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^{15}N Nuclear Magnetic Resonance Studies of Acid−Base Properties of Pyridoxal-5′-Phosphate Aldimines in Aqueous Solution

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By use of ^{15}N NMR spectroscopy, we have measured the pK_a values of the aldimines ^{15}N-(pyridoxyl-5′-phosphate-idine)-methylamine (**2a**), N-(pyridoxyl-5′-phosphate-^{15}N-idine)-methylamine (**2b**), and ^{15}N-(pyridoxyl-idine)-methylamine (**3**). These aldimines model the cofactor pyridoxal-5′-phosphate (PLP, **1**) in a variety of PLP-dependent enzymes. The acid-base properties of the aldimines differ substantially from those of the free cofactor in the aldehyde form **1a** or in the hydrated form **1b**, which were also investigated using ^{15}N NMR for comparison. All compounds contain three protonation sites, the pyridine ring, the phenol group, and the side chain phosphate (**1**, **2**) or hydroxyl group (**3**). In agreement with the literature, **1a** exhibits one of several pK_as at 2.9 and **1b** at 4.2. The ^{15}N chemical shifts indicate that the corresponding deprotonation occurs partially in the pyridine and partially in the phenolic site, which compete for the remaining proton. The equilibrium constant of this ring-phenolate tautomerism was measured to be 0.40 for **1a** and 0.06 for **1b**. The tautomerism is essentially unaltered above pH 6.1, where the phosphate group is deprotonated to the dianion. This means that the pyridine ring is more basic than the phenolate group. Pyridine nitrogen deprotonation occurs at 8.2 for **1a** and at 8.7 for **1b**. By contrast, above pH 4 the phosphate site of **2** is deprotonated, while the pyridine ring pK_a is 5.8. The Schiff base nitrogen does not deprotonate below pH 11.4. When the phosphate group is removed, the pK_a of the Schiff base nitrogen decreases to 10.5. The phenol site cannot compete for the proton of the Schiff base nitrogen and is present in the entire pH range as a phenolate, preferentially hydrogen bonded to the solvent. The intrinsic ^{15}N chemical shifts provide information about the hydrogen bond structures of the protonated and unprotonated species involved. Evidence is presented that the intramolecular OHN hydrogen bond of PLP aldimines is broken in aqueous solution. The coupling between the inter- and intramolecular OHN hydrogen bonds is also lost in this environment. The pyridine ring of the PLP aldimines is not protonated in aqueous solution near neutral pH. The basicity of the aldimine nitrogens would be even lower without the doubly negatively charged phosphate group. Protonation of both the Schiff base and pyridine nitrogens has been discussed as a prerequisite for catalytic activity, and the implications of the present findings for PLP catalysis are discussed.

Introduction

Pyridoxal-5′-phosphate (PLP, vitamin B_6, see Scheme 1) is required as a cofactor in a remarkable variety of enzyme-catalyzed transformations of amines and amino acids, e.g., transamination, decarboxylation, racemization.[1−4] PLP-dependent enzymes bind the cofactor covalently at their active sites.[5−8] In the resting enzyme the cofactor is present as an "internal aldimine" in which it forms a Schiff base with the ϵ-amino group of a lysine residue. When substrate binds to the active site, an "external aldimine" intermediate is formed with the amino group of the substrate, as illustrated in Scheme 1. The cofactor generally forms several hydrogen bonds, the roles of which are not fully understood. First, there is an intramolecular OHN hydrogen bond between the phenolic oxygen and the imino nitrogen. Second, there is intermolecular OHN bond between the ring nitrogen and an aspartate side chain in many enzymes. The ring nitrogen is believed to be protonated in these enzymes.[1,2] Third, there are hydrogen bonds between various amino acid side chains and the phosphate group. The latter is deprotonated under physiological conditions.[9−13] Finally, there are additional intermolecular OHO hydrogen bonds between the phenolic oxygen and neighboring proton-donating side chains.

The hydrogen bonding in the active site appears to be essential for high catalytic activity. For example, it could stabilize the zwitterionic state $O^-\cdots H-N^+$ of the intramolecular hydrogen bond, which is considered a prerequisite for catalytic activity.[1,2] The intermolecular OHN hydrogen bond of the ring nitrogen with the aspartic acid side chain seems to be most important for this stabilization. By use of selected Schiff base-carboxylic acid model complexes in the organic solid state, we found that the intra- and the intermolecular OHN hydrogen bonds exhibit a cooperative coupling.[14,15] This coupling indicates that a zwitterionic state of one OHN hydrogen bond favors the zwitterionic state of the other. In various Schiff base studies,[16−19] the influence of the above-mentioned intermolecular phenolic OHO hydrogen bonds on the stabilization of the zwitterionic structure of the intramolecular OHN hydrogen bond was

