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C−H Activation in Pyridoxal-5′-phosphate Schiff Bases: The Role of the Imine Nitrogen. A Combined Experimental and Computational Study

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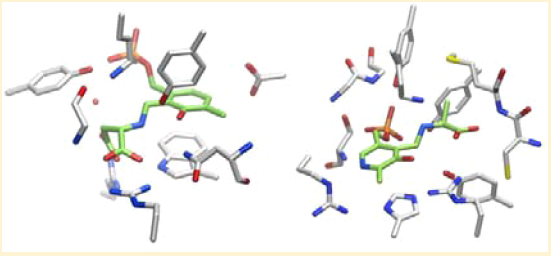
Supporting Information

ABSTRACT: The origins of C−H activation in pyridoxal-5′-phosphate (PLP) Schiff bases and modulation of reaction specificity in PLP-enzymes are still not completely understood. There are no available studies that compare the reactivity of C4′ carbons in ketimine Schiff bases with that of Cα carbons in their aldimine counterparts, which is essential to unravel the mechanisms that govern the evolution of their common carbanionic intermediates. Second-order rate constants for phosphate-catalyzed proton/deuterium exchange reactions in D_2O of C4′ carbons suffer a 10^5-fold increase due to Schiff base formation (k_B = 5.3×10^1 M^{-1} s^{-1}) according to NMR measurements. The C4′ carbon acidity is also increased to pK_a = 9.8, which is significantly higher than that of Cα in PLP-aldimines. DFT calculations reveal the role of each heteroatom in modulating the electrophilicity of C4′ and Cα carbons. Specifically, the protonation state of pyridine nitrogen is the main factor in determining the absolute carbon acidity in aldimines (pK_a of Cα varies from ∼14 to ∼23) and ketimines (pK_a of C4′ varies from ∼12 to ∼18), whereas the protonation state of both imine nitrogen and O3′ phenol oxygen modulates the relative acidities of Cα and C4′ from 1.5 to 7.5 pK_a units. Our results provide an explanation to the modulation of reaction specificity observed in different PLP-enzymes based on the differences in the protonation state of the cofactor and H-bonding patterns in the active site.



INTRODUCTION

Pyridoxal-5′-phosphate (PLP) form of vitamin B6 is a powerful electrophile catalyst of amino acid reactions in biological chemistry. It promotes a broad variety of reactions by forming Schiff base adducts between its carbonyl group and the α-amino group of amino acids, including racemizations, transaminations, α-eliminations, β- and γ-replacements, aldolic cleavages, and decarboxylations. When acting as a cofactor, PLP is covalently bound, in the active site, to the ε-amino group of a lysine residue forming a Schiff base (known as internal aldimine) that is displaced by the reactant amino acid to form the reactive adduct (external aldimine).[1−5] Once the external aldimine is formed, racemizations and transamination reactions proceed just by proton exchanges between the residues of the active site and the Cα/C4′ carbon atoms of the Schiff base adduct (Scheme 1). The first step in β- and γ-replacement reactions also consists in deprotonation of the α carbon (Cα) of the amino acid. Moreover, in all PLP-catalyzed reactions there is at least a secondary step in which either the Cα or the C4′ carbon atoms of the external aldimine are involved in a proton transfer event.[1−5]

What really reveals the importance of carbon acid chemistry in PLP-catalyzed reactions is that each PLP-dependent enzyme drives the evolution of the anionic intermediate by favoring the reprotonation of the proper carbon atom in every specific reaction. Therefore, the modulation of the carbon acidity is crucial for the reaction specificity.[1] In fact, the most popular inactivation strategies for PLP-dependent enzymes are mechanism-based inhibitors. Very often, such inhibitor species are amino acid-like structures that covalently bind PLP forming Schiff base compounds that undergo a series of deprotonation and reprotonation reactions of carbon atoms toward irreversible adducts.[2,6]

The catalytic power of PLP was originally attributed to the ability of the Schiff bases to delocalize the negative charge generated by the heterolytic cleavage of any bond of the Cα atom to the pyridine ring, forming the so-called quinonoid intermediate (Scheme 1B).[4,5] This scenario is general for those enzymes where the pyridine nitrogen of the PLP is protonated by the carboxylate group of an acid residue, as in aspartate aminotransferase (AAT) and dialkylglycine decarboxylase (DGD).[7−10] On the other hand, crystallographic studies revealed that, in the active site of alanine racemase (AlaR), the pyridine nitrogen remains unprotonated, accepting a

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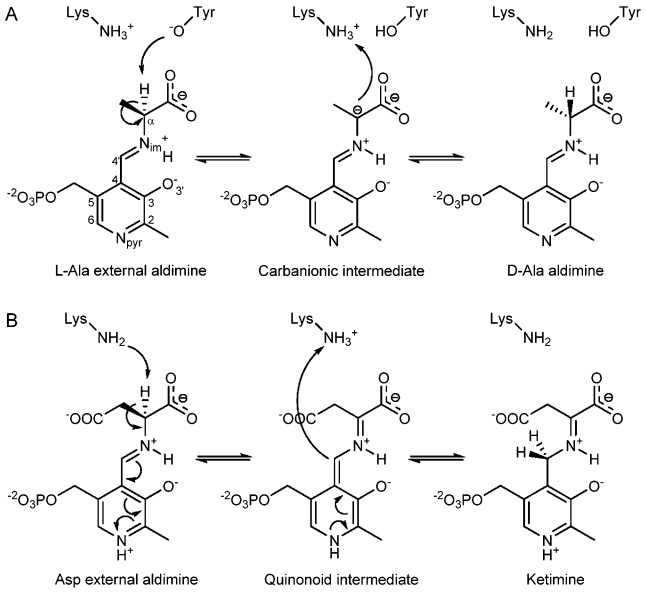
Scheme 1. Evolution of the Carbanionic Intermediate of PLP Schiff Bases According to a Racemization (A) and Transamination (B) Reactions



hydrogen bond from a cationic guaninidium group of an arginine residue, preventing the evolution of the Schiff bases to quinonoid-like structures (Scheme 1B) and challenging the traditional hypothesis for catalysis by PLP.[11]

A comprehensive set of experimental kinetic studies reported by Toney and co-workers[12,13] showed that alanine racemase catalyzes the proton abstraction from the Cα carbon as efficiently as any PLP-dependent aminotransferase, without delocalizing the negative charge in the form of a quinonoid intermediate. Therefore, they suggested that alanine racemase selectively destabilizes such intermediate species in order to achieve high racemization specificity in front of transamination side reactions.[1,13] On the basis of this previous hypothesis, Gao and co-workers[14,15] performed QM/MM molecular dynamics simulations on the PLP-catalyzed racemization of alanine both in aqueous solution and in the active site of alanine racemase. They ascribed the enhancement of the Cα carbon acidity to solvation effects in aqueous solution and to specific interactions in the active site rather than to stabilization by intrinsic delocalization of the negative charge when the pyridine nitrogen of the Schiff bases is unprotonated.

