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Published on Web 10/06/2009

Substituent Effects on the Thermodynamic Stability of Imines Formed from Glycine and Aromatic Aldehydes: Implications for the Catalytic Activity of Pyridoxal-5′-phosphate

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Abstract: Equilibrium constants for addition of glycine to substituted benzaldehydes to form the corresponding imines and pK_a's for ionization of the iminium ions were determined by ^{1}H NMR analysis in D_2O. The introduction of a phenoxide anion substituent into the aromatic ring of benzaldehyde leads to a substantial increase in the pK_a of the iminium ion from 6.3 to 10.2 for *p*-hydroxybenzaldehyde and to 12.1 for salicylaldehyde. An analysis of the differential effect of *ortho*- versus *para*-substitution shows that the iminium ion to salicylaldehyde is stabilized by an intramolecular hydrogen bond in aqueous solution, with an estimated energy ca. 3 kcal/mol larger than can be accounted for by a simple electrostatic interaction. A comparison of the *o*-O^- substituent effect on the acidity of the iminium ions of glycine to benzaldehyde and 4-pyridine-carboxaldehyde provides evidence for the existence of an internal hydrogen bond of similar strength in pyridoxal 5′-phosphate (PLP) iminium ions in water. The effects of other ring substituents on the stability of PLP iminium ions are discussed.

1. Introduction

Many of the enzymes involved in the metabolism of amino acids use the pyridoxal 5′-phosphate (PLP) cofactor.[1−3] It is well-known that covalent catalysis by PLP occurs through formation of an imine linkage between the amino acid and the catalyst. This aldimine intermediate is common to all PLP-dependent transformations of amino acids. The cleavage of one of the bonds to the α-carbon, with elimination of H^+, CO_2, or R^+, results in the formation of a carbanion that is strongly stabilized by delocalization of the electron pair into the π-system of PLP. In a small class of amino acid decarboxylases, a pyruvoyl prosthetic group serves as a cofactor.[4]

We are interested in determining the kinetic and thermodynamic barriers for deprotonation of the α-amino carbon of amino acids[5−7] and in analyzing the effect on those barriers of the covalent modifications used by enzymes to promote amino acid reactions. The simple ketone acetone is an efficient catalyst of deprotonation of the α-amino carbon of glycine methyl ester, and this is the result of the 7-unit lower pK_a for carbon deprotonation of the iminium ion adduct (pK_{CH} = 14) than for deprotonation of *N*-protonated glycine methyl ester (pK_{CH} = 21).[8] This increase in the acidity of the α-protons is a significant fraction of the acidifying effect of forming iminium ion adducts between amino acids and PLP. We have recently analyzed catalysis of deprotonation of glycine by phenylglyoxylate anion, a model for the pyruvoyl prosthetic group, and have estimated that the pK_a for deprotonation of the α-imino carbon of the corresponding iminium ion is 15 pK units lower than pK_{CH} = 29 for the parent carbon acid glycine.[9] This shows that adducts of amino acids to phenylglyoxylate and to the pyridoxal analogue 5′-deoxypyridoxal (DPL)[10] have similar α-amino carbon acidities. On the other hand, DPL is a much better catalyst of deprotonation of glycine than phenylglyoxylate due to the more favorable equilibrium constant for conversion of the amino acid and DPL to the reactive iminium ion. This provides support for the earlier suggestion that one of the reasons enzymes have chosen PLP rather than simpler carbonyl compounds as catalysts of amino acid reactions is the larger affinity of the former as compared to simple ketones and aldehydes for addition to the amino group to form an imine.[11]

There is much known about the stability and acid dissociation constants in aqueous solution of imines derived from amino acids and *o*-hydroxyaryl aldehydes including PLP.[12−19] Earlier data have been proposed to provide support for the existence

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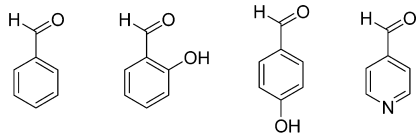
Chart 1



1 **2** **3** **4**

of an intramolecular hydrogen bond between the phenoxide anion and the protonated imino nitrogen in imines of amino acids to PLP.[16] However, recent studies of the acid−base equilibria of PLP aldimines in aqueous solution by ^{15}N NMR have suggested that such an intramolecular hydrogen bond is absent and that instead the phenolic and imino groups are hydrogen bonded to water molecules.[20] We report here a comparison of the equilibrium constants for formation of imines between glycine and the *ortho*- and *para*-hydroxy-substituted benzaldehydes **3** and **4** (Chart 1). This comparison provides evidence for the existence of a stabilizing intramolecular hydrogen bond between the phenolate oxygen and the iminium nitrogen, and an estimate of the strength of this $O^-\cdots H-N^+$ hydrogen bond. Evidence is presented that the glycine iminium ion to PLP is stabilized to a similar extent by this intramolecular $O^-\cdots H-N^+$ hydrogen bond.

2. Experimental Section

2.1. Materials. Benzaldehyde (**1**), 4-hydroxybenzaldehyde (**3**), 4-pyridine- carboxaldehyde (**4**), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 2,2,2-trifluoroethanol (TFE), deuterium chloride (37 wt %, 99.5% D), potassium deuterioxide (40 wt %, 98+% D), and deuterium oxide (99.9% D) were from Aldrich. Glycine and salicylaldehyde (**2**) were purchased from Fluka. All other organic and inorganic chemicals were reagent grade and were used without further purification.

2.2. General Methods. The acidic protons of glycine, K_2HPO_4, and KH_2PO_4 were exchanged for deuterium, as described previously, before preparing solutions of these compounds in D_2O.[21] The aldehydes **2** and **3**, chloroacetic acid, HFIP, and TFE were dissolved directly in D_2O (99.9% D), which resulted in <1 atom % increases in the protium content of this solvent.

