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NMR studies of the protonation states of pyridoxal-5′-phosphate in water

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ABSTRACT

We have measured the ^{13}C NMR spectra of the cofactor pyridoxal-5′-phosphate (vitamin B_6, PLP) at 278 K in aqueous solution as a function of pH. By ^{13}C enrichment of PLP in the C-4′ and C-5′ positions we were able to measure spectra down to pH 1. From the dependence of the ^{13}C chemical shifts on pH, the pK_a values of PLP could be determined. In particular, the heretofore uncharacterized protonation state of PLP, in which the phosphate group as well as the pyridine ring and the phenolic groups are fully protonated, has been analyzed. The corresponding pK_a value of 2.4 indicates that the phosphate group is solely involved in the first deprotonation step. The ^{15}N chemical shifts of the pyridine ring of PLP published previously are in good agreement with the new results. These shifts contain information about the tautomerism of the different protonation states of PLP. The implications of these findings for the biological function of PLP are discussed.

1. Introduction

Pyridoxal-5′-phosphate (PLP, Fig. 1) is a cofactor of enzymes that are responsible for various amino acids transformations such as racemization and transamination [1–3]. A large number of different protonation states and tautomers relevant to its biological function are found. The aldehyde group can be free, hydrated or form Schiff bases with primary amines (e.g., enzymic ε-amino groups of lysine residues or α-amino groups of amino acid substrates). Furthermore, it contains three functional groups which may adopt different protonation states: a phosphate group (AH_2), a pyridine ring (B), and an OH group (XH) near the aldehyde function.

According to Fig. 1 one can conceive five protonation states 0 to IV, which dominate at different pH values. Besides the fully protonated state 0 = AH_2BHXH which contains four protons and the fully deprotonated state ABX, each protonation state can adopt different tautomers. The pK_a values of protonation states I–IV have been determined by UV–Vis [4], ^1H NMR [5], and ^{13}C NMR [6] spectroscopy. The aldehyde form is present in the whole pH range whereas the hydrate is formed only below pH 6, and dominates below pH 4. The interconversion takes place on the second timescale and hence contributes two different sets of signals in the NMR spectra [7].

Recently, some of us have explored the acid–base properties of PLP labeled in the pyridine ring with ^{15}N using ^{15}N NMR spectroscopy [8]. This method is sensitive to the protonation state of the pyridine ring which, in turn, depends on the protonation state and the equilibrium constants K_{II} and K_{III} of tautomerism in protonation states II and III. These could be measured, as well as several pK_a values. However, no information has been obtained to date concerning the first protonation state 0, nor has the ratio between the aldehyde form **1a** and the hydrate **1b** been quantified in this state. Therefore, as part of a series of studies concerning the function of PLP in various environments [8,9] we were interested in detecting the presence of the first protonation state 0 and determining its pK_{a0} value. For that purpose, ^{13}C NMR spectroscopy of ^{13}C labeled PLP is an appropriate stratagem that might also be useful in subsequent studies of model Schiff bases. Therefore, we synthesized PLP labeled at the C-4′ and C-5′ positions with ^{13}C, abbreviated as $^{13}C_2$-PLP (**1**) (Fig. 2). Position C-4′ seemed to us to be the best diagnostic site for the hydration and protonation states of PLP. For the synthesis we used the procedure of O'Leary and Payne [10] by which ^{13}C is also introduced into position C-5′. This later proved to be valuable as both positions were needed to determine the pK_a values of **1a** and of **1b** as a function of pH.

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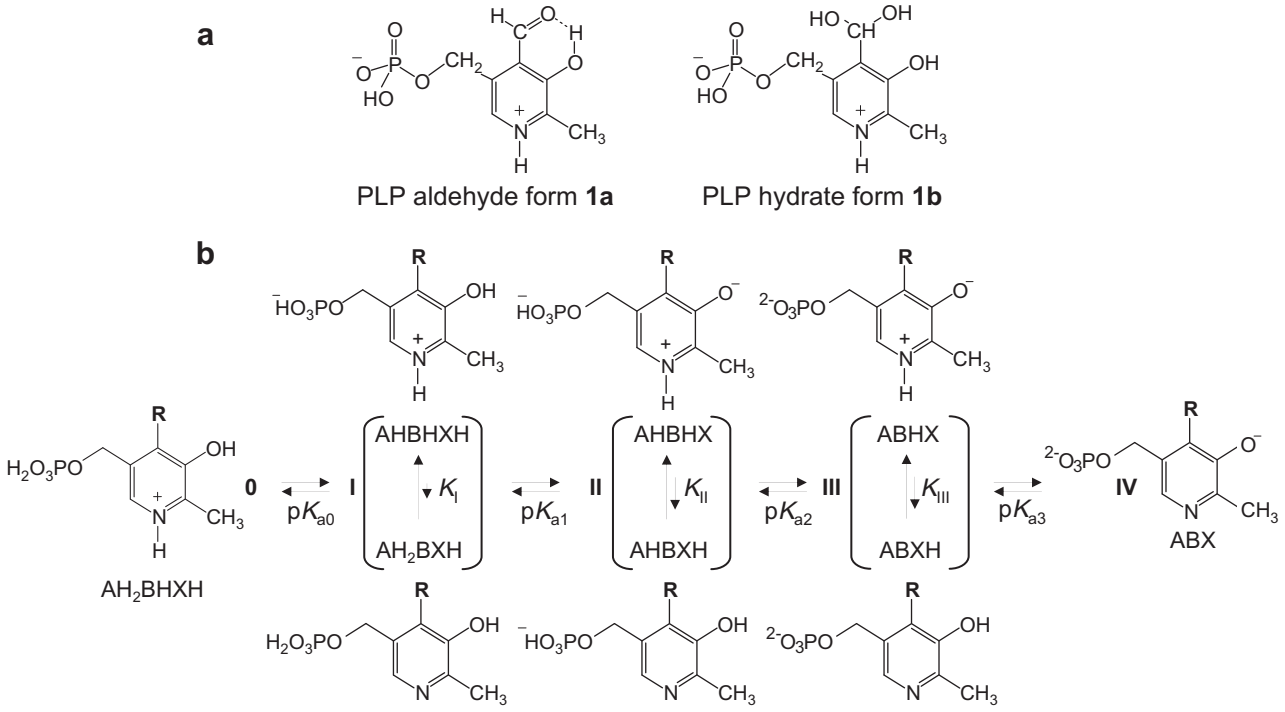

Fig. 1. (a) Aldehyde form **1a** and hydrate form **1b** of pyridoxal-5′-phosphate (PLP). (b) Protonation states of **1a** and **1b**.