Computational studies performed in our group revealed that protonation of the pyridine nitrogen in PLP-alanine Schiff bases stabilizes the negative charge generated upon deprotonation of the Cα carbon by delocalization in the pyridine ring and favors the reprotonation of the carbanionic intermediates at C4′ in front of Cα.[16] This is further supported by NMR studies on the PLP-catalyzed deprotonation of glycine in aqueous solution that show an acidity enhancement from pK_a ~23 to ~17 for the Cα carbon by protonation of the pyridine nitrogen.[17−19] Recent kinetic experiments on aspartate aminotransferase (AAT), alanine racemase (AlaR), and *o*-acetylserine sulfhydrylase (OASS) reconstituted with 1-deazapyridoxal-5′-phosphate (deazaPLP), a PLP analogue lacking pyridine nitrogen, were reported.[20] The absence of pyridine nitrogen in deazaPLP causes a >10^9-fold decrease in reactivity for the external aldimine formed with L-aspartate compared to the external aldimine formed with PLP in the wild-type AAT, whereas mutation of the catalytic base for an alanine residue (K258A AAT) causes a ~10^8 decrease in reactivity.[8,20] This points out that the main catalytic power resides in the PLP cofactor, whereas the role of the enzyme is, more likely, to module the high catalytic versatility of the cofactor promoting the desired reaction specificity through specific interactions.

As mentioned previously, proton transfer reactions involving carbon atoms are ubiquitous in all enzymatic and nonenzymatic PLP-catalyzed reactions of amino acids. In the past years, Richard and co-workers[17−19] carried out a series of remarkable experiments with NMR techniques that allowed the determination of the pK_a values in aqueous solution of the Cα protons of glycine and its Schiff base adducts formed with acetone, 5′-deoxypyridoxal (DPL), and other pyridoxal structure-like compounds. However, the experimental pK_a determinations are limited to the most reactive protonation states in solution of the DPL-glycine Schiff bases so, in some cases, the carbon acidities of specific species had to be estimated by comparison with structure-related compounds.[17] In addition, no experiments have been carried out concerning carbon acidities of the Schiff bases formed between pyridoxamine-5′-phosphate (PMP) and carbonyl compounds (i.e., the pK_a values of C4′ carbon in ketimine Schiff bases).

Crugeiras et al.[21] reported a value of 18.7 as the upper bound pK_a for the C4′ of 4-(aminomethyl)pyridine species (4-PAM), a model compound of pyridoxamine. In this context, we have used the methodology described by Crugeiras et al.[21] to study the phosphate-catalyzed deprotonation of 4-PAM and of its Schiff base (SB) with propanal (PNL). These experiments have allowed us to determine the influence of Schiff base formation on the acidity of C4′ hydrogen atoms. Our experimental results are in agreement with those obtained by theoretical calculations. Further theoretical pK_a calculations were also performed for both the Cα carbon in PLP-Gly Schiff bases and the C4′ carbon of the Schiff bases formed between pyruvate and PMP.

The most biologically relevant protonation states for the pyridine nitrogen, phenolic oxygen and imine nitrogen atoms were considered for all the Schiff bases computationally studied. The results are in very good agreement with the available experimental data and provide an explanation of the mechanism by which PLP-dependent enzymes achieve a high reaction specificity, in particular for the reprotonation of the ubiquitous carbanionic intermediates either at Cα or at C4′ atoms.

EXPERIMENTAL SECTION

Materials. 4-(Aminomethyl)pyridine (4-PAM), D_2O (99.9%), potassium deuteroxide (40 wt %, 98% D), and 3-(trimethylsilyl)pronane-1-sulfonic acid (DSS) were all purchased from Sigma-Aldrich. Propanal (99%) (PNL) and potassium dihydrogen phosphate were obtained from Acros Organics.

Buffer Preparation. Previously to buffer preparation, dihydrogen phosphate was lyophilized twice from a D_2O solution to exchange labile protons for deuterium and avoid exchange diminution in the proportion of D in the resulting buffer solution. Stock phosphate buffer solutions were prepared at 0.2 M in D_2O and I = 1 M (KCl) at pD 6.5, 7.0, and 7.5. pD values were measured with a Crison GLP21$^+$ pH-meter equipped with a Crison 5290 glass electrode. pD values were obtained by adding 0.4 to the meter readings.[22] Stock solutions at each pD were used to prepare diluted buffer solutions at

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0.02, 0.05, and 0.1 M buffer concentrations at each desired pD. The ionic strength was kept constant at 1 M (KCl).

^{1}H NMR Spectroscopy. Solutions of 4-PAM (5 mM) and the reaction mixtures of 4-PAM (5 mM) with PNL (0.4M), which yields the corresponding Schiff base,[23] were prepared at each depicted buffer condition at pH 6.5, 7.0, and 7.5 containing 2 mM DSS. ^{1}H NMR spectra of different mixtures were recorded at different times by using a Bruker AMX NMR spectrometer operating at 300 MHz. Spectra were recorded by using an acquisition time of 4 s and zero-filling data to 64K. Relaxation delay between pulses was 10 times higher than the longest T1 for 4-PAM methyl protons, which was determined by using a 10 mM 4-PAM solution at pH 7.0 (0.2 M phosphate buffer, I = 1 M (KCl). Chemical shifts are reported at 25 °C relative to DSS, which was used as internal standard also for integration purposes.

H/D Exchange Kinetic Measurements. 4-PAM solutions and reaction mixture solutions of 4-PAM with FA were placed in 5 mm diameter NMR tubes and stored at 25 °C in a water bath during the isotope exchange experiments. At timed intervals different ^{1}H NMR spectra of the mixtures were collected at 25 °C. NMR tubes containing reaction mixtures of 4-PAM with PNL were incubated at 25 °C inside the NMR spectrometer at their spectra were collected at different times. The pD of the mixtures was monitored before and after H/D exchange reactions and was found to change ±0.04 pD units.

The H/D exchange of the first C4′ proton of 4-PAM was followed by monitoring the disappearance of its NMR signal at 4.29 ppm. Temporal area variation of the singlet signal corresponding to C4′ proton of 4-PAM (4.27 ppm) was used to study H/D exchange rate of the Schiff base (SB) formed in the reaction between 4-PAM and PNL. Reactions were monitored during the 20−70% of the first C4′ proton exchange.

Progress of H/D exchange was calculated by using the method described by Richard and co-workers[21,24] with minor modifications. Briefly, integrated areas of C4′ methylene group (A_{CH_2}) signal were used to derive the observed first-order rate constants (k_{obs}) for the H/D exchange by using the semilogarithmic plot as shown in eq 1.

$$\ln A_{CH_2} = -k_{obs}t \tag{1}$$

Total exchange reaction occurs twice as fast as the exchange of a single proton of the C4′ CH_2. Therefore

$$k_{ex} = 2k_{obs} \tag{2}$$

where k_{ex} is the first-order rate constant for the exchange of the first C4′ proton. Such k_{ex} values calculated for the Schiff base were corrected by f_{SB} (eq 3), which refers to the fraction of SB formed under the corresponding experimental conditions.

$$k_{ex}^{SB} = \frac{k_{ex}}{f_{SB}} \tag{3}$$

The f_{SB} values were calculated by using the area of the C4′ protons signal for 4-PAM (4.27 ppm) and the area of the imine proton of the resulting SB (9.28 ppm).[23] In all cases, fraction values were calculated 2 min after the reaction was initiated. The corrected first-order rate constants (k_{ex}^{SB}) values were plotted against the concentration of buffer according to the equation

$$k_{ex}^{SB} = (k_{ex}^{SB})_0 + k_{Buff}[\text{Buffer}] \tag{4}$$

where $(k_{ex}^{SB})_0$ in eq 4 stands for the observed first-order rate constant for solvent-catalyzed H/D exchange and k_{Buff} is the observed second-order rate constant for buffer-catalyzed exchange. The fractions of phosphate buffer present in the reactive dianionic form (f_B) were used to correct the k_{Buff} values in order to obtain the apparent second-order rate constants (eq 5).