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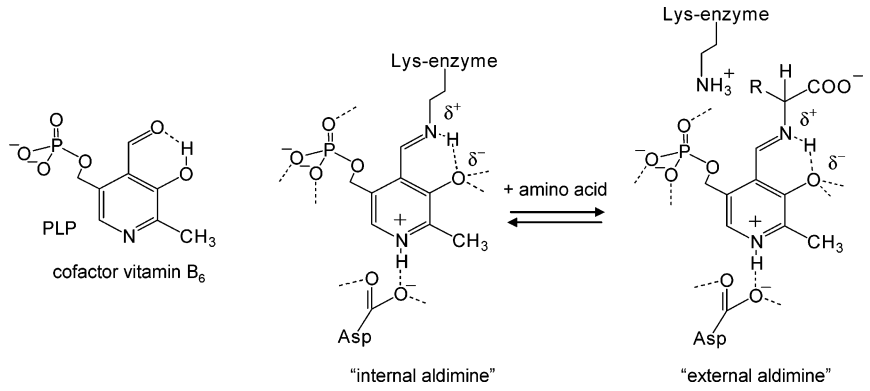
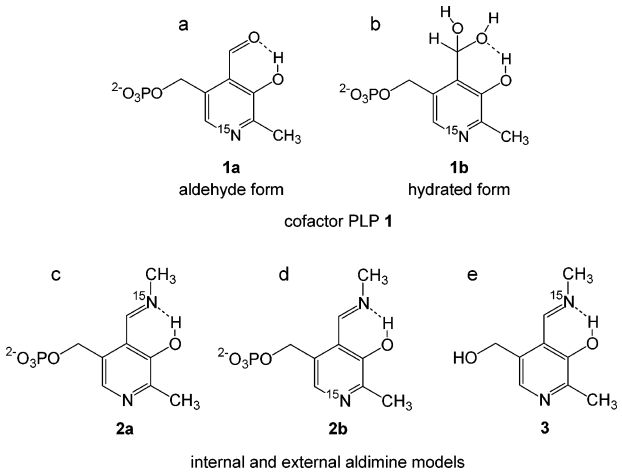
SCHEME 1: Overview of the First Step of the Catalytic Cycle of Vitamin B_6 Dependent Enzymes.



SCHEME 2: (a) ^{15}N-PLP in aldehyde form (1a). (b) ^{15}N-PLP in hydrated form (1b). (c) ^{15}N-(pyridoxyl-5′-phosphate-idine)-methylamine (2a). (d) N-(pyridoxyl-5′-phosphate-^{15}N-idine)-methylamine (2b). (e) ^{15}N-(pyridoxyl-idine)-methylamine (3).



demonstrated. A similar effect was also achieved by increasing the local polarity.[20]

During the course of our cofactor modeling studies the following problem occurred to us. It has been well established that the pyridine nitrogen of the cofactor PLP exhibits a pK_a value of around 8.5.[21−24] Thus, in the aqueous environment it is protonated under physiological conditions. It has been assumed in the literature that the same is true for the reactive liganded cofactors, the external and internal aldimines. However, to our knowledge this assumption has not yet been verified. Such verification is difficult to obtain for the enzyme-bound cofactor and warrants, therefore, suitable model studies. In this paper, we show that the pyridine ring of PLP aldimines is much less basic than of PLP itself and close in basicity to carboxylate groups.

We also address how the phosphate group and its protonation state influences the acid-base properties of the Schiff base and pyridine ring and thereby the reactivity of the cofactor. Several studies on the acid-base properties of the cofactor[21−24] have not yet provided a conclusive answer to this question.

For these reasons, we have studied the Schiff bases **2** and **3** (parts c−e of Scheme 2) as models of aldimine intermediates, which should also give information on the role of the phosphate group, since it is absent in **3**. We have used ^{15}N NMR spectroscopy to study the acid-base properties of these compounds in aqueous solution as a function of pH. For this purpose, it was necessary to label (**2**) specifically with the ^{15}N isotope in the imino position, (**2a**), and in the ring position, (**2b**).

For comparison with previous results, we also reinvestigated the acid-base properties of PLP (**1**) using ^{15}N NMR. In aqueous solution, the latter is present in the aldehyde (**1a**) (favored at high pH) and the hydrated (**1b**) (favored at low pH) forms, as illustrated in parts a and b of Scheme 2.[25−28]

Materials and Methods

Materials. PLP and pyridoxal hydrochloride were purchased from Aldrich and used without further purification. The isotopically enriched compounds deuterium oxide (D_2O, 99% ^{2}H-enriched), sulfuric acid D_2SO_4 (in 96% in D_2O, 96% ^{2}H-enriched), and methylamine hydrochloride (95% ^{15}N-enriched) were purchased from Deutero GmbH. The procedures for preparing the ^{15}N-labeled PLP and the Schiff base, ^{15}N-(pyridoxyl-idine)-methylamine (**3**), are described in ref 14. ^{15}N-(Pyridoxyl-5′-phosphate-idine)-methylamine (**2a**) was formed in vitro in H_2O by reacting unlabeled PLP for 1 h with an excess of the ^{15}N-enriched methylamine hydrochloride at pH 6.0. N-(Pyridoxyl-5′-phosphate-^{15}N-idine)-methylamine (**2b**) was formed in vitro in H_2O by reacting ^{15}N-labeled PLP for 1 h with an excess of the unlabeled methylamine hydrochloride at pH 4.0.

The pH was adjusted at basic pH using 1 M sodium hydrogen carbonate or 1 M sodium hydroxide and at acidic pH using 1 M hydrochloric acid. The pD was adjusted by using a solutions of sodium hydroxide or deuterated sulfuric acid in D_2O. The pD values were obtained using the correction pD = pH + 0.40.[29−31] The sample concentrations were 40 mM for the Schiff bases **2a**, **2b**, and **3** and 80 mM for PLP.