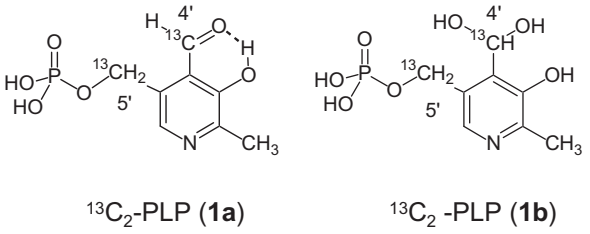

Fig. 2. ^{13}C labeled PLP studied in this work. Isotope enrichment either 25% or 100% in positions C-4′ and C-5′.

2. Experimental

2.1. Synthesis of ^{13}C enriched PLP

$^{13}C_2$-PLP was 25% isotopically enriched at the C-4′ and C-5′ positions according to Fig. 3, which is a modified version of the synthesis by O'Leary and Payne [10]. The main modification concerned the formation of the diethyl maleate ester ***b*** which was obtained by direct esterification of the commercially available 1,4-di-^{13}C-anhydride maleic acid (Eurisotop). In a second modification, *p*-toluidine was used as the amine to react with the oxidized pyridoxine hydrochloride ***g*** instead of *p*-phenitidine, and finally the crude product of the phosphorylation reaction was hydrolyzed at room temperature overnight instead of heating under nitrogen. L-alanine was commercially available from Aldrich Sigma.

2.1.1. Diethyl-di-^{13}C-maleate ester (***b***)

A 1:3 mixture of 1,4-di-^{13}C-anhydride maleic acid ***a*** (2 g, 20 mmol, isotope enrichment 99%) and non ^{13}C enriched maleic acid anhydride was dissolved in a 2:1 mixture of dry ethanol (52 ml) and toluene (26 ml). The reaction was catalyzed with 0.5 ml concentrated H_2SO_4. The mixture was refluxed overnight. After evaporation of the solvent, 20 ml of water was added to the resulting oil. The aqueous phase was then washed with dichloromethane (3 × 30 ml). After evaporation of the solvent, the residue was distilled under vacuum (B_p = 58 °C at 0.3 mbar) to obtain ***b*** as a clear transparent oil. Yield: 2.9 g (17 mmol), 84%, clear transparent oil. R_f = 0.88 (DCM:MeOH, 10:0.1). IR (KBr): ν = 2985, 1685, 1639, 1269, 1200, 1150, 1030, 965, 865, 830, 800 cm^{-1}. ^{13}C {^1H} NMR (125 MHz, chloroform-d): δ = 164.9 (s, COOEt, no $^1J(^{13}C,^{13}C)$ is observed), 129.5 (ABX system, splitting 73 Hz), 60.9 (t, $\underline{C}H_2$–CH_3), 13.7 (q, CH_2–$\underline{C}H_3$). ^1H NMR (270 MHz, chloroform-d): δ = 6.2 (AA′XX′, 2H, splitting 10 Hz and 6 Hz), 4.2 (q, 4H, $C\underline{H}_2$–CH_3), 1.3 (t, $^3J(^1H, ^1H)$ = 7.3 Hz, 6H, CH_2–$C\underline{H}_3$). MS (EI): *m*/*z* (%) = 174.1 ($[M]^+$, 0.1), 145.1 ($[M-CH_2-CH_3]^+$, 4.6), 129.0 ($[M-O-CH_2-CH_3]^+$, 24), 100.0 ($[M-{}^{13}CO_2-CH_2-CH_3]^+$).

2.1.2. Ethyl-N-formyl-D,L-alaninate (***d***)

A mixture containing L-alanine (5 g, 40 mmol, 1 eq) and fresh formic acid (8 ml, 220 mmol, 5.5 eq) in dry ethanol (60 ml) was heated inside a high pressure autoclave HR-100 (made of steel from Berghof GmbH) at 200 °C for 24 h. At the end of the reaction a pressure of ~5 bar had built up. After cooling slowly to room temperature and reaching atmospheric pressure, the solvent was evaporated under vacuum. The residue was distilled under vacuum (B_p = 87–90 °C at 0.3 mbar) to obtain ***d*** as a clear transparent oil and was directly used for the next step without storing. Yield: 5.5 g (38 mmol), 95%, clear transparent oil.

^{13}C {^1H} NMR (125 MHz, chloroform-d): δ = 172.4 (s, COOEt), 160.7 (d, CHO), 61.4 (t, $\underline{C}H_2$–CH_3), 46.6 (d, NH–$\underline{C}H$–CH_3), 18.0 (q, NH–CH–$\underline{C}H_3$), 13.8 (q, CH_2–$\underline{C}H_3$). ^1H NMR (500 MHz, chloroform-d): δ = 5.1 (s, 1H, C$\underline{H}$O), 6.9 (b, 1H, N$\underline{H}$), 4.5 (qt, $^3J(^1H, ^1H)$ = 7.3 Hz, 1H, NH–C$\underline{H}$–CH_3), 4.1 (q, $^3J(^1H, ^1H)$ = 7.1 Hz, 2H, C$\underline{H}_2$–CH_3), 1.3 (d, $^3J(^1H, ^1H)$ = 7.3 Hz, 3H, CH–C$\underline{H}_3$), 1.2 (t, $^3J(^1H, ^1H)$ = 7.1 Hz, 3H, CH_2–$C\underline{H}_3$). MS (FAB (+), matrix chloroform-d/m-NO_2–benzyl–OH): *m*/*z* (%) = 145.8 ($[M+H]^+$, 48), 72.0 ($[M-CO_2-CH_2-CH_3]^+$, 31), 44.1 ($[M-CH_3-CH-CO_2-CH_2-CH_3]^+$, 65).