$$(k_B)_{obs} = \frac{k_{Buff}}{f_B} \tag{5}$$

We assume that the true second-order rate constants, k_B, for the deuterium exchange reactions were calculated in all the cases for the pyridinium species (4-PAM-H_2 and SB-H_2) as it was reported that protonation of pyridine nitrogen results in a 5.3 × 10^4-fold increase in the deprotonation rate of the C4′ carbon.[21,25] We also assumed that Schiff base formation on 4-PAM does not significantly alter the pK_a value of the pyridine nitrogen atom, so we assumed in all the cases a value of 4.93.[24] Therefore, the true second-order rate constants k_B were calculated from the $(k_B)_{obs}$ values according to eq 6.

$$k_B = \frac{(k_B)_{obs}}{f_{N^+}} \tag{6}$$

The carbon acid pK_a values for ionization of the C4′ carbon in pyridine protonated forms of 4-PAM and SB were obtained from the rate constants for its deprotonation by a buffer base to give the free carbanion (k_B) and from the reverse protonation of such carbanion species by the buffer conjugate acid (k_{BH}) according to the equation

$$pK_a = pK_{BH} + \log\left(\frac{k_{BH}}{k_B}\right) \tag{7}$$

where pK_{BH} is the pK_a of the buffer conjugate acid (6.5) in H_2O at I = 1 M.[26] For the proton rate transfer between the buffer conjugate acid $H_2PO_4^-$ and the carbanion (k_{BH}) we initially considered the upper limit corresponding to the diffusion-limited protonation of the carbanion by the buffer conjugate acid species ($k_{BH} \sim 10^9$ M^{-1} s^{-1}) according to the methodology reported by Crugeiras et al.[21] The value $k_{BH} \sim 10^5$ M^{-1} s^{-1} reported by Eigen[27] for reprotonation of carbanions considering a proton transfer-limited reaction was also used to estimate the absolute pK_a values of 4-PAM and SB.

■ COMPUTATIONAL DETAILS

Standard density functional theory (DFT) calculations were carried out with Gaussian09 package.[28] All the structures were fully optimized by using the B3LYP functional[29,30] and the more recent M06-2X functional, developed by Zhao and Truhlar[31] for accurate thermochemical calculations, in combination with the 6-311++G(d,p) basis set. Vibrational analysis was performed to verify all the optimized structures as energy minima by the absence of imaginary frequencies and to calculate the contributions from the vibrational motion of the nuclei to the free energy at 298.15 K without scaling factor correction. The SMD[32] water model was used in all calculations. NBO analysis was used to evaluate delocalization effects on the aldimine, ketimine, and carbanionic intermediate species with module NBO 3.1 in the G09 program.[33]

Carbon acidities were calculated by means of an isodesmic reaction (Scheme 2) in which the pK_a of the acid (AH) is obtained by considering the theoretically calculated free energy

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Scheme 2. Proton Exchange Reaction between an Acid Species (AH) and a Reference Acid Molecule (RefH)[a]

$$AH^{q}_{(aq)} + Ref^{q'-1}_{(aq)} \xrightarrow{\Delta G_{aq}} A^{q-1}_{(aq)} + RefH^{q'}_{(aq)}$$

[a]The global charges of the acids and the conjugate bases are represented by q/q' and $(q-1)/(q'-1)$, respectively.

of proton exchange with respect to a reference acid molecule (RefH) (that accounts for the relative acidity, i.e., $\Delta pK_a RT \ln 10$, between such species) and the experimental pK_a value of the reference species as shown in eqs 8 and 9.

$$\Delta G_{aq} = G_{aq}(RefH) + G_{aq}(A) - G_{aq}(Ref) - G_{aq}(AH) \quad (8)$$

$$pK_a(AH) = \frac{\Delta G_{aq}}{RT \ln 10} + pK_a(RefH) \quad (9)$$

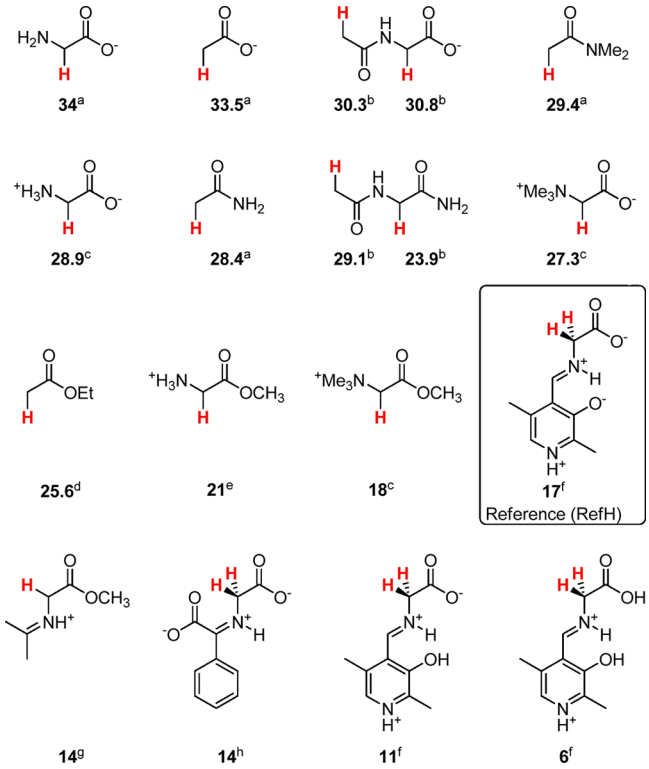
This procedure was proven to predict pK_a values for substituted pyridines and amino acids with mean absolute deviations (MAD) respect to experiment of 0.5 and 0.2 pK_a units, respectively.[34] The achieved accuracy for pK_a predictions of carbon acids resulted in agreement with the uncertainty of the most recent pK_a values provided by NMR experiments (i.e., 0.5−1 pK_a units)[17−19,35] and also with the precision of previous theoretical works that reported successful predictions of carbon pK_a values of ketones, esters, and small peptides.[36−38] It should be noted that an accurate prediction of pK_a values by means of relative acidity calculations is strongly dependent on the structural similarity of the acid AH and the reference molecule RefH.[36] However, the isodesmic reaction approach (Scheme 2) benefits from a better cancellation of errors between the energies used in eq 8, making possible accurate pK_a calculations by using more flexible criteria in the choice of the reference species.[34] This is crucial for the present study since previous experimental studies revealed large pK_a variations in the carbon acidities of glycine in aqueous solution ($pK_a \sim 29$) and several Schiff base adducts formed by glycine and DPL (pK_a values from ~23 to ~6) depending on the protonation state of the Schiff base.[17−19]

Consequently, a Schiff base formed between glycine and 5′-deoxypyridoxal (DPL) of intermediate protonation state and experimentally known pK_a value[18] was used as an acid reference (RefH) in this study (Scheme 3). Moreover, in order to ensure the accuracy of the calculated values in all the pK_a range regardless of the global charges of the acid (AH) and reference molecule (RefH), a set of 17 carbon acids encompassing peptide derivatives and glycine Schiff bases (Scheme 3) was used to fit the calculated free energies of proton exchange (ΔG_{aq}) to their corresponding experimental pK_a values[17−19,24,35,39−43] according to a linear regression based on eq 9.