Phosphate buffers were prepared by mixing stock solutions of K_2DPO_4 and KD_2PO_4 in D_2O at $I = 1.0$ (KCl) to give the desired acid/base ratio. Acetate and pyrophosphate buffers were prepared by dissolving the basic form of the buffer and KCl in D_2O followed by addition of DCl to give the desired acid/base ratio at $I = 1.0$ (KCl). Buffers of chloroacetate, HFIP, and TFE were prepared by dissolving their acidic forms and KCl in D_2O followed by addition of KOD to give the desired acid/base ratio at $I = 1.0$ (KCl).

Solution pD was determined at 25 °C using an Orion model 350 pH meter equipped with an Orion 7103 BN electrode. Values of pD were obtained by adding 0.4 to the observed reading of the pH meter.[22] An apparent pK_a of $pK_{BD} = 8.87$ for **2** in D_2O at 25 °C and $I = 1.0$ (KCl) was determined by potentiometric titration of a 10 mM solution of the substrate with KOD.[23]

2.3. ^{1}H NMR Analyses. ^{1}H NMR spectra at 500 MHz were recorded in D_2O at 25 °C on a Bruker AMX500 NMR spectrometer. The relaxation delay between pulses was at least 8-fold longer than the longest relaxation time of the protons of interest. Spectra were obtained with a sweep width of 6000 Hz, a 90° pulse angle, and an acquisition time of 6 s. Baselines were corrected for drift before the determination of the integrated peak areas. Chemical shifts are reported relative to $(CH_3)_4N^+$ at 2.94 ppm.

2.4. Determination of Equilibrium Constants. The position of the equilibrium for addition of glycine to the aldehydes **1**−**4** to form the corresponding imine in D_2O at 25 °C and $I = 1.0$ (KCl) was determined by ^{1}H NMR spectroscopy. The formation of an imine between glycine and each of these aromatic aldehydes was monitored in solutions that contained: 0.1−2.0 M amino acid and 5−20 mM **1**, 0.1−0.5 M amino acid and 5−10 mM **2**, 0.3−1.0 M amino acid and 50 mM **3**, or 0.001−2.0 M amino acid and 0.005−1.0 M **4**. The pD was maintained by use of 0.10 M of the following buffers: chloroacetic acid, pD 3.2−4.5; acetic acid, pD 4.2−6.0; phosphate, pD 6.0−8.0; pyrophosphate, pD 8.4−8.9; HFIP, pD 9.0−11; and TFE, pD 12.2−13.7. Glycine served as the buffer for some experiments at pD 9.5−11.4.

The ratio of equilibrium concentrations of imine adduct and parent aldehyde or of imine adduct and amino acid was determined 1−2 h after mixing the reactants. This time period was sufficient to reach equilibrium conditions, as shown by control experiments in which the ratio of concentrations was determined again 24 h after starting the reaction. Values of the observed equilibrium constant $(K_{add})_{obsd}$ (M^{-1}) for imine formation to **1**, **2**, or **3** were determined from the ratio of the integrated areas of the peaks for the methine proton of the imine product ($A_H^{X\text{-}IM}$) and of the aldehyde (A_H^X) using eq 1, where $[\mathbf{X\text{-}IM}]_e$, $[\mathbf{X}]_e$, and $[Gly]_e$ are the total equilibrium concentrations of imine, aldehyde, and glycine, respectively. The concentration of amino acid at equilibrium was calculated using eq 2, where $[Gly]_T$ and $[\mathbf{X}]_T$ are the total concentrations of amino acid and aldehyde, respectively.

$$(K_{add})_{obsd} = \frac{[\mathbf{X\text{-}IM}]_e}{[\mathbf{X}]_e[Gly]_e} = \frac{A_H^{X\text{-}IM}}{A_H^X[Gly]_e} \quad \mathbf{X} = \mathbf{1}, \mathbf{2}, \mathbf{3} \qquad (1)$$

$$[Gly]_e = [Gly]_T - [\mathbf{X\text{-}IM}]_e = [Gly]_T - [\mathbf{X}]_T\left(\frac{A_H^{X\text{-}IM}}{A_H^X + A_H^{X\text{-}IM}}\right) \quad \mathbf{X} = \mathbf{1}, \mathbf{2}, \mathbf{3} \qquad (2)$$

Values of the observed equilibrium constant $(K_{add})_{obsd}$ (M^{-1}) for imine formation to **4** were determined from the ratio of the integrated areas of the peaks for the methylene protons of the imine product ($A_{CH_2}^{4\text{-}IM}$) and of the reactant glycine ($A_{CH_2}^{Gly}$) according to eq 3, where $[\mathbf{4\text{-}IM}]_e$ and $[\mathbf{4}]_e$ are the equilibrium concentrations of imine and parent aldehyde, respectively. The concentration of aldehyde at equilibrium was calculated using eq 4, where $[Gly]_T$ and $[\mathbf{4}]_T$ are the total concentrations of amino acid and aldehyde,

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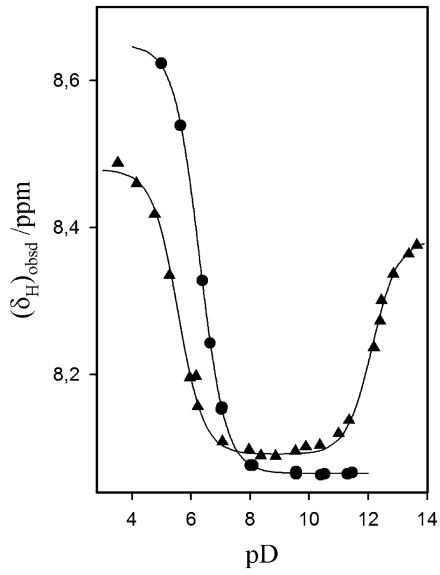
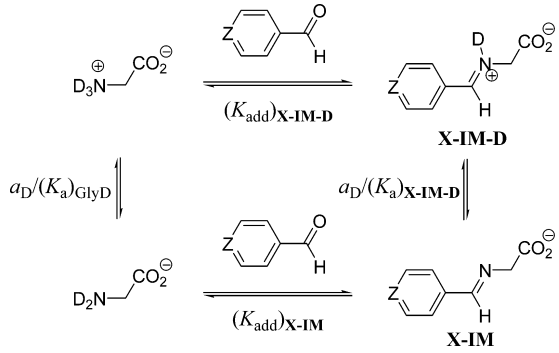