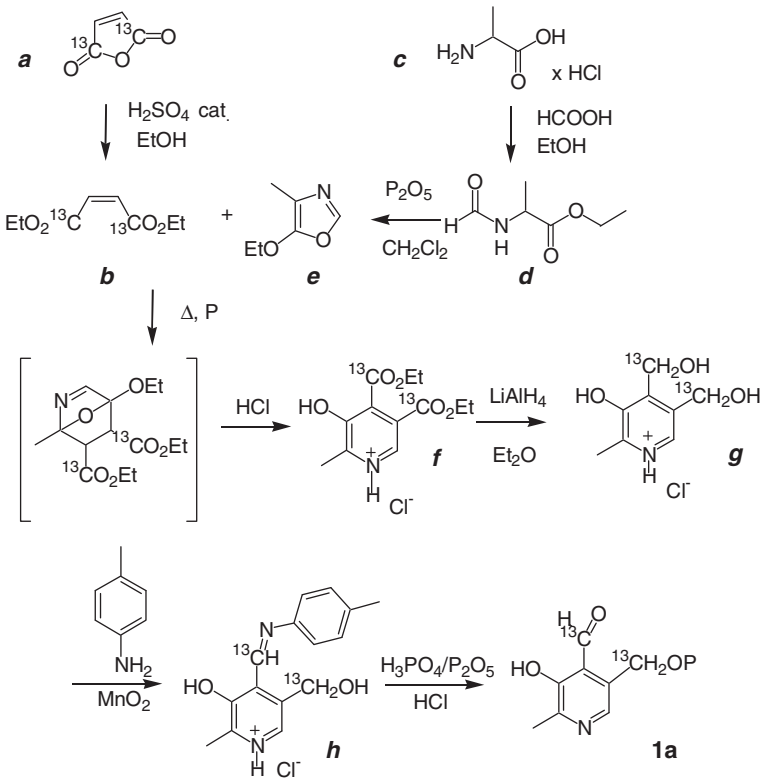

Fig. 3. Synthesis of ^{13}C enriched PLP according to O'Leary and Payne [10].

2.1.3. 5-ethoxy-4-methyloxazole (e)

Small portions of P_2O_5 (23.5 g, 160 mmol, 4 eq) were added to a solution of ethyl-N-formyl-D,L-alaninate (***d***) (5.5 g, 35 mmol, 1 eq) in dichloromethane (250 ml). The reaction mixture was refluxed for 48 h. After cooling to room temperature, a solution of 35% NaOH in water was added slowly through the refrigerant. The aqueous phase was then washed with dichloromethane (3 × 150 ml). After evaporation of the solvent, the residue was distilled under vacuum to obtain ***e*** as a clear transparent oil. Yield: 2.3 g (18 mmol), 50%, clear transparent oil. IR (KBr): ν = 3450, 2985, 1670, 1515, 1335, 1220, 1130, 1020, 655 cm^{-1}. ^{13}C {^1H} NMR (125 MHz, chloroform-d): δ = 154.2 (s, C-5), 142.1 (d, C-2), 112.1 (s, C-4), 70.1 (t, $\underline{C}H_2$–CH_3), 14.9 (q, CH_2–$\underline{C}H_3$), 9.8 (q, $\underline{C}H_3$). ^1H NMR (500 MHz, chloroform-d): δ = 7.4 (s, 1H, C$\underline{H}$), 4.1 (q, $^3J(^1H, ^1H)$ = 7.1 Hz, 2H, C$\underline{H_2}$–CH_3), 2.0 (s, $\underline{C}H_3$), 1.3 (t, $^3J(^1H, ^1H)$ = 7.1 Hz, 3H, CH_2–$\underline{C}H_3$). MS (FAB (+), matrix chloroform-d/m-NO_2–benzyl–OH): *m/z* (%) = 145.8 ([M + H]$^+$, 48), 72.0 ([M−CO_2–CH_2–CH_3]$^+$, 31), 44.1 ([M−CH_3–CH–CO_2–CH_2–CH_3]$^+$, 65).

*2.1.4. [4′,5′-di-^{13}C]-4,5-Bis-ethoxycarbonyl-3-hydroxy-2-methyl-pyridinium chloride (**f**)*

A mixture of 5-ethoxy-4-methyloxazole (***e***) (500 mg, 3.9 mmol, and 1 eq) and diethyldi-^{13}C-maleate ester (***b***) (680 mg, 3.9 mmol, 1 eq) was stirred in a closed vial and heated at 70 °C for 3 days. The reaction mixture turned orange. After cooling down to room temperature, the mixture was dissolved in methanolic HCl (10 ml, 3N) and stirred for 4 h. Pressure crystallization with cold ether was performed to obtain product (***f***) as needle crystals after refrigerating at −23 °C for 24 h. Yield: 670 mg (2.3 mmol), 59%, light yellow needles. R_f = 0.8 (DCM:MeOH, 10:0.4). IR (KBr): ν = 2545, 2045, 1695, 1530, 1265, 1040 cm^{-1}. ^{13}C {^1H} NMR (125 MHz, chloroform-d): δ = 164.5 (s, $^3J(^{13}C, ^{13}C)$ = 1 Hz, $\underline{C}OO$–CH_2–CH_3), 161.8 (s, $\underline{C}OO$–CH_2–CH_3), 154.1 (s, C-3), 149.2 (s, C-2), 130.9 (d, C-6), 128.2 (s, C-4 or C-5), 127.8 (s, C-5 or C-4), 64.1 (t, $\underline{C}H_2$-CH_3), 63.2 (t, $\underline{C}H_2$–CH_3), 15.6 (q, C-2′), 13.8 (q, CH_2–$\underline{C}H_3$), 13.5 (q, CH_2–$\underline{C}H_3$). ^1H NMR (500 MHz, chloroform-d): δ = 8.3 (s, 1H, H-6), 4.4 (dq, $^3J(^1H, ^1H)$ = 7.3 Hz, $^3J(^1H, ^{13}C)$ = 2.6 Hz, 4H, C$\underline{H_2}$–CH_3), 2.9 (s, 3H, H-2′), 1.4 (dt, $^3J(^1H, ^1H)$ = 7.3 Hz, $^3J(^1H, ^{13}C)$ = 2.7 Hz, 6H, CH_2–C$\underline{H_3}$). MS (FAB (+), matrix acetone/m-NO_2–benzyl–OH): *m/z* (%) = 256.3 ([M−Cl]$^+$, 100), 209.8 (31), 182.0 (36). MS (FAB (−), matrix acetone/m-NO_2–benzyl–OH): *m/z* (%) = 288.2 ([M−H]$^-$, 2), 254.4 ([M−H–HCl]$^-$, 100), 226.0 (9.65).