RESULTS

H/D Exchange NMR Experiments Provide pK_a Values of C4′ in Ketimine Schiff Bases. ^{1}H NMR spectroscopy has been used to determine the pK_a value of the C4′ carbon of 4-(aminomethyl)pyridine (4-PAM) and its Schiff base (SB) formed with propanal (PNL). H/D exchange experiments were carried out in phosphate buffered D_2O (pD 6.5−7.5) at 25 °C and I = 1 M (KCl) to study the exchange rate of the first α-pyridyl proton of those compounds. Figure 1 shows the DSS referenced overlapping of the time variation C4′-H_2 signal of a

Scheme 3. Set of Carbon Acids and Their Experimental pK_a Values Used for the Linear-Regression Fit with the Calculated Proton Exchange Energies with Respect to the Acid Reference Species (RefH)[a]



[a]The experimental pK_a values were taken from refs 34 (a), 35 (b), 33 (c), 28 (d), 32 (e), 18 (f), 36 (g), and 37 (h). Acidic protons are depicted in bold red.

solution of 4-PAM (Figure 1A) and of a mixture of 4-PAM and PNL (Figure 1B), in 0.05 M phosphate buffer, pD 6.5 and 25 °C. It is clear that C4′ protons of 4-PAM exchange slower than those of the Schiff base of 4-PAM and PNL (e.g., ~20% of exchange degree was accomplished after 16 days in the solution of 4-PAM, whereas the same percentage was reached after 50 min in the 4-PAM/PNL mixture).

The ^{1}H NMR spectra of an additional reaction mixture of 4-PAM (5 mM) and PNL (0.4M) in the presence of phosphate buffer (0.02M) at pD 7.5 were recorded after 5 min of preparation and after 4 days kept at 25 °C (see Supporting Information Figure S1). The appearance of a broad upfield-shifted signal, indicative of the H/D coupling after deuterium incorporation to C4′,[21,40] together with the absence of additional peaks, discarded the formation of products other than the Schiff base adduct.

The temporal variation of the area of the NMR signal of the C4′ proton of 4-PAM was analyzed by using the methodology put forward by Richard and co-workers,[21,24] as it is described in the Experimental Section. Table 1 displays the first-order rate constant for the exchange of the first C4′ proton of SB-H_2 (k_{ex}^{SB}) calculated from eq 3. Figure 2 shows the linear relationship between k_{ex}^{SB} and the total concentration of phosphate buffer at several pD values.

The k_{ex}^{SB} value decreases upon pD increase, suggesting that H/D exchange occurs mainly on pyridinium species, as it was already pointed out by other authors.[21,25] Table 1 also reports the second-order rate constants (k_B) for the buffer-catalyzed H/

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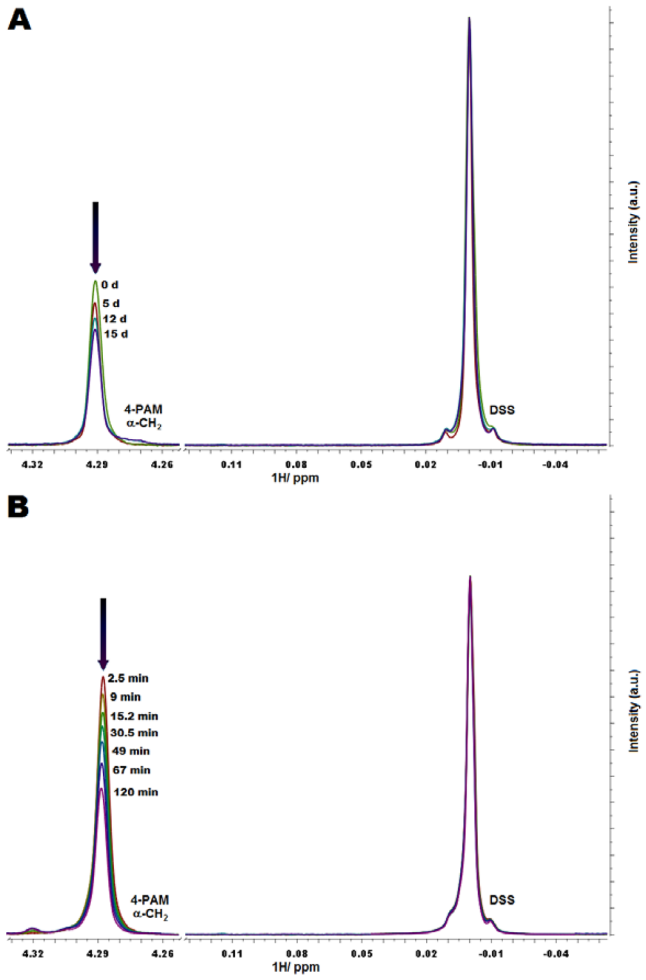

Figure 1. Stack plot of the ^{1}H NMR spectra during H/D exchange kinetics. (A) Temporal variation of α-CH_2 signal intensity of 4-PAM in phosphate buffered solution 0.05 M at pH 6.5 and 25 °C. (B) Temporal variation of α-CH_2 signal intensity of 4-PAM (5 mM) incubated in presence of PNL (0.4M) in phosphate buffered solution 0.05 M at pH 6.5 and 25 °C. Scaled signal intensity corresponding to DSS methyl protons is used in (A) and (B) as reference.

D exchange reaction of the dicationic SB-H_2 calculated from the values of $(k_B)_{obs}$ and corrected by f_B and f_{N^+}. Finally, k_B values were used to calculate the pK_a values of C4′ in SB-H_2 and 4-PAM-H_2 (eq 7).

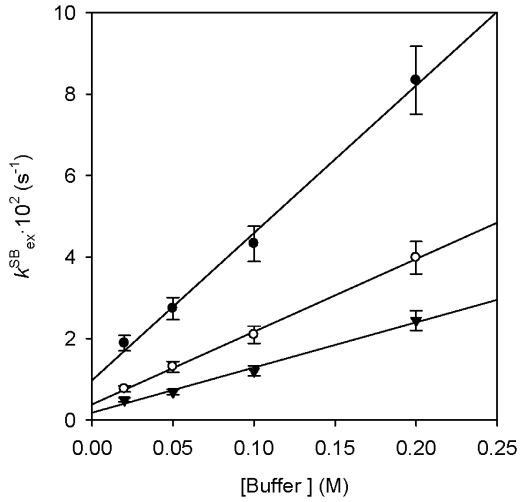

Figure 2. Variation of the rate constant for H/D exchange (k_{ex}^{SB}) of Schiff bases (SB-H_2) formed by 4-(aminomethyl)pyridine and propanal as a function of the total concentration of phosphate buffer at pD 6.5 (●), pD 7.0 (○), and pD 7.5 (▼) at 25 °C, I = 1 M KCl. The error bars indicate an uncertainty of 10% resulting as an estimation from the errors during the integration of the NMR peaks.