Figure 1. The dependence on pD of $(\delta_H)_{obsd}$ (ppm), the chemical shift of the methine hydrogen of the imines formed in D_2O that contains: (●) 0.1−2.0 M glycine and 5−20 mM **1**; and (▲) 0.1−0.5 M glycine and 5−10 mM **2** at 25 °C and $I = 1.0$ (KCl). The solid lines show the fit of these data to eq 5 derived from Scheme 1 (●), or to eq 6 derived from Scheme 2 (▲).

Scheme 1



respectively, and K_{hyd} is the apparent equilibrium constant for aldehyde hydration, which was determined by ^{1}H NMR under the conditions of each experiment.

$$(K_{add})_{obsd} = \frac{[\mathbf{4\text{-}IM}]_e}{[\mathbf{4}]_e[Gly]_e} = \frac{A^{\mathbf{4\text{-}IM}}_{CH_2}}{A^{Gly}_{CH_2}[\mathbf{4}]_e} \quad (3)$$

$$[\mathbf{4}]_e = \frac{1}{(1 + K_{hyd})}\left([\mathbf{4}]_T - \frac{A^{\mathbf{4\text{-}IM}}_{CH_2}}{A^{Gly}_{CH_2} + A^{\mathbf{4\text{-}IM}}_{CH_2}}[Gly]_T\right) \quad (4)$$

3. Results

Figure 1 (●) shows the change with changing pD in the chemical shift $(\delta_H)^{\mathbf{1\text{-}IM}}_{obsd}$ (ppm) of the signal for the methine proton of **1-IM** (the benzaldehyde-glycine imine, Scheme 1, Z = CH, X = **1**), which forms in D_2O that initially contains glycine (0.1−2.0 M) and **1** (5−20 mM) at 25 °C and $I = 1.0$ (KCl). The chemical shift of the methine proton of **1-IM** increases from 8.07 to 8.62 ppm on moving from high pD to pD 5.0, due to protonation of the aldimine nitrogen. The dependence of $(\delta_H)^{\mathbf{1\text{-}IM}}_{obsd}$ on pD is described by eq 5, derived for Scheme 1, where $\delta^{\mathbf{1\text{-}IM}}_H$ and $\delta^{\mathbf{1\text{-}IM\text{-}D}}_H$ are the chemical shifts of the methine hydrogen of **1-IM** and **1-IM-D**, respectively, and $(K_a)_{\mathbf{1\text{-}IM\text{-}D}}$ is the acidity constant of the iminium nitrogen. The solid line in Figure 1 shows the nonlinear least-squares fit of the data to eq 5, determined using $\delta^{\mathbf{1\text{-}IM}}_H = 8.07$ ppm observed at high pD, and treating $\delta^{\mathbf{1\text{-}IM\text{-}D}}_H$ and $(K_a)_{\mathbf{1\text{-}IM\text{-}D}}$ as variable parameters. The value of $p(K_a)_{\mathbf{1\text{-}IM\text{-}D}}$ obtained from this fit is reported in Table 1.

Figure 1 (▲) shows the change with changing pD in the chemical shift $(\delta_H)^{\mathbf{2\text{-}IM}}_{obsd}$ (ppm) of the signal for the methine proton of **2-IM** (the *o*-hydroxybenzaldehyde-glycine imine, Scheme 2, X = **2**), which forms in D_2O that initially contains glycine (0.1−0.5 M) and *o*-hydroxybenzaldehyde (5−10 mM) at 25 °C and $I = 1.0$ (KCl). This figure shows that the chemical shift changes with protonation of the aldimine nitrogen and the phenoxide oxygen. The solid line shows the fit of the experimental data to eq 6, derived for Scheme 2, using values of: (a) $\delta^{\mathbf{2\text{-}IM\text{-}D}}_H = 8.09$ ppm for the chemical shift for the methine proton of **2-IM-D** determined from data at intermediate pD; (b) $\delta^{\mathbf{2\text{-}IM\text{-}D_2}}_H = 8.48$ ppm, $\delta^{\mathbf{2\text{-}IM}}_H = 8.38$ ppm for the chemical shifts for the methine protons of **2-IM-D$_2$** and **2-IM**, respectively, determined from the nonlinear least-squares fit of the experimental data to eq 6; and (c) the acidity constants $(K_a)_{\mathbf{2\text{-}IM\text{-}D_2}}$ and $(K_a)_{\mathbf{2\text{-}IM\text{-}D}}$ for **2-IM-D$_2$** and **2-IM-D** reported in Table 1.

$$\delta^{\mathbf{X\text{-}IM}}_{obsd} = \frac{\delta^{\mathbf{X\text{-}IM}}(K_a)_{\mathbf{X\text{-}IM\text{-}D}} + \delta^{\mathbf{X\text{-}IM\text{-}D}}a_D}{((K_a)_{\mathbf{X\text{-}IM\text{-}D}} + a_D)} \; \mathbf{X = 1, 4} \quad (5)$$