*2.1.5. Pyridoxine HCl (**g**)*

[4′,5′-di-^{13}C]-4,5-Bis-ethoxycarbonyl-3-hydroxy-2-methyl-pyridinium chloride (***f***) (500 mg, 1.7 mmol, 1 eq) was added *via* a Soxhlet extractor overnight to a solution of $LiAlH_4$ (520 mg, 14 mmol, 8 eq) in 100 ml of dry ether. The reaction mixture was treated with 40 ml of water, added drop-wise initially. The mixture was filtered through a frit and washed with boiling water (2 × 60 ml). After evaporation of half of the volume, CO_2 was bubbled through the aqueous clear yellow solution for 1 h. After drying under vacuum, the white powder was extracted with boiling methanol (2 × 50 ml), and a clear yellow solution is obtained. The solvent was evaporated and methanolic HCl (10 ml, 3 N) was added to the yellowish oil. After evaporation of the solvent and drying, pyridoxine hydrochloride (***g***) was obtained as a powder. Yield: 325 mg (1.6 mmol), 92%, white powder. R_f = 0.5 (unprotonated form), 0.1 (protonated form) (DCM: MeOH, 10: 0.2). IR (KBr): ν = 3325, 2825, 1625, 1545, 1385, 1280, 1215 cm^{-1}. ^{13}C {^1H} NMR (125 MHz, D_2O): δ = 155.2, 145.1, 142.8, 139.0 (s, C-2 or C-3 or C-4 or C-5), 131.9 (d, C-6), 60.4 (t, C-4′ or C-5′, $^3J(^{13}C, ^{13}C)$ = 4 Hz), 59.1 (t, C-5′ or C-4′, $^3J(^{13}C, ^{13}C)$ = 4 Hz). ^1H NMR (500 MHz, D_2O): δ = 8.0 (s, 1H, H-6), 4.9 (d, $^1J(^1H, ^{13}C)$ = 150 Hz, 2H, C$\underline{H_2}$–OH), 4.7 (d, $^1J(^1H, ^{13}C)$ = 150 Hz, 2H, C$\underline{H_2}$–OH), 2.5 (s, 3H, C$\underline{H_3}$). MS (FAB (+), matrix H_2O/glycerol): *m/z* (%) = 206.9 ([M]$^+$, 1), 172.3 ([M−Cl]$^+$, 29), 153.8. MS (FAB (−), matrix H_2O/glycerol): *m/z* (%) = 205.9 ([M−H]$^-$, 29), 170.3 ([M−H–HCl]$^-$, 51), 151.8, 129.

*2.1.6. N-(pyridoxylidene)-tolylamine hydrochloride (**h**)*

Pyridoxine HCl (**g**) (1.6 g, 7.9 mmol, 1 eq) was dissolved in ca. 30 ml of water then oxidized with manganese dioxide (MnO_2) which was freshly prepared from potassium permanganate ($KMnO_4$, 1.2 g, 7.6 mmol, 1 eq), sodium bisulfite ($NaHSO_3$, 1.6 g, 15 mmol, 10 eq) and 50% sulfuric acid H_2SO_4 (4 ml) diluted in 60 ml of water. After stirring for 4 h, the solution was diluted by adding 500 ml of water and *p*-toluidine (1.2 g, 11 mmol, 1.4 eq) was added. The pH was adjusted to 7.5 with a 1 N $NaHCO_3$ solution and left to stir overnight. The Schiff base was filtered and washed with water then with ether. The product was dried under high vacuum. Yield: 1 g (3.5 mmol), 44%, dark yellow powder. $R_f = 0.2$ (DCM:MeOH, 10:0.2). IR (KBr): $\nu = 3115, 2825, 1405, 1005, 815, 495\ cm^{-1}$.$^{13}C$ $\{^1H\}$ NMR (125 MHz, DMSO-d_6): $\delta = 160.7$ (d, C-4′, $^3J(^{13}C, ^{13}C) = 2$ Hz), 153.2, 148.2, 144.6, 138.2, 137.7, 133.6, 119.9, 114.0, 110.5 (aromatic carbons), 58.9 (t, C-5′, $^3J(^{13}C, ^{13}C) = 2$ Hz), 18.7 (q, CH_3).^1H NMR (500 MHz, DMSO-d_6): $\delta = 9.17$ (s, 1H, H-4′), 7.98 (s, 1H, H-6), 7.42 and 7.32 (d, H-aromatic of toluidine part), 4.77 (d, $^1J(^1H, ^{13}C) = 150$ Hz, 2H, C$\underline{H_2}$-OH), 3.33 (s, 3H, CH_3). MS (FAB (+), matrix H_2O/glycerol): m/z (%) = 259.4 ($[M + H]^+$, 6), 153.7, 151.8 ($[M-N-Toluene-H]^+$, 100), 116.9. MS (FAB (−), matrix H_2O/glycerol): m/z (%) = 257.4 ($[M-H]^-$, 2), 221.8 ($[M-H-HCl]^-$, 2), 152.8 ($[M-N-Toluene]^-$, 100).

*2.1.7. ^{13}C-pyridoxal-5′-phosphate (**1a**)*

A mixture of phosphorus pentoxide (P_2O_5, 12 g, 84 mmol, 13 eq) and phosphoric acid (85%, 16 g, 140 mmol, 21 eq) was prepared and cooled down to room temperature since the mixing evolves heat. Subsequently, N-(pyridoxylidene)–tolylamine hydrochloride (**h**) (1.6 g, 6.6 mmol, 1 eq) was added. The honey-like mixture was incubated at 45 °C for 7 h. The reaction was quenched with an aqueous solution of HCl (3.5 ml, 0.1 M) and left overnight. The crude product was loaded on an ion exchange column (Amberlite IR 120) and eluted using water as solvent. After lyophilizing, PLP was obtained as a yellow solid. Yield: 470 mg, 24%, yellow solid. IR (KBr): $\nu = 3365, 2925, 1630\ cm^{-1}$. ^{13}C $\{^1H\}$ NMR (125 MHz, D_2O): $\delta = 195.6$ (C-4′, $^3J(^{13}C, ^{13}C)$ not observable, aldehyde form), 163.6 (d, C-6), 151.3 (s, C-5), 135.4 (s, C-4), 125.5 (s, C-2), 123.6 (s, C-3), 89.4 (d, C-4′, $^3J(^{13}C, ^{13}C)$ not observable, acetal form), 61.7 (t, C-3′), 15.6 (q, C-2′). ^1H NMR (500 MHz, D_2O): $\delta = 10.4$ (d, $^1J(^1H, ^{13}C) = 185$ Hz, 1H, H-4′ – aldehyde form), 8.1 (s, 1H, H-6 – aldehyde form), 6.4 (d, $^1J(^1H, ^{13}C) = 170$ Hz, 1H, H-4′ – hydrate form), 5.2 (dd, $^1J(^1H, ^{13}C) = 150$ Hz, $^2J(^1H, ^1H) = 5$ Hz, 2H, H-3′ – aldehyde form), 5.1 (dd, $^1J(^1H, ^{13}C) = 150$ Hz, $^2J(^1H, ^1H) = 5$ Hz, 2H, H-3′ – hydrate form), 2.6 (s, 3H, H-2′ – aldehyde form), 2.5 (s, 3H, H-2′ – hydrate form).