NMR Experiments. NMR spectra were measured using a Bruker AMX 500 spectrometer (500.13 MHz for ^{1}H, 50.68 MHz for ^{15}N) at room temperature (300 K). Standard ^{1}H spectra were recorded in D_2O at 300 K. Inverse-gated ^{1}H-decoupled ^{15}N NMR spectra were recorded in H_2O at 300 K with field locking on a D_2O-containing capillary with a recycle time of 10 s. We recorded under the same ^{2}H field locking conditions and temperature ^{15}N spectra of neat nitromethane containing a capillary with D_2O in order to reference the ^{15}N chemical shifts. The relation $\delta(CH_3NO_2, \text{liquid}) = \delta(^{15}NH_4Cl, \text{solid}) - 341.168$ ppm was used to convert the ^{15}N chemical shifts from the nitromethane scale into the solid external $^{15}NH_4Cl$ scale.[32]

Analysis of ^{15}N NMR Titration Curves. The general scheme shown at the top of Figure 1, adapted from ref 1, was employed to determine the acid-base properties of PLP. The three ionizable groups of each compound studied are labeled as AH (phosphate group), BH (pyridine ring), and XH (phenolic group) in their protonated states, and as A, B, and X in their unprotonated states. The group containing the observed ^{15}N nucleus is underlined. We take four protonation states i = I−IV into account, where

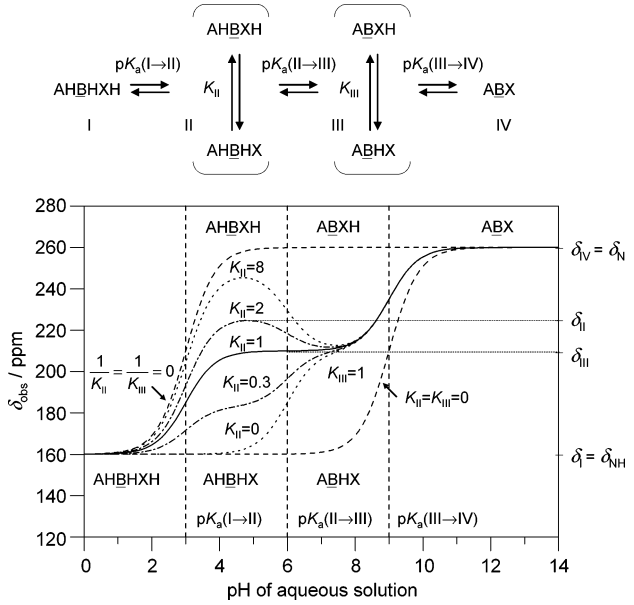


Figure 1. Simulation scheme for a four-state Henderson−Hasselbalch equation. The acid−base equilibria scheme is presented on the top. Here are the three ionizable groups labeled as AH (phosphate group), BH (pyridine ring), and XH (phenolic group) in their protonated states, and as A, B, and X in their unprotonated states.

II and III are subject to a ring-phenolate tautomerism characterized by the equilibrium constants K_{II} and K_{III}, as illustrated in Figure 1. We assume that the intrinsic ^{15}N chemical shifts δ_{NH} and δ_N only depend on the protonation state of the nitrogen atom of interest but not on the protonation states of the neighboring groups.

The observed average nitrogen chemical shifts δ_{obs} of **1**−**3** can then be expressed as a function of pH in terms of the Henderson−Hasselbalch equation,[33,34] adapted for NMR spectroscopic measurements[35] in the fast proton-transfer regime

$$\delta_{obs} = \delta_I + \sum_i (\delta_{i+1} - \delta_i) \frac{10^{pH-pK_a(i\rightarrow i+1)}}{1 + 10^{pH-pK_a(i\rightarrow i+1)}},\ i = I-III \quad (1)$$

Here, $pK_a(i\rightarrow i+1) = -\log K_a(i\rightarrow i+1)$, where $K_a(i\rightarrow i+1)$ represents the equilibrium constant of the formation of protonation state $i + 1$. δ_N represents the ^{15}N chemical shift in state $i + 1$, which is the limiting value for the deprotonated nitrogen atom. δ_{NH} represents the ^{15}N chemical shift in state i, which is the limiting value of the protonated nitrogen atom. See also Figure 1 for further details. For I ≡ AHBHXH, it is obvious that $\delta_I = \delta_{NH}$, and for IV ≡ ABX that $\delta_{IV} = \delta_N$. However, the ^{15}N chemical shifts of II ≡ {AHBHX, AHBXH} and of III ≡ {ABHX, ABXH} represent averages over the two associated tautomeric states, i.e.

$$\delta_i = \frac{1}{1 + K_i}\delta_{NH} + \frac{K_i}{1 + K_i}\delta_N,\ i = II,\ III \quad (2)$$

where K_i represents the equilibrium constant of the ring−phenolate tautomerism. The different curves depicted in Figure 1 were simulated for a set of given arbitrary parameters using the eqs 1 and 2. These curves exhibit various plateaus at $\delta_I = \delta_{NH}$, δ_{II}, δ_{III}, and $\delta_{IV} = \delta_N$, where the two central plateaus give information about the equilibrium constant K_{II} and K_{III}.

Results

NMR Spectroscopy. The complete set of ^{15}N NMR spectra recorded is given in the Supporting Information. The ^{15}N chemical shifts are listed in Table 1. Typical ^{15}N spectra of aqueous solutions of the ^{15}N-labeled cofactor PLP are given in Figure 2. At high pH values, only one ^{15}N signal is observed, whereas below pH 7.5 a second signal appears, shifted to high field. In previous ^1H NMR studies[25−28] it has been shown that PLP in aqueous solution at high pH preferentially forms the aldehyde form (**1a**), whereas the hydrated form (**1b**) is favored at low pH, leading to the signal assignment shown in Figure 2. The assignment was supported by standard two-dimensional $^1H/^{15}N$ HSQC NMR experiments with the ^{15}N-labeled PLP in D_2O at pH 7.2, which is presented in the Supporting Information. Both ^{15}N signals shift to high field when the pH is decreased to 0.4.

