4.

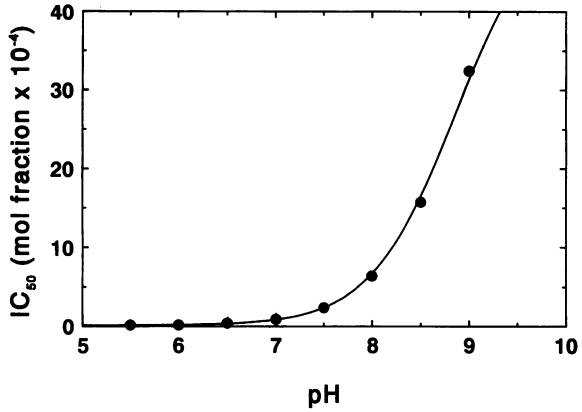

FIG. 5. Effect of pH on phospholipase A_2 inhibition by phosphonate transition-state analogue. IC_{50} values were determined by using substrate consisting of 0.5 mM thio-PC in Triton X-100 mixed micelles with an R_e of 8:1 (Triton X-100/phospholipid). Solid line was drawn by fitting the data to Eq. 4.

The pH dependence of phospholipase A_2 inhibition by the phosphonate analogue is shown in Fig. 5 and again the inhibition data were fit to Eq. 4. The affinity of the phosphonate analogue for the enzyme is strongest in the acidic region and diminishes with increasing pH. Using a pK_a of 6.1 and an α value of 1.3, the difference in the dissociation constants for binding of the phosphonate to the deprotonated enzyme and to the protonated enzyme is 1620-fold. In contrast to the amide analogue, the phosphonate transition-state analogue binds much tighter to the protonated enzyme than to the deprotonated enzyme.

DISCUSSION

Previous chemical modification (21, 22) and x-ray crystallographic studies (4–6) have established the importance of His-48 in phospholipase A_2 catalysis. Although the pK_a of this residue could be altered in the active site of the enzyme due to a hydrophobic environment or hydrogen bonding with other residues, His-48 is shown in this study to have an effective pK_a of 6.1, perhaps reflecting such interactions. It is generally considered to act as a general base in the hydrolysis catalyzed by phospholipase A_2 (23–25). In our current work, we have shown that the interaction of phospholipase A_2 with the substrate and transition-state analogues is strongly influenced by the ionic state of the enzyme. These pH effects implicate the presence in the enzyme of a group having a pK_a of 6.1 that is important for inhibitor binding. This implies a strong correlation between the ionization of His-48 and the inhibition potency of the amide and phosphonate analogues.

The amide analogue inhibits phospholipase A_2 most effectively under basic conditions, in which the catalytic histidine residue is not protonated. Recently, the x-ray crystal structure of the complex of a mutant bovine phospholipase A_2 with a different amide substrate analogue has been solved and suggested that the inhibitor is hydrogen bonded to His-48 through the proton of the inhibitor's amide group (4). In the hydrogen bond formed between the enzyme and the amide analogue, the former functions as a proton acceptor and the latter functions as a proton donor as shown in Fig. 6*A*. Therefore, the inhibition potency of the amide substrate analogue depends critically on its ability to form a hydrogen bond with the enzyme, which in turn depends on the ionization of His-48. In the pH range used in the present study, the catalytic histidine residue with a pK_a of 6.1 exists in both protonated and deprotonated forms. Among these two ionic states, only the uncharged His-48 residue can function as a

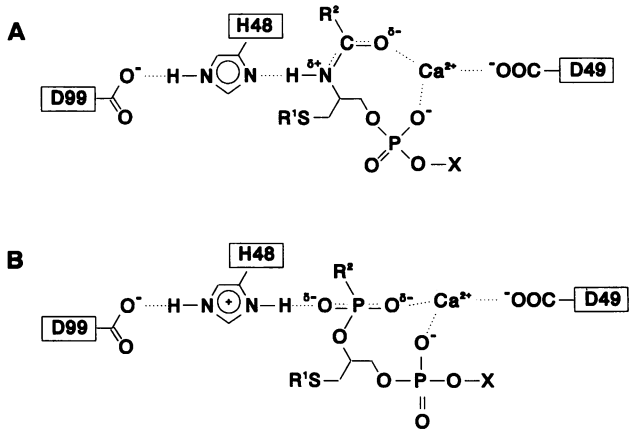

FIG. 6. Hydrogen bonding of the amide substrate analogue (*A*) and the phosphonate transition-state analogue (*B*) with phospholipase A_2.

proton acceptor. After protonation of the Nδ1 of His-48, the enzyme can no longer function as a proton acceptor and loses the ability to form a hydrogen bond with the amide. The reduction of inhibition potency with decreasing pH is most likely due to the lack of the capacity of the protonated imidazole of His-48 to form a hydrogen bond with the amide analogue. The formation of a hydrogen bond between the amide inhibitor and the unprotonated enzyme provides $\approx$1.7 $\pm$ 0.3 kcal/mol (1 cal = 4.184 J), which is consistent with the values reported for the uncharged hydrogen bond donor and acceptor in a protein–ligand complex (26).