2.2. Sample preparation

Aqueous solutions of PLP (5 mM) were prepared using water or heavy water degassed and stored under argon in order to remove oxygen and carbon dioxide. The pH values of the solutions were adjusted before each spectroscopic measurement by addition of degassed 3 M, 1 M or 0.1 M sodium hydroxide or hydrochloric acid solutions. For that purpose we used a HANNA HI 9025 pH meter equipped with a HAMILTON Spintrode P electrode. The pH values were measured after the experiments and showed an average error of ±0.15.

2.3. Spectroscopic methods

NMR spectra of pyridoxal-5′-phosphate were measured using a Bruker AMX 500 spectrometer (500.13 MHz for ^1H, 125 MHz for ^{13}C) at 278 K. The ^{13}C spectra were recorded in the inverse gated ^1H-decoupled mode using H_2O as solvent, with field locking using a D_2O containing capillary. The recycle delay was set to 10 s. TMS was used as external reference. Some routine NMR spectra of **b** to **g** were measured using a 270 MHz NMR spectrometer.

3. Results

A ^{13}C NMR titration of $^{13}C_2$-PLP in H_2O (5 mM) was performed between pH 1 and 12. Typical $\{^1H\}^{13}C$ spectra obtained are depicted in Fig. 4. Only the peaks arising from the isotopically enriched carbon sites C-4′ and C-5′ were analyzed, not the small peaks arising from the carbon sites containing ^{13}C at natural abundance. **1a** gives a signal typical for the aldehyde position C-4′, around 194.5 ppm, and a second signal of equal height around 61.0 ppm. The signal of C-4′ of **1b** is shifted to about 87 ppm because of the change of the hybridization at this carbon. By contrast, the chemical shift of C-5′ is altered little. At low pH, the hydrate form dominates and hence the two signals of **1b** are larger than those of **1a**, in agreement with previous ^{15}N NMR studies [8]. By contrast, the signal of **1a** dominates above pH 4. The equilibrium constants for hydration were calculated from the mole fraction ratios, which were derived by signal integration. The results are assembled in Table 1.

$$K_h = \frac{[\mathbf{1b}]}{[\mathbf{1a}]} \tag{1}$$

Table 2 collects all ^{13}C chemical shifts measured at the different pH values. Generally, average chemical shifts of species subject to different protonation states can be expressed as a function of pH using the Henderson–Hasselbalch equation [11] adapted for NMR spectroscopic methods in the fast proton exchange regime [12]. For PLP, which has five protonation states 0 to IV according to Fig. 1, this equation can be written in the following form [8].

$$\delta_{obs} = \delta_0 + \sum_i (\delta_{i+1} - \delta_i) \frac{10^{pH-pK_{ai}}}{1 + 10^{pH-pK_{ai}}}, \quad i = 0 \text{ to IV} \tag{2}$$

As usual, pK_{ai} represents the pH values where protonation states i and $i+1$ are at the same concentration. δ_i represents the limiting chemical shift of protonation state i. Eq. (2) is valid for all nuclei of PLP.

The ^{13}C chemical shifts of the aldehyde form **1a** and of the hydrated form **1b** of PLP in Table 2 are plotted in Fig. 5 as a function of pH. The solid lines were calculated using Eq. (2), optimizing the pK_a values and the limiting ^{13}C chemical shifts. The results are given in Tables 3 and 4.

4. Discussion

In the first part of this section, we discuss the pH-dependent equilibrium between the aldehyde form **1a** and the hydrate form **1b**. In the second part, we discuss the different protonation states observed here, and compare these results with those obtained previously [8] using ^{15}N NMR of PLP labeled with ^{15}N in the pyridine ring.

4.1. Equilibrium between aldehyde and hydrate form

In Fig. 6, the mole fractions of the aldehyde form **1a** and of the hydrated form **1b** are plotted as a function of pH. Qualitatively, it has been known for a long time that **1b** dominates at low pH and **1a** at high pH. However, the ^{13}C experiments here allowed us to elucidate the pH of 4.2 where the equilibrium constant of hydration K_h is unity.

A consequence of the hydration equilibrium is that it is difficult to determine the ^{13}C chemical shifts of C-5′**1a** at low and of **1b** at high pH.