TABLE 1: ^{15}N Chemical Shifts of Specifically ^{15}N-Labeled PLP and Related Model Systems in Aqueous Solution at 298 K as a Function of pH

	^{15}N site	pH	$\delta(^{15}N)^a$		^{15}N site	pH	$\delta(^{15}N)^b$
1a	ring	13.8	264.06	**2a**	imino	13.4	272.30
		12.8	263.99			12.0	255.05
		12.0	263.95			11.0	173.79
		11.0	263.74			10.6	150.65
		9.2	259.42			10.0	141.32
		8.7	249.97			9.0	141.28
		8.2	233.00			6.0	143.04
		7.7	211.14	**3**	imino	13.4	264.01
		6.5	199.56			11.6	255.98
		6.0	198.36			10.8	227.17
		5.6	197.22			10.3	193.86
		5.0	197.71			9.9	173.07
		4.3	196.77			9.3	155.64
		4.0	194.98			8.9	151.91
		3.5	191.62			5.8	145.89
		3.0	186.42	**2b**	ring	11.2	264.17
		2.4	176.48			10.4	264.94
		2.1	172.59			9.9	265.15
		0.4	170.34			9.4	265.23
1b	ring	6.5	167.94			9.0	265.14
		6.0	167.82			8.5	264.59
		5.6	168.00			7.6	262.53
		5.0	167.50			7.4	261.67
		4.3	165.74			6.8	254.06
		4.0	164.32			6.6	248.02
		3.5	163.37			5.7	214.10
		3.0	162.88			5.5	205.72
		2.4	162.56			5.2	190.91
		2.1	162.43			5.0	186.46
		0.4	162.56			4.5	173.83

[a] Margin of error ±0.2 ppm between pH 5 and 7, otherwise ±0.05 ppm. [b] Margin of error ±0.2 ppm between pH 9 and 12, otherwise ±0.05 ppm.

Typical ^{15}N spectra of aqueous solutions of the Schiff bases **2a**, **2b**, and **3** are given in Figure 3. Since these bases decompose in acidic media, only the pH range from about 4 to 14 was accessible. The stability of the Schiff bases in this range was checked by ^1H NMR of the corresponding solutions in D_2O. The ^1H spectra of **2a** and **3** are included in the Supporting Information.

As illustrated in Figure 3a, when the pH is decreased, the imino nitrogen signal of **2a** shifts to high field from 272 ppm at pH 13.4 to 141 ppm at pH 6. The imino nitrogen signal is sharp at high pH, broadens somewhat around pH 11 and sharpens again below this value. Similar changes are observed for **3** (Figure 3b). Finally, the pyridine ring nitrogen signal of **2b** shifts from 264 ppm at pH 11.2 to 174 ppm at pH 4.5 (Figure 3c), but no intermediate line broadening was observed.

Data Analysis. The observed ^{15}N chemical shifts of the cofactor in the aldehyde form (**1a**) and in the hydrated form

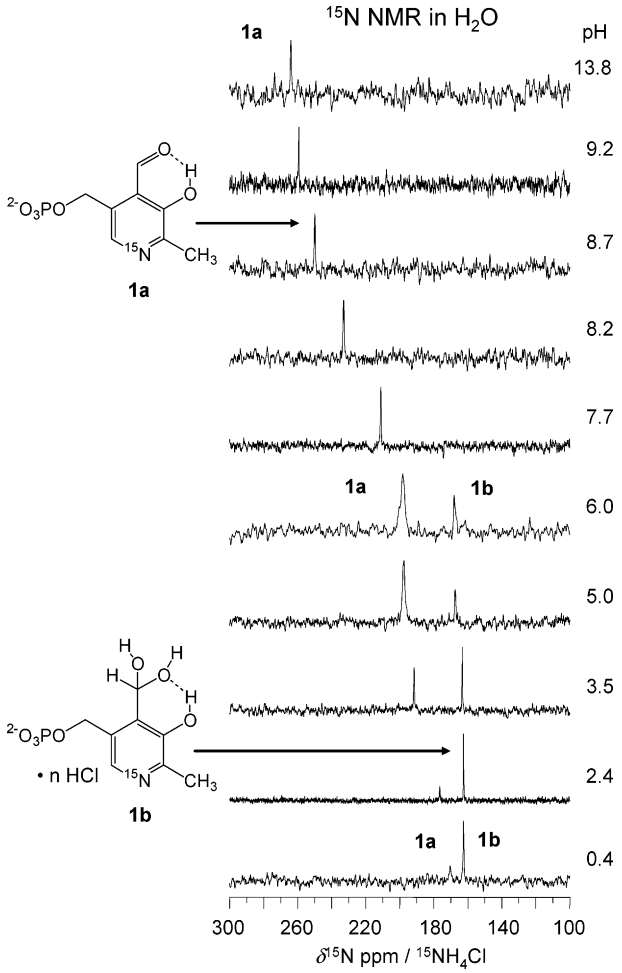


Figure 2. $^{15}N\{^1H\}$ NMR spectra of the ^{15}N-labeled cofactor PLP in aldehyde form (**1a**) and hydrated form (**1b**) recorded in aqueous solution by locking to a D_2O-containing capillary at room temperature. Values are listed in Table 1.

(**1b**) as a function of pH are plotted in parts a and b of Figure 4, respectively. Figure 5 illustrates the overall acid-base equilibria of the cofactor, adapted from ref 1, whose essential features were used to prepare Figure 1.

The experimental data are described in terms of the four-step Henderson−Hasselbalch equation (eq 1 in combination with eq 2) and the pK_a values and equilibrium constants included in Figure 5 and Table 2. In particular, the equilibrium constants K_{II} and K_{III} of the ring-phenolate tautomerism were obtained from the ^{15}N chemical shifts of the central plateaus. Since both constants are the same within the margin of error, it is not possible to establish the value of p$K_a = 6.1$ by ^{15}N NMR. Therefore, this value was taken from refs 1, 36, and 37. In addition, since the hydrated form is not stable at high pH, we used the reported value of 8.7, taken from refs 38 and 39.