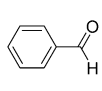
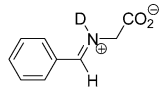
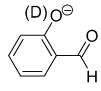
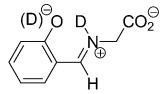
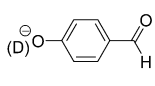
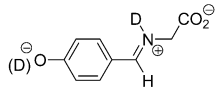
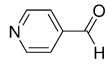
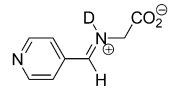
$$\delta^{\mathbf{X\text{-}IM}}_{obsd} = \frac{\delta^{\mathbf{X\text{-}IM}}(K_a)_{\mathbf{X\text{-}IM\text{-}D}}(K_a)_{\mathbf{X\text{-}IM\text{-}D_2}} + \delta^{\mathbf{X\text{-}IM\text{-}D}}(K_a)_{\mathbf{X\text{-}IM\text{-}D_2}}a_D + \delta^{\mathbf{X\text{-}IM\text{-}D_2}}a_D^2}{((K_a)_{\mathbf{X\text{-}IM\text{-}D}}(K_a)_{\mathbf{X\text{-}IM\text{-}D_2}} + (K_a)_{\mathbf{X\text{-}IM\text{-}D_2}}a_D + a_D^2)} \; \mathbf{X = 2, 3} \quad (6)$$

Figure 2 (▼) shows the change with changing pD in the chemical shift $(\delta_{CH_2})^{\mathbf{3\text{-}IM}}_{obsd}$ (ppm) of the signal for the methylene hydrogens of **3-IM** (the *p*-hydroxybenzaldehyde-glycine imine, Scheme 2, X = **3**), which forms in D_2O that initially contains 0.3−1.0 M glycine and 0.05 M **3** at 25 °C and $I = 1.0$ (KCl). This figure shows that the chemical shift changes with protonation of the aldimine nitrogen and the phenoxide oxygen. The solid line through the data shows the fit to eq 6, derived for Scheme 2, where $\delta^{\mathbf{3\text{-}IM\text{-}D_2}}_{CH_2} = 4.20$, $\delta^{\mathbf{3\text{-}IM\text{-}D}}_{CH_2} = 4.00$, and $\delta^{\mathbf{3\text{-}IM}}_{CH_2} = 3.90$ ppm are the ^{1}H NMR chemical shifts for the α-CH_2 hydrogens of the different ionized forms of **3-IM**, and $(K_a)_{\mathbf{3\text{-}IM\text{-}D_2}}$ and $(K_a)_{\mathbf{3\text{-}IM\text{-}D}}$ are the acidity constants for **3-IM-D$_2$** and **3-IM-D** (Table 1) determined from the nonlinear least-squares fit of the experimental data to eq 6.

Figure 2 (■) also shows the change with changing pD in the chemical shift $(\delta_{CH_2})^{\mathbf{4\text{-}IM}}_{obsd}$ (ppm) of the signal for the α-CH_2 hydrogens of **4-IM** (the 4-pyridine aldehyde-glycine imine, Scheme 1, Z = N, X = **4**), which forms in D_2O that initially contains 0.001−2.0 M glycine and 0.005−1.0 M **4** at 25 °C and $I = 1.0$ (KCl). The solid line shows the fit of the data to eq 5, derived for Scheme 1, where $\delta^{\mathbf{4\text{-}IM}}_{CH_2} = 4.09$ ppm is the chemical shift for the α-CH_2 hydrogens of **4-IM** determined at high pD and using values of $\delta^{\mathbf{4\text{-}IM\text{-}D}}_{CH_2} = 4.27$ ppm for the α-CH_2 hydrogens of **4-IM-D** and $(K_a)_{\mathbf{4\text{-}IM\text{-}D}}$(Table 1) determined from the nonlinear least-squares fit of the experimental data to eq 5.

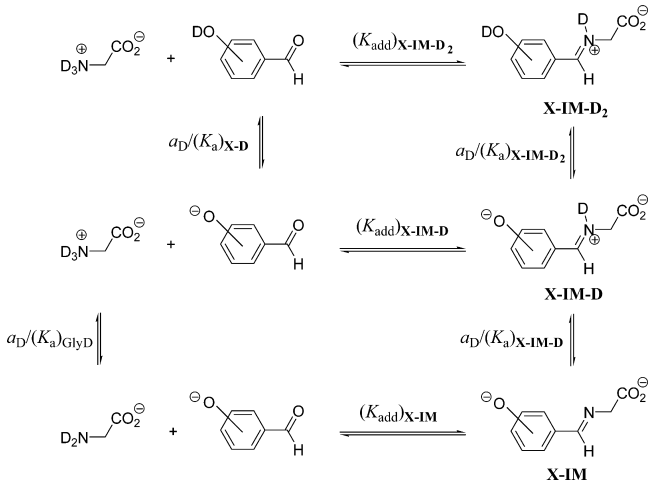
The apparent equilibrium constants $(K_{add})_{obsd}$ (M^{-1}) for addition of glycine to the aromatic aldehydes **1**−**4** to form the corresponding aldimines in D_2O at 25 °C were determined by ^{1}H NMR analysis as described in the Experimental Section. Figure 3 shows the change, with changing pD, in log $(K_{add})_{obsd}$

Table 1. Equilibrium Constants in D_2O for Addition of Glycine to the Aromatic Aldehydes **1**−**4**[a]

aldehyde	iminium ion	$p(K_a)_{IM}$ [b]	$p(K_a)_{OD}$ [c]	(K_{add}) [d] (M^{-1})	$(K_{add})^{+}$ [e] (M^{-1})	$(K_{add})^{++}$ [f] (M^{-1})
		6.29 ± 0.01		44 ± 8	$(3.3 \pm 0.4) \times 10^{-3}$	
		12.13 ± 0.05	5.55 ± 0.04	1.76 ± 0.02	72 ± 8	$(3.4 \pm 0.1) \times 10^{-2}$
		10.16 ± 0.03	6.61 ± 0.02	0.49 ± 0.03	0.29 ± 0.03	$(8.7 \pm 0.9) \times 10^{-3}$
		4.95 ± 0.06		(500 ± 20)	$(2.0 \pm 0.4) \times 10^{-3}$	

[a] At 25 °C and $I = 1.0$ (KCl). [b] Apparent pK_a of the iminium ion nitrogen in D_2O, determined by NMR titration as described in the text. [c] Apparent pK_a of the iminium ion phenol group in D_2O, determined by NMR titration as described in the text. [d] Equilibrium constant for addition of the basic amino form of the amino acid to the basic form of the aldehyde. [e] Equilibrium constant for addition of the amino acid zwitterion to the basic form of the aldehyde. [f] Equilibrium constant for addition of the amino acid zwitterion to the protonated form of the aldehyde.