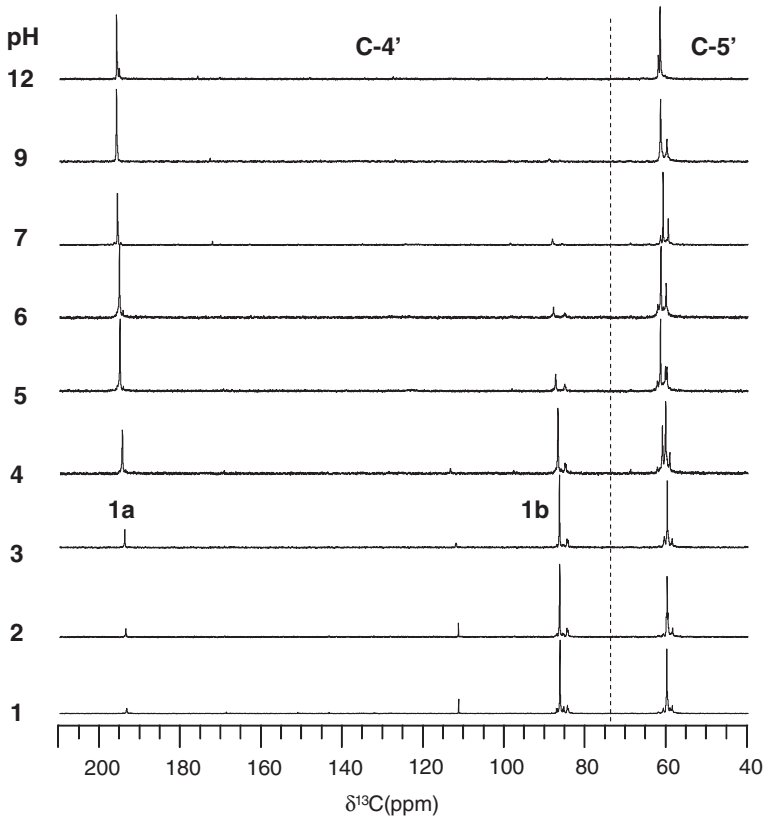

Fig. 4. $\{^1H\}^{13}C$ NMR spectra of $^{13}C_2$-PLP (**1**) in H_2O at 278 K as a function of pH.

Table 1
Mole fractions of the aldehyde **1a** and hydrate **1b** obtained by ^{13}C NMR.

pH	Mole fraction		K_h
	C-4′ **1a**	C-4′ **1b**	
1.2	0.13	0.87	6.70
2.0	0.14	0.86	6.03
3.1	0.19	0.81	4.2
3.6	0.32	0.68	2.15
3.9	0.41	0.59	1.42
5.0	0.77	0.23	0.30
5.9	0.83	0.17	0.21
7.0	0.87	0.13	0.15
8.0	0.92	0.08	0.09
9.0	0.97	0.03	0.03
10.1	0.96	0.04	0.04
11.1	0.96	0.04	0.04
12.0	1.0	0	0

Table 2
^{13}C chemical shifts in ppm of $^{13}C_2$-PLP at the position C-4′ and C-5′.

pH	C-4′		C-5′	
	1a	**1b**	**1a**	**1b**
1.0	193.5	86.5		60.1
1.5	193.5	86.5		60.1
2.0	193.6	86.6		60.2
3.2	193.9	86.6	60.8	60.2
3.6	194.5	87.0	61.0	60.4
3.9	194.7	87.1	61.2	60.5
5.0	195.0	87.6	61.7	60.5
5.9	195.2	88.1	61.6	60.3
7.0	195.7	88.4	61.1	59.8
8.0	195.9	88.6	61.2	59.8
9.0	196.0	89.1	61.7	60.1
10.1	195.9	89.8	61.9	61.0
11.1	196.0	89.7	61.9	62.1
12.0	196.0	89.7	61.9	62.3

4.2. Determination of the pK_a values of PLP by ^{13}C NMR

The results of our ^{13}C NMR titration study of PLP in aqueous solution are illustrated in Fig. 5. Two ^{13}C spin probes were present, at C-4′ and C-5′ (Fig. 2). For the aldehyde form **1a**, the solid lines reproduce well the experimental data. Between two pK_a values, illustrated by vertical dashed lines, the chemical shifts exhibit little dependence on pH, while near the pK_a values a strong dependence is observed. Position C-4′ is most diagnostic both in the case of **1a** and of **1b** since its signals can be observed even when the corresponding species represents a minor form. An exception is **1a** at high pH: the chemical shifts of III and IV are very similar, but the corresponding pK_{aIII} value could be well determined from the signal of position C-5′. Problems arise with the determination of the chemical shifts of C-5′ of the minor forms, i.e. of **1a** at low pH and of **1b** at high pH. However, all four pK_a values could be determined by the combination of the spin probes C-4′ and C-5′.

4.3. Determination of the equilibrium constants of tautomerism of PLP by ^{15}N NMR

In a previous study [8], Eq. (2) was used to analyze the pH-dependent ^{15}N chemical shifts of PLP labeled with ^{15}N in the pyridine ring. It was shown that the chemical shift δ_i of a given protonation state i obtained by the Henderson–Hasselbalch fit of the experimental data to Eq. (2) depends on the equilibrium constants K_i of the OH/NH tautomerism (Fig. 1b) given by