The ^{15}N chemical shifts of the ^{15}N-enriched Schiff bases **2a**, **2b**, and **3** as a function of pH are shown in Figure 6. The chemical shift titration curves were calculated in terms of the corresponding two-state acid-base equilibria presented on the right-hand side. The parameters used to describe these curves are included in Table 2. Again, it was assumed that the ^{15}N chemical shift of a given nitrogen atom only depends on the protonation state of this atom not of the protonation state of the other nitrogen atom. This assumption is well justified as indicated in Figure 6, where the pyridine nitrogen chemical shift is not sensitive to protonation of the Schiff base nitrogen and vice versa.

Discussion

We have studied the ^{15}N chemical shifts of the cofactor PLP (**1**) and the related Schiff bases **2** and **3** in aqueous solution as a function of pH. These Schiff bases represent models of enzyme-bound PLP, whose acid-base properties are not completely understood. Our studies also complete our knowledge about the acid-base properties of **1**. The pK_as of the various ionization states are in good agreement with those reported in the literature (see Table 2). Therefore, it is now possible to discuss the changes in the acid-base properties of PLP after binding to the enzyme, including potential catalytic implications. However, we first discuss the values of the ^{15}N chemical shifts of the model compounds as they provide information about their hydrogen bond properties. This knowledge is necessary to understand their acid-base properties.

^{15}N Chemical Shifts and Geometries of the Inter- and Intramolecular OHN Hydrogen Bonds. At high pH, the intrinsic ^{15}N chemical shifts of the deprotonated pyridine ring nitrogen of the free cofactor **1** and of the model aldimines **2** and **3** in aqueous solution are the same, i.e., $\delta_N = 264.0$ ppm. This value differs from the value of 282.5 ppm found for similar molecules in the organic solid state.[14] This significant difference arises from hydrogen bonding to water. By use of the hydrogen bond correlation determined previously,[14] we estimate an average H···N hydrogen bond length of about 1.77 ± 0.02 Å and an average O···N distance of 2.77 ± 0.04 Å. Similar ^{15}N chemical shifts were found for hydrated pyridine in mesoporous silica.[40,41] At low pH the intrinsic pyridinium ^{15}N chemical shifts of **1a**, **1b**, and **2b** are also the same, i.e., $\delta_{NH} = 170.4$ ppm, exhibiting an average H···N bond length of about 1.08 ± 0.01 Å, and an average O···N distance of 2.67 ± 0.02 Å. These distances also indicate that the pyridinium forms a hydrogen bond to water.

The question arises whether our measurements provide also insights in the hydrogen bond pattern of the aldimine moieties. Unfortunately, we can discuss this question only qualitatively as we have not yet established a quantitative correlation between the ^{15}N chemical shifts and the H-bond geometries of aldimines.[14,20] The ^{15}N chemical shifts of the deprotonated molecules **2a** and **3** at high pH (272.3 and 264.0 ppm) are substantially smaller than the value of 280 ppm typical for non-hydrogen-bonded model Schiff bases in aprotic solvents.[20] This result indicates the formation of hydrogen bonds to a surrounding water cluster in the deprotonated forms. After protonation of the aldimine nitrogen ^{15}N chemical shift values of $\delta_{NH} =$ 139.0 and 146.0 ppm are observed for **2a** and of **3** (Figure 6a). A comparison of these values with those of model compounds[20] indicates that the imino nitrogen atoms are fully protonated and that the proton involved in a hydrogen bond.

The question remains whether the protonated aldimines form intermolecular hydrogen bonds to water or whether they form an intramolecular OHN hydrogen bond as observed in aprotic solvents or in the organic solid state. Information about this question can come from the graph in Figure 6. Above pH 7 the ring nitrogen is deprotonated. The deprotonation of the aldimine nitrogen atoms only occurs above pH 10. This means that the protonation of the ring nitrogen and of the imino nitrogen are independent of each other. However, in our previous study of aldimine models in the organic solid state we had shown using solid-state NMR that the inter- and the intramolecular OHN-hydrogen bonds exhibit a strong coupling to each other. Thus, if a proton is transferred to the pyridine ring, the tautomerism in the intramolecular OHN hydrogen bond (if it is intact) is shifted toward the zwitterionic structure where H is located on