The upper bound pK_a values were calculated considering the carbanion reprotonation by the buffer conjugate acid as a diffusion-limited step (k_{BH} ~1 × 10^9 M^{-1} s^{-1}).[21,27] The absolute pK_a values (i.e., pK_a = 14.8 ± 0.1 for 4-PAM-H_2 and pK_a = 9.8 ± 0.1 for SB-H_2) were obtained by considering k_{BH} ~1 × 10^5 M^{-1} s^{-1}, which corresponds to a proton-transfer-limited process for reprotonation of the carbanionic species.[27] The results show the relevant quantitative increase in the C4′ acidity by Schiff base formation in 4-(aminomethyl)pyridines. In this case, ketimine formation reduces the pK_a value of C4′ in 5 units respect to the former amine compound.

Computational pK_a Calculations of Carbon Acids. As shown in Figure 3, there is a good correlation between the DFT-calculated free energies of proton exchange reactions (ΔG_{aq}) and the experimental pK_a values for the carbon acids depicted in Scheme 3 in a range of 30 pK_a units. The fitting procedure provides the regression equations for B3LYP and M06-2X functionals (eqs 10 and 11, respectively) required to

Table 1. H/D Exchange Rate Constants of the First α-Methylene Proton of the SB-H_2 in D_2O

[buffer] (M)	pD	k_{ex} × 10^4 (s^{-1})	f_{SB}	k_{ex}^{SB} × 10^2 (s^{-1})	$(k_B)_{obs}$ (M^{-1} s^{-1})	f_B	f_{N^+} × 10^3	k_B (M^{-1} s^{-1})	pK_a[a]
0.02	6.49	3.192	0.017	1.866	0.363	0.2	28.0	64.8	9.7
0.05	6.46	2.900	0.011	2.737					
0.10	6.48	2.666	0.006	4.327					
0.20	6.50	1.831	0.002	8.339					
0.02	6.97	1.634	0.021	0.763	0.178	0.5	8.0	44.5	9.9
0.05	6.96	1.370	0.011	1.304					
0.10	6.98	1.042	0.005	2.094					
0.20	7.01	0.795	0.002	3.983					
0.02	7.47	1.540	0.031	0.493	0.111	0.8	2.8	49.5	9.8
0.05	7.49	0.728	0.010	0.689					
0.10	7.50	0.404	0.003	1.204					
0.20	7.53	0.304	0.002	2.445					

[a]Absolute pK_a values by considering the reprotonation reaction limited by the proton transfer process between the buffer acid donor and the carbanion acceptor (k_{BH} ~ 10^5 M^{-1} s^{-1}).

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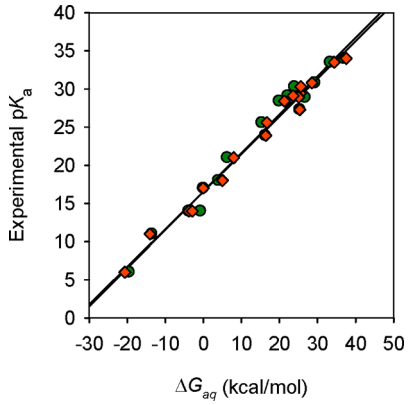


Figure 3. Experimental pK_a values vs the calculated free energies of proton exchange for the carbon acids depicted in Scheme 3. In orange diamonds and green circles are respectively B3LYP and M06-2X calculated free energies.

predict the carbon acidities of the PLP Schiff bases. The coefficients of determination (R^2) obtained for the free energies calculated with B3LYP and M06-2X functionals (0.988 and 0.977, respectively) are consistent with a previous study performed by Klamt et al.[44] in which a coefficient of determination of 0.984 is reported for a set of 64 organic and inorganic acid species of broad chemical diversity.

$$\mathrm{p}K_a(\mathrm{AH}) = 0.67(\pm 0.02)\frac{\Delta G_{aq(\mathrm{B3LYP})}}{RT\ \ln 10} + 16.56(\pm 0.30) \quad (10)$$

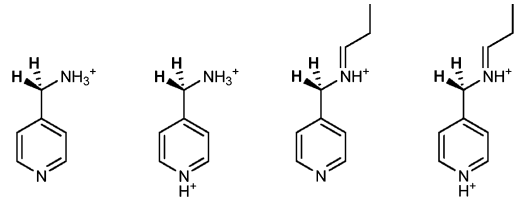
$$\mathrm{p}K_a(\mathrm{AH}) = 0.69(\pm 0.03)\frac{\Delta G_{aq(\mathrm{M06\text{-}2X})}}{RT\ \ln 10} + 16.58(\pm 0.40) \quad (11)$$

In both cases (B3LYP and M06-2X), the *y*-interceptions provided by the regression are very close to the expected value that corresponds to the experimental pK_a value of the acid reference, RefH, despite the interception at this point has not been forced. On the other hand, the slopes are somewhat lower (ca. 30%) than the expected $1/RT$ ln 10 value (eqs 10 and 11). Klamt et al.[44] also noticed this behavior to be frequent when correlating theoretical free energies of dissociation and experimental pK_a values. Concerning the present study, it is of interest to note that the correction of the slope on the calculated free energies becomes important for the species that exhibit a very different proton affinity respect to the reference species (i.e., large ΔG_{aq} values) but becomes negligible when considering species with similar proton affinities (i.e., ΔG_{aq} values close to 0). Therefore, the acid reference RefH in the present study is chosen to be almost centered in the expected range of pK_a values for the PLP Schiff bases in the different protonation states. It is also worthy to note that the mean absolute deviations (MAD) of the pK_a values predicted by the regression equations are 0.8 and 1.1 pK_a units, thus comparable with the experimental uncertainties for carbon acid species.[17−19,24,35,39−43]

Theoretical and experimental pK_a values are shown in Scheme 4 for 4-PAM-H_2 and SB-H_2 species. A very good agreement is observed between those experimental and theoretical values, independently of the functional (B3LYP or M06-2X) used in the calculations.

On the other hand, the theoretical pK_a values for the Schiff bases of PLP and glycine in the zwitterionic form (i.e., deprotonated phenolic O3′ oxygen and protonated imine (N_{im}) nitrogen atoms, labeled as **Cα-2** and **Cα-2H** in Scheme 5), are the only values from our study that can be directly compared with previous experimental results of aldimine Schiff bases.[17,18] A pK_a value of ∼17 is calculated for the Schiff base protonated at the pyridine nitrogen (**Cα-2H**). The result was expected because this species is the 5′-phosphorylated counterpart of the acid reference (RefH in Scheme 3) reported by Toth and Richard.[18] In addition, the pK_a value of the nonprotonated zwitterionic Schiff base (**Cα-2**) is predicted to be 22.6, which is in excellent agreement with the estimations of Richards and coworkers[17] based on experimental data (pK_a ∼ 23), which supports the consistency of our pK_a calculations since neither **Cα-2** species nor its experimental pK_a was included in the training set of carbon acids (Scheme 3) when performing the linear regressions.

Scheme 4. Experimental pK_a (p$K_{a(NMR)}$) and Theoretical pK_a Values of 4-(Aminomethyl)pyridine (4-PAM) and Schiff Base (SB) Formed by 4-PAM and Propanal[a]



	4-PAM-H	**4-PAM-H_2**	**SB-H**	**SB-H_2**
p$K_{a(NMR)}$		14.8		9.8
p$K_{a(B3LYP)}$	25.1	15.1	18.4	10.5
p$K_{a(M062X)}$	24.2	13.9	18.4	10.2

[a]Acidic protons at C4′ position are depicted in bold.