$$\delta_i = \frac{1}{1+K_i}\delta_{NH} + \frac{K_i}{1+K_i}\delta_N \quad (3)$$

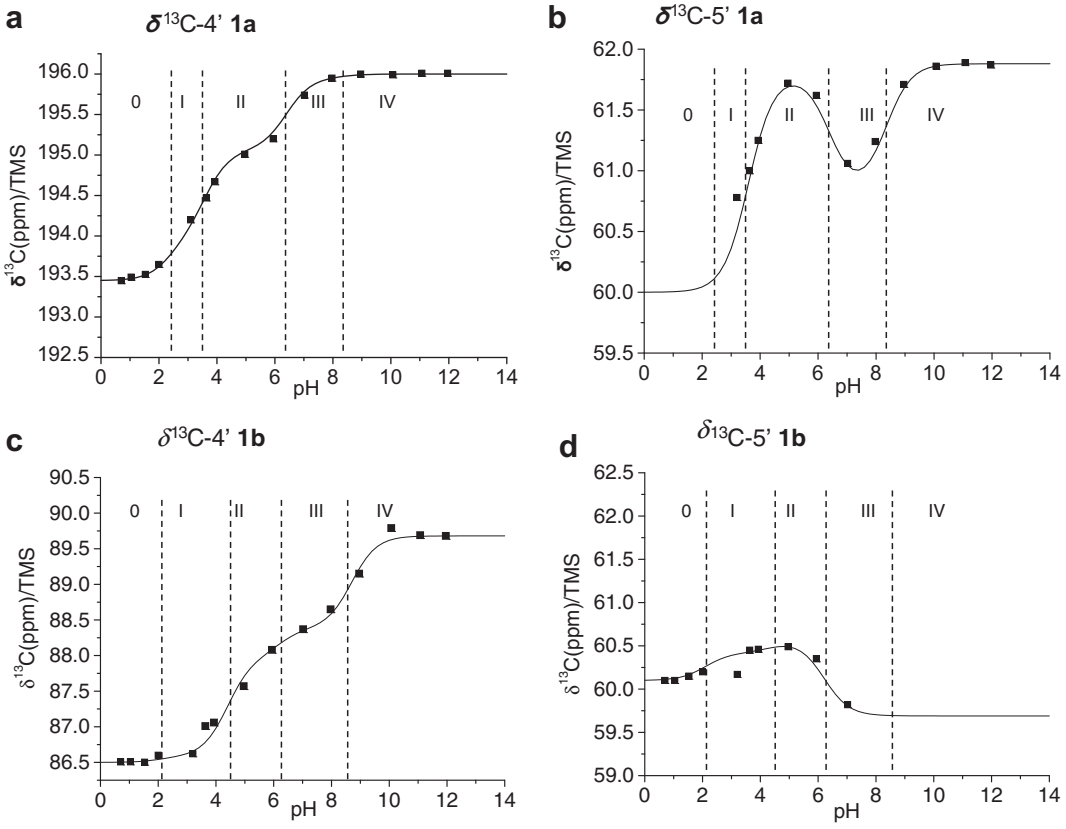

Fig. 5. ^{13}C chemical shifts of the C-4′ and C-5′positions of the aldehyde form **1a** and the hydrate form **1b** in water as a function of pH. The dashed vertical lines indicate pK_a values. The solid lines were calculated using the Henderson–Hasselbalch equation (2) and the parameters reported in Tables 2 and 3.

Table 3
pK_a values of PLP in water.

PLP	pK_{a0}	pK_{aI}	pK_{aII}	pK_{aIII}
1a				
^{15}N NMR corrected[a]	2.4	3.6	6.4	8.3
^{13}C-4′ NMR[b]	2.4	3.6	6.4	n.o.
^{13}C-5′ NMR[b]	n.o.	3.6	6.4	8.3
Literature		3.1–3.7[c]	6.1[d]	8.3–8.9[e]
1b				
^{15}N NMR	n.o.	4.4	n.o	8.7
^{13}C-4′ NMR	2.1	4.4	6.2	8.7
^{13}C-5′ NMR	2.1	4.4	6.2	8.7
Literature		4.1[c]	6.1[b]	8.7[c]

n.o.: Not observed.
[a] Ref. [8].
[b] This study.
[c] Refs. [4,14].
[d] Refs. [2,15].
[e] Ref. [16].

Table 4
Limiting ^{13}C and ^{15}N NMR chemical shifts (in ppm) of PLP obtained from Fig. 5.

PLP	δ_0	δ_I	δ_{II}	δ_{III}	δ_{IV}
1a					
^{15}N	170.4	184.4	198.4	199.4	264
C-4′	193.4	194.0	195.1	196.0	196.0
C-5′	60.0	60.0	61.8	60.8	61.9
1b					
^{15}N	162.6	162.6	168.1	168.1	264
C-4′	86.5	86.5	87.9	88.3	89.6
C-5′	60.1	60.4	60.5	59.6	62.2

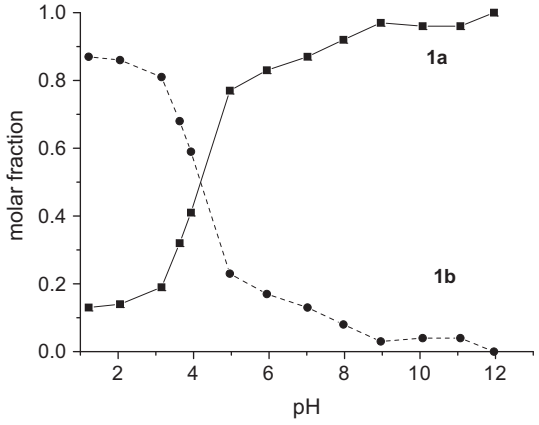

Fig. 6. Mole fractions evaluated by integration of the C-4′ signals of the aldehyde form **1a** and of the hydrate form **1b** of PLP in water at 278 K as a function of pH.

δ_{NH} and δ_N represent the chemical shifts of the protonated and deprotonated ring nitrogen, respectively. The two values are very different, but exhibit little dependence on the protonation state. Thus, only if K_i differs for two adjacent protonation states, the corresponding pK_a value can be obtained by ^{15}N NMR.

By inspection of Fig. 1b, it is clear that K_I is very small, i.e. the assumption is plausible [8] that the tautomeric form AH_2BXH of protonation state I is not present in view of the large acidity of phosphoric acid. For protonation state IV the equilibrium does not exist. Protonation state 0 was not considered previously [8], but it is clear that only a single tautomer can be formed. On the other hand, K_{II} and K_{III} could be obtained previously [8] by fitting the ^{15}N chemical shifts to Eqs. (2) and (3).

By contrast, it is difficult to derive the equilibrium constants of tautomerism K_i of a given protonation state i from its average ^{13}C chemical shift δ_i listed in Table 4, as the ^{13}C chemical shift changes caused by the tautomerism depend on the protonation state, in contrast to the ^{15}N chemical shifts. However, by the combination of ^{13}C and ^{15}N NMR this problem can be solved. We took the pK_a values obtained by ^{13}C NMR (dashed vertical lines) and reanalyzed the ^{15}N chemical shifts reported previously [8]. The results are depicted in Fig. 7. The solid curves were calculated using the chemical shifts δ_{NH} and δ_N (dashed horizontal lines) as well as the equilibrium constants of tautomerism of protonation states II and III of **1a** and **1b**, K_{II} and K_{III} previously [8] (see also caption of Fig. 7). For reference, we added the dashed curves which were calculated assuming that the equilibrium constants of tautomerism $K_{II} = K_{III} = 0$ (lower curve) or infinite (upper curve).