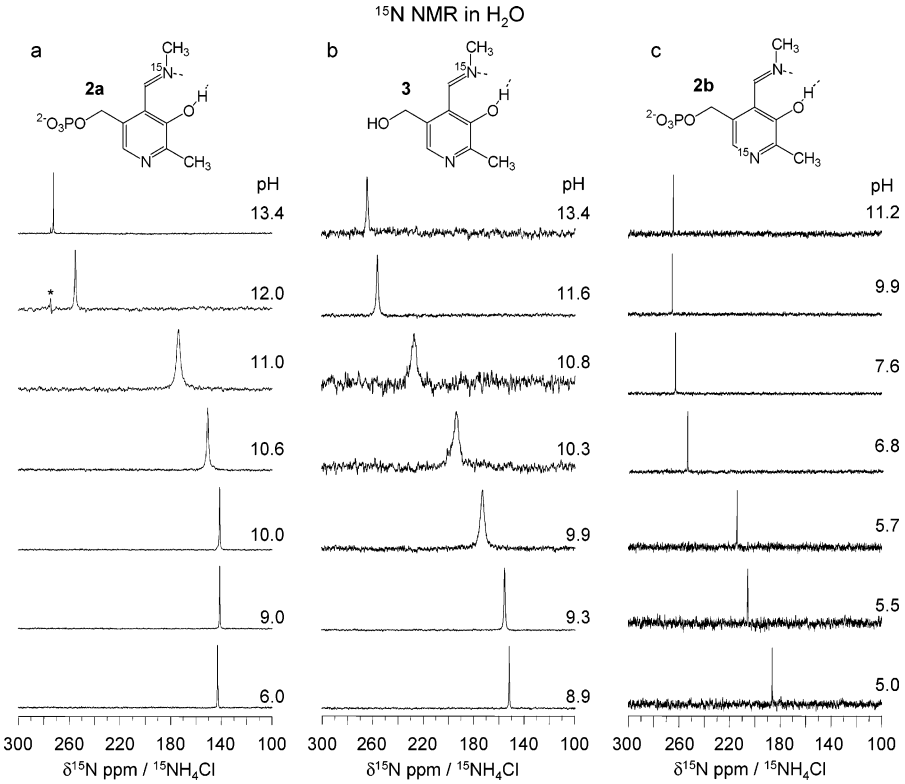


Figure 3. ^{15}N {^{1}H} NMR spectra of the ^{15}N-labeled Schiff bases recorded in aqueous solution by locking to a D_2O-containing capillary at room temperature. (a) ^{15}N-(Pyridoxyl-5′-phosphate-idine)-methylamine (**2a**), formed in vitro. (b) ^{15}N-(Pyridoxylidine)-methylamine (**3**). (c) N-(Pyridoxyl-5′-phosphate-^{15}N-idine)-methylamine (**2b**), formed in vitro. Values are listed in Table 1. Asterisk signifies an artifact.

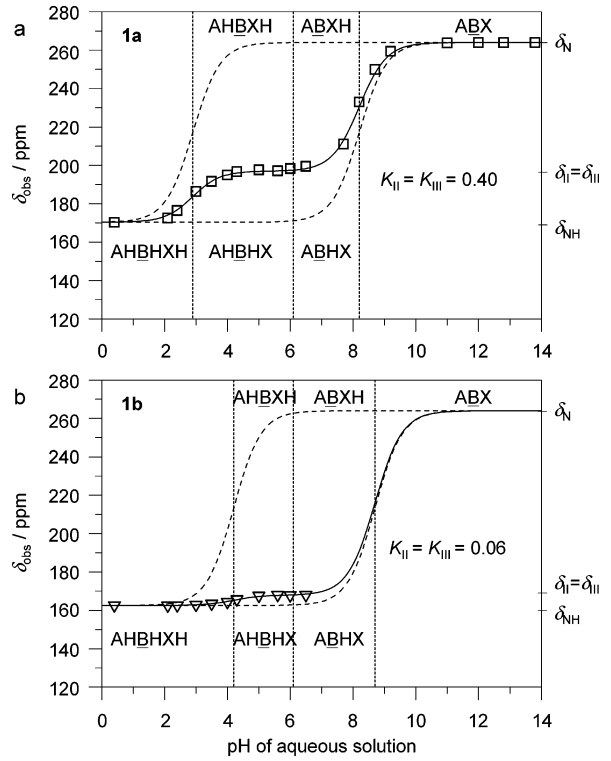


Figure 4. ^{15}N chemical shifts of the ^{15}N-labeled cofactor PLP as a function of pH. (a) PLP in the aldehyde form (**1a**, open squares). (b) PLP in the hydrated form (**1b**, open down triangles). The solid lines were obtained by fitting the experimental points by using eqs 1 and 2. The fitting parameters are assembled in Table 2.

nitrogen.[14,20] Apparently, this coupling is lost in water since the imino nitrogen remains protonated until pH 10, in a region where the pyridine ring is no longer protonated. This means that the intramolecular OHN hydrogen bond must be disrupted and that the imino and the phenolate moieties form hydrogen bonds to water molecules. This may be the result of the high concentration of water, and of the circumstance that the intramolecular OHN-hydrogen bond is nonlinear whereas the intermolecular ones can be linear which may favor the latter from an energetic point of view. When the intramolecular bond is formed a water molecule may be released, which could lead to an entropy increase. However, this entropy increase will be strongly reduced by the formation of hydrogen bonds to other water molecules.

Acid−Base Properties of PLP and Related Aldimine Models. According to Figure 5, at low pH, the three ionizable groups of PLP contain each a single proton: the phosphate group is present as the monoanion, and the pyridine N and phenolic O are protonated. The first observable proton dissociation at pH 2.9 (**1a**) and 4.2 (**1b**) occurs partially at the pyridine N and partially at the phenol O, with both competing for the remaining proton. The equilibrium constant for this pyridine N/phenolic O tautomerism was measured and demonstrates similar basicity for both sites in **1a** and a slightly higher basicity for the pyridine N in **1b**. At pH 6.1 the phosphate group is deprotonated to the dianion.[9,11,13] The remaining proton in the pyridine/phenolic sites is released only above pH 8. Thus, at physiological pH, PLP contains in the aldehyde form (**1a**) a single proton located about 60% at the pyridine N and about 40% at the phenolic O. In the hydrated form (**1b**) protonation of the pyridine ring dominates strongly. The main change in these acid-base properties after Schiff base formation is that the new imino nitrogen of **2**, which is hydrogen bonded to water, exhibits high basicity since its pK_a is increased to 11.4. This is consistent with the absence of an intramolecular OHN hydrogen bond, where oxygen can compete for the proton, reducing the proton occupancy at nitrogen. The protonated, positively charged imino function is