DISCUSSION

Kinetic and Thermodynamic Acidities of Ketimine Schiff Bases. The kinetic constant of deprotonation of SB-H_2 species in phosphate buffer, $k_B = 5.3 \times 10^1$ M^{-1} s^{-1} (average of the values depicted in Table 1), is 5 orders of magnitude higher than that of 4-PAM-H_2 species ($k_B = 4.9 \times 10^{-4}$ M^{-1} s^{-1}, average of the values shown in Table S1 of Supporting Information), which corresponds to a reduction of ∼6.8 kcal/mol in the free energy barrier of proton abstraction and assess the effect of charge delocalization during the ionization process. This effect is ∼1.3 kcal/mol lower than that reported for PLP-Gly aldimines,[17] which reflects the importance of the 3′-OH group both in the aldimines of PLP and ketimines of PMP and in the stabilization of structures with the protonated imine nitrogen by electrostatic interactions and formation of intramolecular hydrogen bonds N−H···O.[19] Additionally, the hydrogen bond formation restrains the rotation of the methylene C4′ group and favors the delocalization, through the π system, of the generated negative charge in the transition state.

The increase in reactivity for proton abstraction due to Schiff base formation with 4-PAM is lower than that reported for Schiff base formation with PLP.[17] However, the SB-H_2 species shows larger second-order kinetic constants for deprotonation by hydrogen phosphate (k_B) than PLP aldimines under the same pH conditions. The reported k_B value for the PLP

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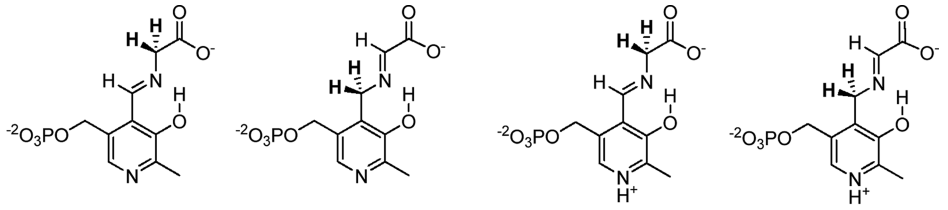
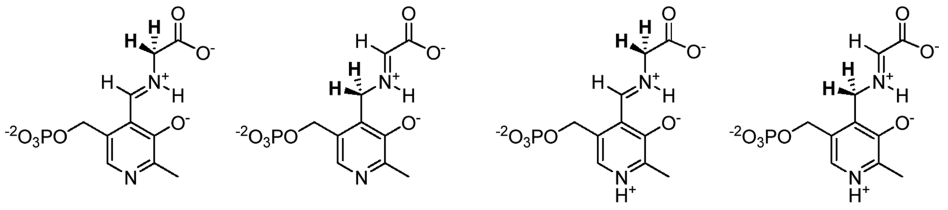
Scheme 5. Schiff Bases of Pyridoxal-5′-phosphate (PLP) and Glycine[a]



	Cα-1	C4′-1	Cα-1H	C4′-1H
pK_a(B3LYP)	**21.1**	**18.1**	**14.3**	**12.8**
pK_a(M06-2X)	**21.6**	**19.2**	**14.5**	**13.0**



	Cα-2	C4′-2	Cα-2H	C4′-2H
pK_a(B3LYP)	**22.6**	**15.1**	**17.0**	**12.1**
pK_a(M06-2X)	**22.6**	**16.5**	**17.2**	**13.8**

[a]Cα and C4′ stand for the aldimine and ketimine Schiff bases, respectively, whereas labels "1" and "2" stand for the tautomers protonated at the phenolic oxygen (O3′) or at the imine nitrogen atoms (N_{im}), respectively. The "H" label designates the structures protonated at the pyridine nitrogen (N_{pyr}). Acidic protons of aldimines and ketimines at Cα and C4′ positions, respectively, are depicted in bold.

aldimines protonated in the imine and pyridine groups is 1.7×10^{-3} M^{-1} s^{-1} while for the PLP aldimines protonated in the imine, phenol, and pyridine groups, $k_B = 4.3 \times 10^{-1}$ M^{-1} s^{-1}.[17] Therefore, SB-H_2 is respectively ~10^4-fold and ~10^2-fold more reactive.

The pK_a values of C4′ carbons of 4-PAM-H_2 and SB-H_2 have been calculated by using eq 7, from the NMR kinetic experiments. Crugeiras et al.[21] used the value 10^9 M^{-1} s^{-1} for the reprotonation constant of the carbanion of 4-PAM-H_2 by $H_2PO_4^-$ (k_{BH}), assuming that such reaction is diffusion-limited, and obtained a value of 18.7 for the upper bound pK_a of 4-PAM-H_2.[21] In this work, the obtained pK_a limits of 4-PAM-H_2 and SB-H_2 are 18.8 and 13.8, respectively.

Nevertheless, it should be noted that the encounter of the reactant acid and base does not involve the instantaneous formation of a reactive complex for those chemical species that do not establish well-defined hydrogen bonds or undergo large structural modifications during proton transfer. Therefore, in such cases the proton transfer is not diffusion-controlled but exhibits an energy barrier associated with bond rearrangement and solvent reorganization.[27] In fact, Eigen[27] demonstrated that the reprotonation kinetic constants (k_{BH}) of the carbanion of acetylacetone by a set of buffer species do not reach the diffusion limit over a ΔpK_a range of 20 units. Indeed, the higher k_{BH} values cluster around 10^5 M^{-1} s^{-1}, and only reprotonation by water (solvent) exhibits a larger value (i.e., $k_{BH} \sim 10^7$ M^{-1} s^{-1}).

DFT calculations performed within the standard transition state theory framework (data not shown) provide the values ~3×10^5 and ~5×10^4 M^{-1} s^{-1}, respectively, for the reprotonation kinetic constants (k_{BH}) of 4-PAM-H_2 and SB-H_2 by $H_2PO_4^-$. The experimental pK_a values of 4-PAM-H_2 and SB-H_2 are provided in Scheme 4, assuming the value $k_{BH} = 10^5$ M^{-1} s^{-1}. In Scheme 4 are also shown the pK_a values obtained from thermodynamic DFT calculations. The good agreement between all values supports the assumption that the reprotonation of the carbanionic species of 4-PAM-H_2 and SB-H_2 is not diffusion-controlled.

Regardless of the chosen value for k_{BH} constant, both the experimental and theoretical results predict a pK_a reduction of 5 units as a consequence of the formation of SB-H_2 species. However, previous studies show that Schiff base formation between glycine and DPL originates a larger decrease in the pK_a of Cα (ca. 12 units),[17,18] which suggests that the increase in reactivity by formation of PLP-aldimines is larger than by formation of PMP-ketimines. Besides, when the pyridine ring is unprotonated, the pK_a reduction caused by formation of SB-H is 6.7−5.8 units (Scheme 4), similar to that observed after Schiff base formation between glycine and acetone (~7 pK_a units).[17] These results point out that protonation of the pyridine nitrogen and Schiff base formation (1) are two different mechanisms of carbanion stabilization, (2) do not reduce the pK_a of C4′ cooperatively, and (3) affect the pK_a of C4′ and Cα to a different extent.

pK_a of PLP and PMP Schiff Bases. The calculated pK_a values for the Cα and C4′ carbons of PLP and PMP Schiff bases depicted in Scheme 5 point out that the acidity of Cα ranges from pK_a ~ 14 to pK_a ~ 23, while the acidity of C4′ ranges from pK_a ~ 12 to pK_a ~ 19. It is also observed that the pK_a values of Cα and C4′ show significant differences for some Schiff bases with equivalent protonation states. Additionally, the same changes in the protonation state of the heteroatoms have different effect on the acidity of Cα and C4′.