Scheme 2



for addition of glycine to **1** (●), **2** (▲), **3** (▼), and **4** (■). The line through the circles (●) shows the fit of data for the first reaction to eq 7 (X = **1**), derived for Scheme 1, using $(K_a)_{1\text{-IM-D}} = 5.13 \times 10^{-7}$ M (Table 1) and treating $(K_a)_{GlyD} = (3.9 \pm 0.5) \times 10^{-11}$ M and $(K_{add})_{1\text{-IM-D}} = (3.3 \pm 0.4) \times 10^{-3}$ M^{-1} as variable parameters. The data for formation of the imine of glycine and **2** (▲) were fit to eq 8 (X = **2**), derived for Scheme 2, using $(K_a)_{2\text{-IM-D}_2} = 2.8 \times 10^{-6}$ M and $(K_a)_{2\text{-IM-D}} = 7.4 \times 10^{-13}$ M (Table 1) and treating $(K_{add})_{2\text{-IM-D}_2} = (3.4 \pm 0.1) \times 10^{-2}$ M^{-1}, $(K_a)_{GlyD} = (3.1 \pm 0.2) \times 10^{-11}$ M, and $(K_a)_{2\text{-D}} = (1.32 \pm 0.06) \times 10^{-9}$ M as variable parameters. This fit is shown by the solid line through the "▲" in Figure 3. The observed values of the equilibrium constant for addition of glycine to **3** to form the corresponding imine (▼) were also fit to eq 8 (X = **3**), using $(K_a)_{GlyD} = 3.9 \times 10^{-11}$ M and $(K_a)_{3\text{-D}} = 7.4 \times 10^{-9}$ M for the acidity constants of glycine zwitterion and **3-D**, respectively, $(K_a)_{3\text{-IM-D}_2} = 2.5 \times 10^{-7}$ M and $(K_a)_{3\text{-IM-D}} = 6.9 \times 10^{-11}$ M (Table 1) for the acidity constants of the iminium ion, and treating $(K_{add})_{3\text{-IM-D}_2} = (8.7 \pm 0.9) \times 10^{-3}$ M^{-1} as a variable parameter. The line through the "■" shows the fit of the experimental data for the reaction of glycine with **4** to eq 7 (X = **4**), with $(K_a)_{GlyD} = 3.9 \times 10^{-11}$ M, $(K_a)_{4\text{-IM-D}} = 1.1 \times 10^{-5}$ M (Table 1), and $(K_{add})_{4\text{-IM-D}} = (2.0 \pm 0.4) \times 10^{-3}$ M^{-1} as the only variable parameter.

$$(K_{add})_{obsd} = (K_{add})_{X-IM-D}\left(\frac{a_D + (K_a)_{X-IM-D}}{a_D + (K_a)_{GlyD}}\right) \mathbf{X = 1, 4} \quad (7)$$

$$(K_{add})_{obsd} = (K_{add})_{X-IM-D_2} \times \left(\frac{a_D^2 + a_D(K_a)_{X-IM-D_2} + (K_a)_{X-IM-D_2}(K_a)_{X-IM-D}}{a_D^2 + a_D((K_a)_{GlyD} + (K_a)_{X-D}) + (K_a)_{GlyD}(K_a)_{X-D}}\right) \mathbf{X = 2, 3} \quad (8)$$

4. Discussion

The imines formed from amino acids and substituted aromatic aldehydes have been of particular interest to chemists and biochemists because they constitute useful simple models for characterizing the behavior of the more complex imines to the enzymatic cofactor PLP. Detection of imines in aqueous solution below pH 7 is generally difficult because of the very low equilibrium constants for conversion of the carbonyl and amine species to the corresponding imine adduct. In fact, most of the available information on the stabilities of imines in aqueous solution refers to the unprotonated species. The data determined in this work are in fair agreement with the more limited thermodynamic data reported earlier by Leussing and Bai on the addition of glycine to salicylaldehyde anion[24,25] and by

(24) Leussing and Bai reported a value of 3.3 M^{-1} for the equilibrium constant for addition of glycinate to salicylaldehyde anion to form the unprotonated imine **2-IM** in H_2O at 25 °C and $I = 0.5$ (KCl) (ref 25). Analysis of their data also allows the calculation of $(K_{add})_{2\text{-IM-H}} = 1.1 \times 10^2$ M^{-1} and $p(K_a)_{2\text{-IM-H}} = 11.22$. Assuming that solvent deuterium isotope effects on the equilibrium constants for imine formation are close to 1, these earlier values are in fair agreement with $(K_{add})_{2\text{-IM}} = 1.76$ M^{-1} and $(K_{add})_{2\text{-IM-D}} = 72$ M^{-1} determined in this work (Table 1). A value of $pK_a = 11.8$ for ionization of **2-IM-D** in D_2O at 25 °C and $I = 0.5$ (KCl) can be calculated by adding $\Delta pK_a = 0.6$ for the solvent deuterium isotope effect on the ionization of the iminium ion (ref 26) to the pK_a value in H_2O given above. This is ca. 0.3 units lower than $p(K_a)_{2\text{-IM-D}} = 12.13$ reported here (Table 1).