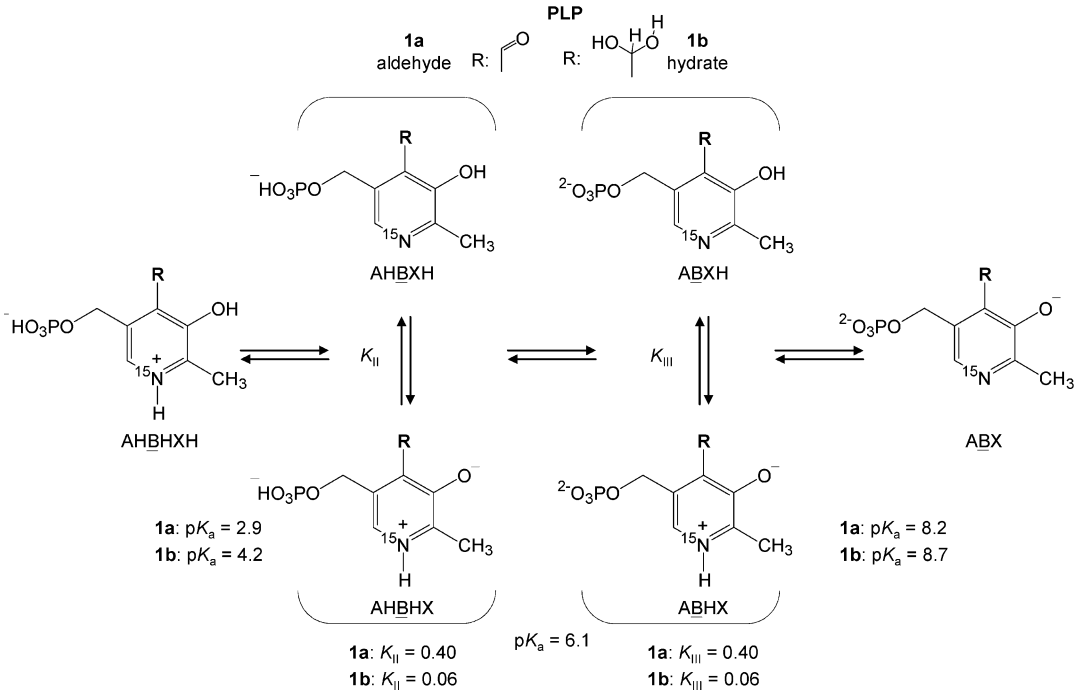


Figure 5. Acid−base equilibria scheme and pK_a values of the cofactor PLP in aqueous solution.

TABLE 2: pK_a Values and Intrinsic ^{15}N Chemical Shifts of Specifically ^{15}N-Labeled PLP and Related Model Systems in Aqueous Solution at 298 K (Nomenclature Is According to Figure 1)[a,b,c,d,e]

	acid−base equilibria	^{15}N site	pK_a	pK_a ± 0.2	K^d	δ_N/ppm ± 0.5	δ_{NH}/ppm ± 0.5	δ^e/ppm ± 0.5
1a	AHBHXH ⇄ AHBXH	ring	3.1−3.7[a]	2.9	0.40	264.0	170.4	
	AHBXH ⇄ ABXH	ring	6.1[b]	6.1	0.40	264.0	170.4	197.0
	ABHX ⇄ ABX	ring	8.3−8.9[a]	8.2	0.40	264.0	170.4	
1b	AHBHXH ⇄ AHBXH	ring	4.1[c]	4.2	0.06	264.0	162.6	
	AHBXH ⇄ ABXH	ring	6.1[b]	6.1	0.06	264.0	162.6	167.9
	ABHX ⇄ ABX	ring	8.7[c]	8.7	0.06	264.0	162.6	
2a		imino		11.4		272.3	139.0	
3		imino		10.5		264.0	146.0	
2b		ring		5.8		264.0	170.4	

[a] pK_a for the aldehyde form **1a** obtained from refs 21−24. [b] pK_a for **1a** and **1b** are obtained from refs 1, 36, and 37. [c] pK_a for the hydrated form **1b** obtained from refs 38 and 39. [d] $K \equiv K_{II} = K_{III}$ represent the equilibrium constants of the ring-phenolate tautomerism reactions AHBHX ⇄ AHBXH and ABHX ⇄ ABXH. [e] $\delta \equiv \delta_{II} = \delta_{III}$.

strongly electron withdrawing and therefore increases the acidity of the other sites. For example, it decreases the pK_a of the phosphate site from 6.1 in PLP to less than 4, see Figure 6. Unfortunately, this value could not be measured because the aldimines are not stable in acidic solution. On the other hand, the doubly negatively charged phosphate group slightly enhances the basicity of the imino nitrogen, since removal of the phosphate group in **3** leads to a decrease in the corresponding pK_a from 11.4 to 10.5. The pK_a values for the imino nitrogens of **2a** and **3** obtained here correspond closer to the apparent pK_a values of external aldimines, which are usually 9−11,[42−44] than to those of internal aldimines, which are in order of 6−7.[45−47] The electron-withdrawing effect of the imino function affects the ring nitrogen whose pK_a decreases to 5.8, a normal value for pyridine derivatives.[48−50] One anticipates an even larger drop in the absence of the doubly negatively charged phosphate group. As mentioned above, in the case of PLP, the pyridine/phenolate site loses its proton only above pH 8. This means, that the pyridine ring of the aldimines is not protonated above pH 6, although the Schiff base nitrogen is protonated.

Implications for the Biological Function. The protonation of the imino nitrogen of the aldimines has been regarded as a prerequisite to the catalytic activity of the cofactor,[1,2] which requires protonation of the pyridine ring. In other words, the intra- and the intermolecular OHN hydrogen bonds are cooperative coupled.[14,15] In the solid state or assumed for polar aprotic solvents, under conditions where solvating water molecules are absent and where the intramolecular OHN hydrogen bond is intact, the protonation of the ring nitrogen leads to a shift toward the zwitterionic form O$^-$···H−N$^+$ of the intramolecular OHN hydrogen bond.[14,15,20] Our results imply that this coupling would be unnecessary if the aldimines behaved in enzymes as they do in aqueous solution, where the intramolecular OHN hydrogen bond is most likely absent.