Overall, the Cα carbons are less acidic than C4′ ones (Scheme 5). Additional calculations (not shown) performed over molecular structures analogues to the species depicted in Scheme 5, with the same π structure but without heteroatoms, clearly point out that the lower acidity of Cα carbons is due to the higher stability of aldimine species caused by the more delocalized π system.

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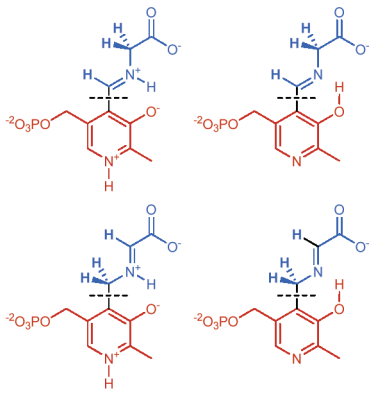
The pK_a values shown in Scheme 5 also point out that for those species where the phenol and imine groups are not ionized (i.e., species **C*x*-1**), protonation of pyridine nitrogen has a larger effect over the pK_a of Cα than over the pK_a of C4′. Specifically, for Cα carbons, the pK_a is reduced by 6.8−7.1 units (by comparing **Cα-1** and **Cα-1H**, according to B3LYP and M06-2X calculations, respectively), whereas the pK_a of C4′ carbons is reduced by 5.3−6.2 units, as observed when comparing **C4′-1** and **C4′-1H**.

The protonation of pyridine nitrogen in the species with ionized phenol and imine groups (i.e., species **Cx-2**) has lower effects on the pK_a of Cα and C4′ than that observed for **Cx-1** species. As observed in Scheme 5, the pK_a difference between **Cα-2** and **Cα-2H** is 5.6−5.4 units and the pK_a difference between **C4′-2** and **C4′-2H** is just of 3.0−2.7 units. This is due to a destabilization of the carbanionic intermediates of **Cx-2** species as a result of the π-back-donation of the phenoxide anion (Table 2).

Table 2. Charge Transferred from the Imine Moiety to the Pyridine Moiety[a]

Species	Delocalised charge
Cα-1	0.06
Cα-1H	0.12
Cα-2	-0.26
Cα-2H	-0.19
C4'-1	0.09
C4'-1H	0.13
C4'-2	-0.06
C4'-2H	-0.03
anion-1	0.36
anion-1H	0.65
anion-2	0.03
anion-2H	0.24



[a]First, the NBO atomic partial charges of the all atoms were summed up into two groups, namely imine moiety (blue atoms) and pyridine moiety (red atoms). The charge transferred from the imine moiety to the pyridine moiety was calculated as the difference between the summation of partial charges of the imine moiety and the global charge of a hypothetical state with no delocalization. Positive/negative values indicate charge delocalization toward/from the pyridine ring, respectively. The labels anion-x stand for the carbanionic intermediates of the "x" species. Further information about the methodology used in this analysis is available in ref 16.

The proton transfer from the phenol to imine nitrogen decreases the acidity of Cα carbon by 1.5−1.0 pK_a units as seen when comparing **Cα-1** and **Cα-2** (Scheme 5). Oppositely, the acidity of C4′ increases by 3.0−2.7 pK_a units (**C4′-1** vs **C4′-2**). This difference is indicative of the negative charge delocalization from the phenoxide anion to the cationic imine nitrogen, via π-system in **Cα-2** and **Cα-2H** species (PLP Schiff bases) which is not possible in **C4′-2** and **C4′-2H** species due to the presence of C4′-H_2 methylene group (Table 2). In the latter case, the acidity increase of C4′ is attributed to the destabilization of the C4′-hydrogens by the presence of the positively charged iminium nitrogen.

The effects of the intramolecular hydrogen bond inversion are enhanced when the pyridine nitrogen is protonated, as it increases the π-backdonation of the phenoxide group. The overall effect is the increase of 2.7 units in the pK_a of Cα (**Cα-1H** vs **Cα-2H** in Scheme 5). However, such increase is not observed for the pK_a of C4′ when comparing **C4′-1H** and **C4′-2H**, since the destabilization of C4′-hydrogens by the iminium cation counteracts the destabilization of the corresponding carbanion by the π-backdonation of the phenoxide group.

In summary, the results of Scheme 5 point out that the main role of pyridine nitrogen is to modulate the magnitude of the pK_as of Cα and C4′ atoms, whereas the main role of phenol oxygen and imine nitrogen is to modulate the relative pK_a of Cα and C4′.

Implications on Enzyme Catalysis. The active sites of alanine racemase (AlaR)[45] and aspartate aminotransferase (AAT)[8] taken from the crystallographic structures (PDB codes 1L6F and 3QPG, respectively) are depicted in Figure 4. We have considered the X-ray structure of AAT crystallized with the external aldimine of deaza-PLP-L-aspartate because it has been demonstrated that the deaza-PLP occupies the same position as the PLP.[8] Additionally, the side chain of Lys258 is

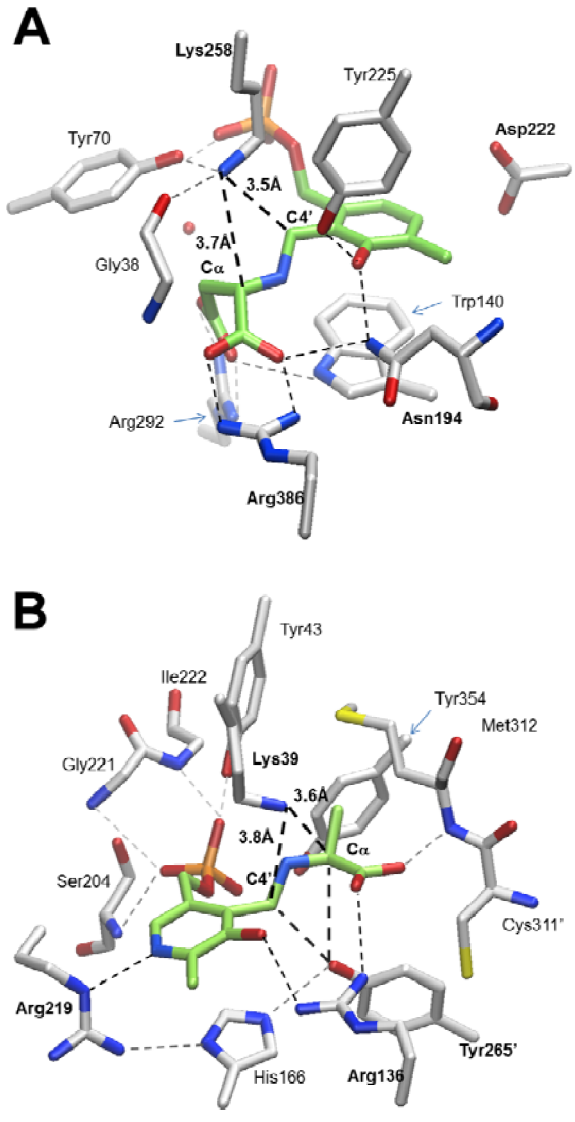


Figure 4. Active site interactions of the external aldimines of deaza-PLP in aspartate aminotransferase (A) and PLP in alanine racemase (B) from the X-ray crystal structures (PDB codes 3QPG and 1L6F, respectively).