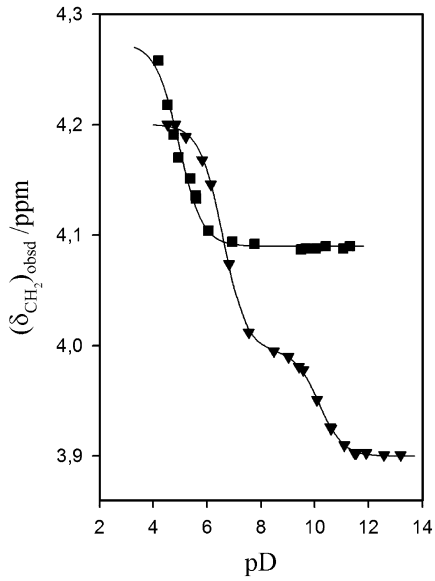


Figure 2. The dependence on pD of $(\delta_{CH_2})_{obsd}$ (ppm) for the $-CH_2-$ hydrogens of the imine formed in D_2O that contains: (▼), 0.3−1.0 M glycine and 50 mM **3**; (■), 0.001−2.0 M glycine and 0.005−1.0 M **4** at 25 °C and $I = 1.0$ (KCl). The solid lines show the fit of the data to eq 5 derived from Scheme 1 (■), or to eq 6 derived from Scheme 2 (▼).

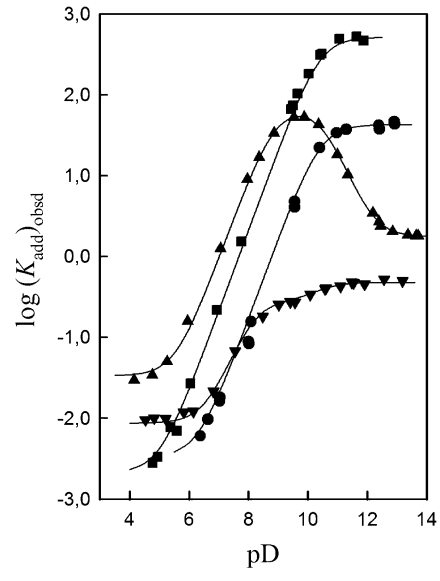


Figure 3. Logarithmic dependence of $(K_{add})_{obsd}$ (M^{-1}) on pD for conversion of glycine to the corresponding aldimines in D_2O at 25 °C and $I = 1.0$ (KCl). Key: Reaction of glycine with **1** (●), **2** (▲), **3** (▼), and **4** (■). The solid lines through the experimental data were calculated using (●) eq 7 (X = **1**), (▲) eq 8 (X = **2**), (▼) eq 8 (X = **3**), (■) eq 7 (X = **4**), and the values of the corresponding equilibrium constants given in Table 1.

Bruice and co-workers on the reaction of glycinate with pyridine-4-carboxaldehyde.[27,28]

The equilibria for imine formation from glycine and PLP,[18] DPL,[16,18] or the simple analogue of the coenzyme, 3-hydroxypyridine-4-aldehyde,[13] have been well characterized. Table 2 summarizes the literature values for the equilibrium constants for formation of the individual ionic forms of these imine adducts together with their pK_a values.

Strength of the Intramolecular Hydrogen Bond in PLP Aldimines. It is well-known that amino acids form highly stable imines to PLP, which exist mainly as the iminium ions in aqueous solution below pH 10 (Table 2). This contrasts with the much lower stability of the structurally simpler iminium ion formed between glycine and benzaldehyde, which has a pK_a of ca. 6 (Table 1). The low thermodynamic acidity of PLP iminium ions has generally been attributed to the existence of a strong hydrogen bond between the iminium ion and the phenolate oxygen. However, in a recent examination of the pH dependence of ^{15}N NMR chemical shifts of PLP aldimines in aqueous solution, Limbach and co-workers concluded that this intramolecular hydrogen bond is probably absent, and the imino and phenolate groups are preferentially hydrogen bonded to water molecules.[20] This raises the question of whether *o*-hydroxyaryl aldimines are in fact stabilized by an intramolecular hydrogen bond in aqueous solution, and, if so, what is the strength of this interaction, or if the large stability of these iminium ions can be fully accounted for by resonance and electrostatic effects of the *ortho*-phenoxide substituent. This problem can be clarified by comparing the effect of an *ortho*- versus a *para*-phenoxide anion substituent on the basicity of the imine adduct formed from glycine and benzaldehyde (Scheme 3), because the *para*-phenoxide substituent effect provides an estimate for the resonance stabilization of the iminium ion in the absence of any direct hydrogen-bonding interaction.

Table 3 summarizes the effect of changing substituents X at the aromatic ring of benzaldehyde on the equilibrium constant for addition of glycine to this aldehyde (K_{add}) and on the pK_a of the corresponding iminium ion. The observed 3.9 unit effect of a *p*-O^- substituent on the pK_a of the iminium ion of benzaldehyde (Scheme 3B) corresponds to the sum of stabilizing resonance and electrostatic interactions of this substituent with the imino nitrogen, which are greater in the iminium ion than in the neutral imine product. By comparison, there is a 1.9 unit larger effect of an *o*-O^- (Scheme 3A) than of a *p*-O^-substituent (Scheme 3B) on iminium ion acidity, which could reflect additional stabilization of the iminium ion due to formation of an intramolecular hydrogen bond between the oxygen anion and the iminium nitrogen. The observed global substituent effects can be used to estimate the strength of this hydrogen bond after correction for the differential stabilization of the imino nitrogen by *o*-O^- and *p*-O^- substituents through resonance and electrostatic interactions.