Previous studies (1) of phospholipase A_2 inhibition have been carried out under conditions that may not have produced the correct ionic form of the enzyme to optimally interact with the phosphonate analogue. In the presumed transition state, the hydrated carbonyl carbon of the substrate is in a tetrahedral configuration (Fig. 7). The enzyme catalyzes the hydrolysis of phospholipid by stabilizing the transition state partially through the formation of a hydrogen bond with the hydroxyl group arising from the addition of a water molecule to the substrate carbonyl group. The primary role of the catalytic histidine in the transition state is to act as a proton acceptor. The addition of a water molecule to the substrate carbonyl group provides the most important bridging proton through which the enzyme forms a strong interaction with the transition state. Although the tetrahedral phosphonate moiety resembles the tetrahedral transition state, it lacks the bridging hydrogen atom under basic conditions through which the oxygen atom of a phosphonate transition state analogue can form a hydrogen bond with the Nδ1 of His-48 (Fig. 6*B*). To form this hydrogen bond, either the histidine residue or the phosphonate group of the inhibitor has to be protonated. Since the phosphonate ($pK_a \approx 2$) (27, 28) is much more acidic than His-48 (pK_a 6.1), the bridging hydrogen presumably comes from the protonation of His-48 rather than from the phosphonate.

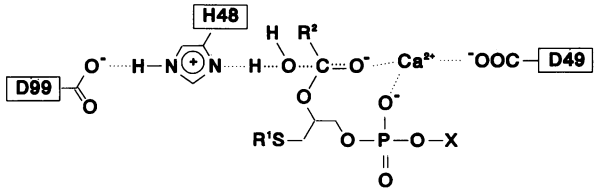

FIG. 7. Postulated interactions of the catalytic residues of phospholipase A_2 with the tetrahedral transition state arising from addition of a water molecule to the substrate carbonyl group.

Under the assay condition used previously (pH 8.5), <1% of the catalytic histidine residue is protonated. This can account for the relatively poor behavior of the phosphonate analogue under basic conditions. At the optimal condition, however, the phosphonate analogue indeed appears to be a very potent phospholipase A_2 inhibitor with an IC_{50} of 1.6×10^{-5} mol fraction (70 nM). At acidic pH, this molecule is one of the best inhibitors reported to date that binds to the enzyme 10^5 tighter than the natural substrate, dipalmitoyl phosphatidylcholine.

The observation that the transition-state analogue binds to the positively charged enzyme 3 orders of magnitude tighter than to the neutral enzyme demonstrates the vital role the hydrogen bond plays in the transition-state stabilization. The difference in the binding affinity of the phosphonate analogue to the two ionic states of the enzyme represents on average 4.4 ± 0.2 kcal/mol in binding energy. This hydrogen bond strength is consistent with the values reported for the hydrogen bond interactions between a charged hydrogen bond donor and a charged acceptor in biological systems (26, 29, 30). Under physiological conditions, only a small percentage of the His-48 residue is protonated. Thus, the phosphonate analogue does not fully manifest its potential to interact with the enzyme. Interestingly, the amide has the binding proton to form the hydrogen bond but it is more planar than tetrahedral and its orientation must be twisted or distorted from that expected for the substrate in order to form this hydrogen bond. To design a molecule that can function most effectively under physiological conditions, these results suggest that a phospholipid analogue that has the tetrahedral features as well as the bridging proton to form a hydrogen bond with the enzyme should act as the most potent inhibitor.

In summary, the inhibition of phospholipase A_2 by the amide and phosphonate analogues is dramatically affected by pH. Both analogues form hydrogen bonds with His-48. In the case of the amide substrate analogue, the hydrogen binding proton must be donated by the amide. In the case of the phosphonate transition-state analogue, the proton must come from His-48. Thus, the binding affinity of the enzyme for these analogues is critically dependent on the ionic state of His-48. This single hydrogen bond plays a central role in the interaction of phospholipase A_2 with these inhibitors and suggests a new consideration in designing potent inhibitors.

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