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expected to be placed in the proper position for the catalysis, since the external aldimine is formed with the natural substrate.

In the active site of AAT, the ε-amino group of Lys258 is approximately equidistant from carbon Cα (3.7 Å) and carbon C4′ (3.5 Å) of the external aldimine,[8] which is expected as the ε-amino group is responsible for both abstracting the proton from Cα and reprotonating the intermediate at C4′. In the case of AlaR, the proton abstraction from Cα is carried out by the phenoxide group of Tyr265′ while the residue Lys39 (equivalent to Lys258 in AAT) reprotonates the Cα on the opposite side of the carbanionic intermediate. Therefore, it is worthy to note that the ε-amino group of Lys39 in AlaR is also equidistant from both carbon Cα (3.6 Å) and carbon C4′ (3.8 Å), as observed in the crystal structures.[45] This means that the reaction specificity should not be exclusively attributed to proximity or preorganization entropic effects, but also to the intrinsic reactivity of the external aldimine.

The most significant difference in the active sites of AAT and AlaR is the interaction of the pyridine nitrogen of the PLP cofactor with the carboxylate group of Asp222 in AAT or with the guanidinium group of Arg219 in AlaR. This particular feature of AlaR has been extensively discussed as the key factor of the reaction specificity since the protonation state of the pyridine nitrogen strongly influences the acidity of Cα carbon.[1−3,7,8,10−20] However, our results point out that the relative acidity of Cα and C4′ atoms depends on the protonation state of the imine nitrogen and phenol oxygen (Scheme 5).

Accordingly, it should be noted that there are also differences in the enzyme−PLP interactions at the phenol oxygen and imine moiety. In AlaR there is a second guanidinium group (Arg136) which displays very close interactions with the phenol oxygen O3′ and the carboxylate group of the external aldimine (Figure 4).[45] Oppositely, in AAT, the phenol group interacts with the neutral phenol and amide groups of Tyr225 and Asn194 (Figure 4).[8] Recent NMR experiments have demonstrated that the proton transfer from the phenol oxygen to the imine nitrogen is promoted by protonation of the pyridine nitrogen and, also, by the presence of hydrogen bond donors interacting with the O3′ phenol oxygen.[46−51] These results indicate that in AlaR, where the pyridine nitrogen is not protonated, cationic groups close to the phenol oxygen are required to stabilize the phenoxide anion and the proton transfer to the imine nitrogen (Figure 4).

The calculated pK_a difference between carbons Cα and C4′ ranges ∼1.5 (**C4′-1H** vs **Cα-1H**) and ∼2.4−3.0 (**C4′-1** vs **Cα-1**) for those Schiff bases with neutral phenol and imine groups (Scheme 5). However, inversion of the intramolecular hydrogen bond notably increases the pK_a difference, up to ∼3.4−5.0 for **C4′-2H** vs **Cα-2H** and ∼6.1−7.5 for **C4′-2** vs **Cα-2** (Scheme 5). Such results point out that AlaR favors reprotonation of the intermediate at Cα (i.e., racemization) in front of transamination, by stabilization of the ionized phenol oxygen and imine nitrogen, by the interaction with the guanidinium group of Arg136.

In fact, Kurokawa et al.[52] reported that AlaR only catalizes a single transamination event every 2×10^7 turnovers of normal racemization. This corresponds to a ∼10 kcal/mol more energetic transition state for transamination with regard to racemization or, alternatively, a pK_a difference of ∼7.3 units between **Cα-2** and **C4′-2** (assuming a Brønsted relationship with $\beta = 1$), in agreement with our results (Scheme 5).

Computational studies performed by Gao and co-workers[14,15] revealed that both guanidinium groups of Arg136 (interacting with O3′) and Arg219 (interacting with pyridine nitrogen) contribute equally to the stabilization of the transition state. However, the Arg136 residue stabilizes the carbanionic intermediate in a larger extent than residue Arg219. Quantum mechanics/molecular mechanics simulations performed by Rubinstein and Major[53] show that the Arg219Glu mutation makes the transamination product thermodynamically more stable than the racemization one. This mutation also reduces the barrier of reprotonation at C4′ and increases that of reprotonation at Cα, but with no inversion of the barriers, so that racemization remains as the more kinetically favorable. Moreover, the interaction between the guanidinium group of Arg136 and the Schiff base carboxylate is strengthened as the intermediate is formed, which avoids delocalization of the negative charge to the pyridine moiety.[53]

Arginine racemase (EC 5.1.1.9) is also interesting as an example of another PLP-enzyme that catalyzes transamination as a side reaction, once every 4×10^5 racemization turnovers.[52,54] This means that the activation energy for transamination is ∼7.6 kcal/mol higher than that of racemization or, in other words, that the carbon Cα is ∼5.6 pK_a units more basic than the C4′. Although the crystallographic structure of arginine racemase has not been resolved, the relative acidity calculated for **Cα-2H** and **C4′-2H** (Scheme 5) suggests that the external aldimine is protonated at the pyridine nitrogen. If so, it would be an evidence of the fact that the protonation state of the pyridine nitrogen is not crucial to obtain high reaction specificity for racemization, as long as the phenol and imine groups are ionized. However, this remains as an open question and further work is required to test this hypothesis.

The oxygen O3′ has been reported to catalyze PLP and PMP Schiff base formation by abstracting or donating protons from the incoming or to the leaving groups[55−59] to stabilize the formed Schiff base by charge donation via π-system and by a strong intramolecular hydrogen bond interaction with the protonated imine nitrogen[19] and to increase the pK_a of the imine group.[60] However, in aspartate aminotransferase, the enzyme interactions with the phenol group displayed in the X-ray structures[8] suggest that the ionization to phenoxide anion is less favored than in alanine racemase.[45] Nevertheless, a lower degree of ionization in O3′ may be beneficial for transamination; our results suggest that the optimum conditions for reprotonation at C4′ in front of Cα are those of phenol oxygen remaining protonated (**C4′-1H** and **Cα-1H** in Scheme 5). It is important to note that the evolution of the reaction depends of the protonation state of the carbanionic intermediate, which does not necessarily coincides with that of the external aldimine. In fact, for the same reason that the phenoxide anion hinders the delocalization of the negative charge onto the pyridine ring, formation of the carbanion increases the phenoxide basicity. This may cause the proton transfer from the imine nitrogen to neutralize the phenoxide anion and favor the reprotonation of the intermediate at C4′. Chen et al.[61] also proposed that the proton bound to the imine nitrogen transfers to the phenol oxygen during the formation of the carbanionic intermediate, after observing great similitude between the UV−vis spectra of the intermediates of PLP and 3′-*O*-methylpyridoxal-5′-phosphate. In addition, inspection of the AAT active site[8] reveals that the imine moiety is not completely coplanar to the pyridine ring, probably due to the interactions between the

two carboxylate groups of the substrate L-aspartate in the external aldimine and the NH groups of Arg292, Arg386, and Trp140 (Figure 4A). These interactions should also promote the reprotonation at C4′ by both destabilization of the planar intermediate and stabilization of the resulting twisted Schiff base due to the quaternization of the C4′ carbon.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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