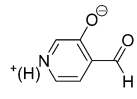
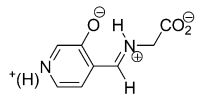
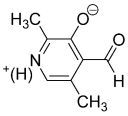
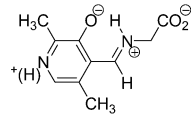
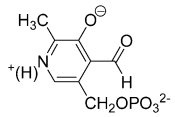
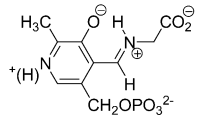
The 0.5 unit effect of the *p*-O^- substituent on the pK_a of benzylamine (Chart 2),[29−31] which corresponds to 0.7 kcal/mol stabilization energy, provides an estimate of the polar interaction

(25) Leussing, D. L.; Bai, K. S. *Anal. Chem.* **1968**, *40*, 575–81.
(26) Laughton, P. M.; Robertson, R. E. In *Solute−Solvent Interactions*; Coetzee, J. F., Ritchie, C. D., Eds.; Dekker: New York, 1969; p 399.
(27) An equilibrium constant, $(K_{add})_{\mathbf{4\text{-}IM}} = 377\ M^{-1}$, has been reported previously for reaction of glycine anion with the neutral aldehyde **4** to form the corresponding imine **4-IM** in H_2O at 30 °C and $I = 1.0$ (KCl) (ref 28). This is in fair agreement with $(K_{add})_{\mathbf{4\text{-}IM}} = 500\ M^{-1}$ in D_2O at 25 °C and $I = 1.0$ (KCl) reported here. However, there is no information in the literature on the stability of the protonated forms of this imine adduct.
(28) French, T. C.; Bruice, T. C. *Biochemistry* **1964**, *3*, 1589–96.
(29) A pK_a of 9.94 for ionization of *p*-O^--benzylammonium ion in aqueous solution at 25 °C was calculated from the linear Hammett correlation of pK_a for ionization of *p*-X-benzylammonium ions (X = CH_3O, CH_3, H, Cl, CF_3, CN) (ref 30) given by eq 9, using $\sigma_n = -0.5$ for an oxygen anion substituent (ref 31, p 72).

$$p(K_a)_X = -(0.99 \pm 0.05)\sigma_X + (9.44 \pm 0.02) \quad (9)$$

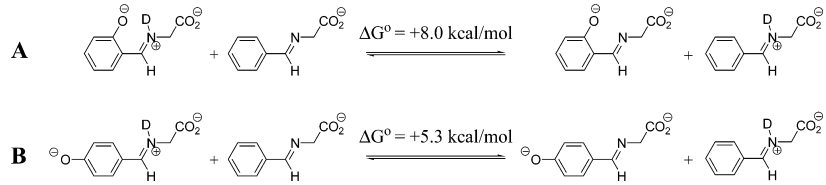
(30) Bunting, J. W.; Stefanidis, D. *J. Am. Chem. Soc.* **1990**, *112*, 779–86.

Table 2. Equilibrium Constants in H_2O for Addition of Glycine to 3-Hydroxypyridine-4-aldehyde, DPL, and PLP

aldehyde	iminium ion	$p(K_a)_{IM}$ [a]	$p(K_a)_{Pyr}$ [b]	(K_{add}) [c] (M^{-1})	$(K_{add})^{+}$ [d] (M^{-1})	$(K_{add})^{+-}$ [e] (M^{-1})
		9.12 [f]	5.50 [f]	49.3 [f]	10.1 [f]	0.88 [f]
		11.22 [g] (11.30) [h]	6.43 [g] (6.59) [h]	12.3 [g] (13.2) [h]	339 [g] (437) [h]	8.7 [g] (15.9) [h]
		11.35 [g]	6.36 [g]	21.4 [g]	833 [g]	8.9 [g]

[a] Apparent pK_a of the iminium ion nitrogen in H_2O. [b] Apparent pK_a of the iminium ion pyridine group in H_2O. [c] Equilibrium constant for addition of the basic amino form of the amino acid to the basic form of the aldehyde. [d] Equilibrium constant for addition of the amino acid zwitterion to the basic form of the aldehyde. [e] Equilibrium constant for addition of the amino acid zwitterion to the *N*-protonated form of the aldehyde. [f] Data in H_2O at 30 °C and $I = 1.0$ (KCl) from ref 13. [g] Data in H_2O at 25 °C and $I = 0.1$ (KCl) from ref 18. [h] Data in H_2O at 25 °C and $I = 0.2$ from ref 16.

Scheme 3



between this substituent and the positive charge at the iminium nitrogen. A value of 4.6 kcal/mol for the differential stabilization of the iminium ion compared to the neutral imine by a resonance interaction with the *p*-oxygen anion can be calculated by subtraction of the estimated electrostatic contribution from the observed overall 5.3 kcal/mol stabilizing effect of the *p*-O^- substituent (Scheme 3B, Chart 2).

This gives an upper limit for the difference in resonance stabilization of the electron-deficient iminium ion as compared to the imine provided by the *o*-O^- substituent. However, analysis of the data in Table 3 shows that the effect of an *o*-O^- on the equilibrium constant for addition of glycine anion to benzaldehyde to form the neutral imine (Scheme 4A) is ca. 70% of the effect of a *p*-O^- substituent on the same equilibrium constant (Scheme 4B). This reflects a stronger stabilization of the carbonyl group of the aldehyde than of the imine group of the product by resonance electron donation from a *p*-O^- as compared to an *o*-O^- substituent. An estimate of the difference in resonance stabilization of the benzaldehyde iminium ion as compared to the neutral imine by an *ortho*-oxygen anion can be calculated as 70% of the observed resonance effect of the *para*-oxygen anion, which corresponds to $(0.70) \times (4.6) = 3.2$ kcal/mol.