4.4. Acid–base properties of PLP and biological function

What do the pK_a values and equilibrium constants of tautomerism tell us about the acid–base properties of the two forms of PLP? At pH values below 2.4 both **1a** and **1b** are fully protonated, i.e. the protonation state 0 (Fig. 1b) is formed. This state has not been observed before. The new pK_{a0} values of 2.4 for **1a** and of 2.1 for **1b** are very close to the corresponding value of 2.15 for H_3PO_4 [13]. Thus, when pH is increased, at pH 3 the phosphoric group is monoanionic in **1a** and **1b**, and protonation state I is formed, but the pyridine ring still protonated. When pH is further increased, first **1a** looses a second proton below and **1b** above pH 4, i.e. protonation state II is formed. This is also the region where the hydrate **1b** looses its dominance. Thus, **1b** dominates only in protonation state I, whereas 1b dominates in the higher protonation states. In a narrow pH region around 4, the conversion of **1a** to **1b** is then associated with a protonation, i.e. a conversion from II to I. By ^{15}N NMR it is shown that the tautomeric equilibrium of **1b** is shifted almost entirely into the direction of the protonated pyridine form, whereas **1a** forms a substantial amount of the deprotonated pyridine form. This observation can be rationalized in terms of a stronger intramolecular OHO-hydrogen bond in **1a** as compared to **1b** as illustrated in Fig. 8. We speculate that this hydrogen bond plays a decisive role for the dominance of **1a** at higher pH, but not at low pH. At physiological pH 7.35 **1b** is negligible, i.e. protonation state III of **1a** seems to be of most physiological importance. The remaining proton is almost equally distributed between the pyridine ring and the phenolic function. Complete deprotonation and formation of state IV does not seem to play a physiological role.

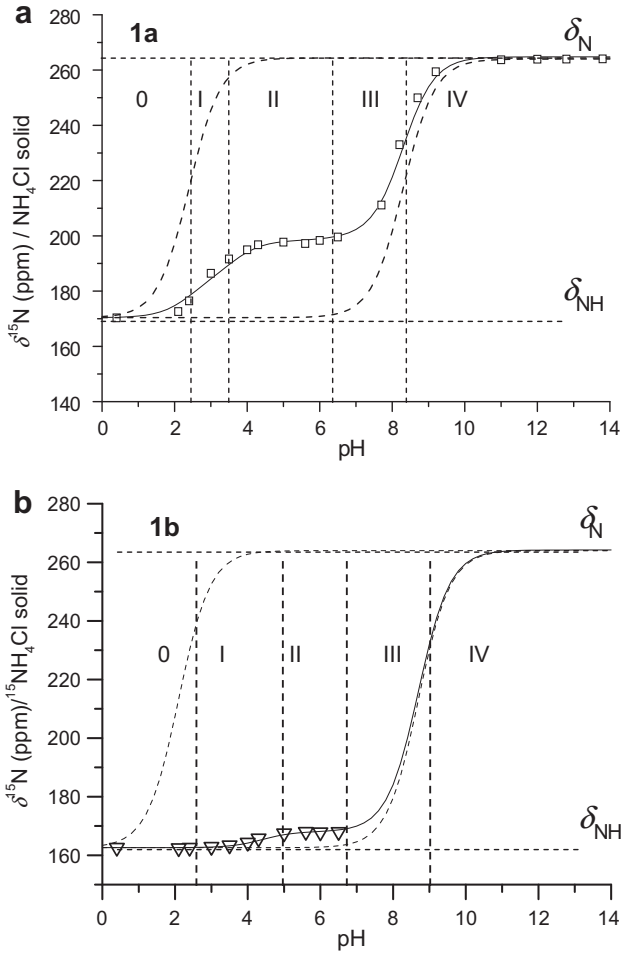

Fig. 7. ^{15}N chemical shifts measured previously [8] of the ^{15}N-labeled pyridine ring of **1a** and **1b** as a function of pH. The dashed vertical lines indicate pK_a values, the dashed horizontal lines the limiting ^{15}N chemical shifts of the non-protonated and the protonated pyridine ring. The solid curves were obtained by using the pK_a values of PLP determined here by ^{13}C NMR (Table 3) and the following parameters determined previously [8]: δ_N(**1a**) = δ_N(**1b**) = 264 ppm, δ_{NH}(**1a**) = 170.4 ppm, δ_{NH}(**1b**) = 162.6 ppm (dashed horizontal lines), K_{II}(**1a**) = K_{III}(**1a**) = 0.4, K_{II}(**1b**) = K_{III}(**1b**) = 0.06. The dashed curves were calculated assuming that the equilibrium constants of tautomerism $K_{II} = K_{III} = 0$ (lower curve) or infinite (upper curve).

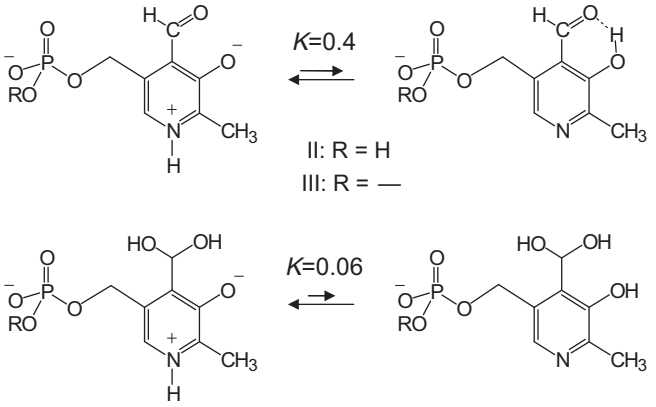

Fig. 8. Tautomerism of **1a** and of **1b** in protonation states II and III. For further discussion see text.

However, in the active site of PLP dependent enzymes the acid–base behavior can be modified compared to the aqueous environment. Thus, recently [9d] it was shown that the Schiff base formed by PLP with the ε-amino group of a lysine residue in aspartate aminotransferase behaves as if it were embedded in a highly polar but aprotic organic solvent. In this environment, the acid–base properties of PLP are unique. Thus, further studies of the interaction of PLP with amines in water, model environments and in enzymes will be fruitful in the future.

5. Conclusions

Using ^{13}C NMR of the cofactor pyridoxal-5′-phosphate (PLP) labeled with ^{13}C in positions C-4′ and C-5′ it has been possible to determine the pK_a values of the different protonation states 0 to IV depicted in Fig. 1b for the aldehyde form **1a** as well as for the hydrate form **1b**. The results are in good agreement with those published previously as illustrated by Table 3. Here, for the first time, measurements could be performed at lower pH where PLP is not very soluble. Because of the ^{13}C spin probes which exhibit a high signal intensity and a high sensitivity to pH changes we were able to detect protonation state 0 which is of the AH_2BHXH type. Furthermore, we speculate that the dominance of **1a** at high-

er pH is associated with the stabilizing effect of the intramolecular OHO-hydrogen bond, and that this effect is less important in protonation states 0 and I. The new results lead to a consistent picture of the protonation states of PLP in aqueous environments. The present work will guide future work aimed at understanding better the interaction of PLP with amines in model systems and in enzyme environments.

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