On the other hand, enzymes generally exclude all but localized, tightly bound waters from the active site in order to promote catalysis and reaction specificity. This problem is resolved by enzymes via specific hydrogen bonding interactions in the nonaqueous active site environment. They ensure the protonation of the imino nitrogen via the cooperative coupling of the inter- and intramolecular OHN hydrogen bonds.

The reduced basicity of the pyridine ring in the aldimines compared to PLP is compensated by having the primary proton donor to the pyridine ring be a carboxylate group of an aspartate side chain in many enzymes. The acidity of the latter can be strongly enhanced in enzymes by specific, local H-bond interactions. Interestingly, the doubly negatively charged phosphate group of PLP may play a significant electrostatic role by

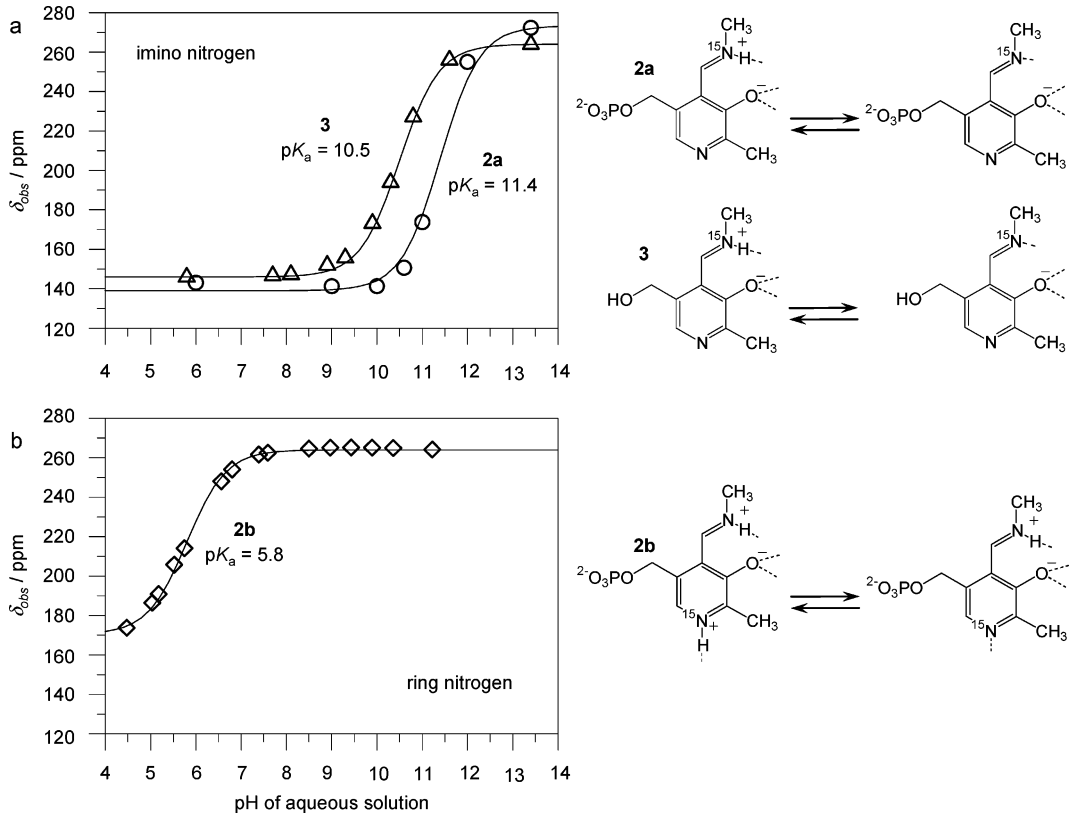


Figure 6. ^{15}N chemical shifts of the ^{15}N-labeled Schiff bases as a function of pH. (a) ^{15}N imino-labeled Schiff bases containing the side chain phosphate group (**2a**, open circles) and hydroxyl group (**3**, open up triangles). (b) ^{15}N-ring-labeled Schiff bases containing the side chain phosphate group (**2b**). Solid lines were obtained by fitting the experimental points by using the eq 1. The fitting parameters are assembled in Table 2.

enhancing the basicity of the pyridine and imino nitrogens, a function only recently recognized experimentally with the enzyme dialkylglycine decarboxylase.[9]

Conclusion

In conclusion, ^{15}N NMR spectroscopy of aqueous solutions of ^{15}N-labeled PLP (**1**) and corresponding aldimines **2** and **3** representing models for the cofactor bound to enzymes provide important new information about the acid-base properties of central aldimine intermediates in PLP-dependent enzymes. Schiff base formation reduces the basicities of the pyridine ring and of the side chain phosphate group. On the other hand, removal of the phosphate group decreases the basicities of the imino and pyridine nitrogen atoms. Furthermore, our results show that the intramolecular OHN hydrogen bond in the aldimines is most likely absent in aqueous solution. Rather, the protonated aldimine nitrogen is solvated by hydrogen bonds to surrounding water molecules. Consequently, aldimines in aqueous solution behave quite differently than aldimines in the nonaqueous active site environment, where specific, local hydrogen bonding interactions are employed to ensure the correct protonation state for high catalytic activity. One might conclude that evolution has provided nature with a cofactor whose properties are optimized for reaction in a nonaqueous environment. Thus, further studies on aldimines and their reactions in nonaqueous polar solvents are justified to probe more accurately the coupling of hydrogen bonding interactions critial to PLP catalysis in enzyme active sites.

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Supporting Information Available: In the Supporting Information, the complete set of ^{15}N spectra of the cofactor **1** and **2b** are presented as well as the ^{1}H spectra of **2a** and **3** solution in D_2O. In addition, the standard two-dimensional ^{1}H/^{15}N HSQC NMR of the cofactor is shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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