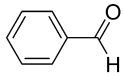
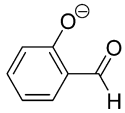
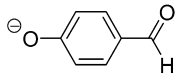
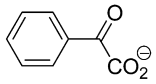
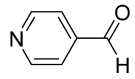
The salicylaldehyde iminium ion (**5**) may be further stabilized by a through-space Coulombic interaction between the positively charged nitrogen and the negatively charged phenoxide anion. A simple application of Coulomb's law to determine the ratio of electrostatic energies in the two iminium ions **5** and **6**, assuming that there is no difference in the effective dielectric constant and magnitude of the charges in both interactions, gives $E_5/E_6 = r_6/r_5$, where r_5 and r_6 are the distances between the opposed charges in **5** and **6**, respectively. The computed chemical structures for **5** and **6** (Chart 3) show that the two charged centers are at a distance of 2.6 and 6.5 Å, respectively, and, therefore, $E_5/E_6 = 2.5$. If we use the value of 0.7 kcal/mol estimated above for the interaction between the positively charged nitrogen and the *p*-phenoxide anion in **6**, we obtain a value of 1.8 kcal/mol for the interaction between these two groups in **5**. Steric effects are assumed to be negligible given the substantially planar conformation of these sterically uncrowded molecules. If the contributions of resonance and through-space electrostatic effects are subtracted from the overall 8.0 kcal/mol effect of an *o*-O^- substituent on the benzaldehyde iminium ion acidity (Scheme 3A), the remaining energy ($8.0 - 3.2 - 1.8) = 3.0$ kcal/mol, may be attributed to the contribution of hydrogen bonding to the greater stabilization of the iminium ion as compared to the neutral imine.

Intermolecular hydrogen bonds in aqueous solution are generally very weak because water molecules efficiently compete in the solvation of the acid and base groups.[32] However, hydrogen-bonding interactions in water may become significant when the acid donor and base acceptor are in the same molecule and forced to the appropriate geometry by molecular constraints.[33,34] This seems to be the case for the iminium ion **5**, which we have shown to be stabilized by an intramolecular hydrogen bond in aqueous solution. This hydrogen bond is ca. 3 kcal/mol stronger than predicted for a simple electrostatic interaction

(31) Hine, J. *Structural Effects on Equilibria in Organic Chemistry*; John Wiley & Sons: New York, 1975.

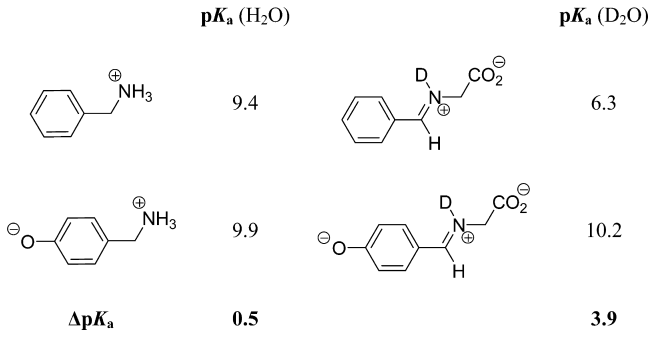
(32) Stahl, N.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108*, 4196–205.
(33) Frey, P. A.; Cleland, W. W. *Bioorg. Chem.* **1998**, *26*, 175–192.
(34) Hibbert, F.; Emsley, J. *Adv. Phys. Org. Chem.* **1990**, *26*, 255–79.

Table 3. Effect of Changing Ring Substituents, or the Substitution of C-4 of the Ring by N, on the Equilibrium Constant for Addition of Glycine to Benzaldehyde To Form the Corresponding Imine[a]

aldehyde	$(K_{add})_X$ [b]	$\frac{(K_{add})_X}{(K_{add})_H}$	$\Delta\Delta G_X$ [c] (kcal/mol)	$(K_{add})^+_X$ [d]	$\frac{(K_{add})^+_X}{(K_{add})^+_H}$	$\Delta\Delta G_X$ [e] (kcal/mol)	$p(K_a)_{X\text{-}IMD}$ [f]	$\Delta(pK_a)_X$ [g]
	44	1.0	0	0.0033	1.0	0	6.29	0
	1.76	0.040	1.9	72	22,000	-5.9	12.13	5.8
	0.49	0.011	2.7	0.29	88	-2.7	10.16	3.9
	1.5[h]	0.034	2.0	1×10^{-4} [h]	0.030	2.1	6.19[h]	-0.1
	500	11.4	-1.4	0.002	0.61	0.3	4.95	-1.3

[a] Data from Table 1 for reactions in D_2O, unless noted otherwise. [b] Equilibrium constants for addition of glycine anion to the aldehyde in the first table row to form the corresponding unprotonated imine. [c] The effect of the substituent X on the change in Gibbs energy for formation of the imine from the free aldehyde and amino acid. [d] Equilibrium constants for addition of glycine zwitterion to the aldehyde in the first table row to form the corresponding iminium ion. [e] The effect of the substituent X on the change in Gibbs energy for formation of the iminium ion from the free aldehyde and amino acid. [f] Apparent acidity constants for the substituted iminium ions. [g] The effect of the substituent X on the pK_a for ionization of the iminium ion. [h] Data from ref 9 for reactions in D_2O at 25 °C and $I = 1.0$ (KCl).

Chart 2



between the interacting iminium cation and phenoxide anion. This is not much different from the estimated ca. 4 kcal/mol stabilization energy involved in formation of an intramolecular hydrogen bond in the salicylate anion (**7**) in water.[35] In both molecules, the special nature of the internal hydrogen bond appears to be marked by the existing direct conjugation between the hydrogen bond donor and acceptor groups (Chart 4). However, despite the large number of investigations on this kind of structures,[36] there is no general agreement as to whether the observed extra strength of the intramolecular hydrogen bond is due to stabilization by resonance[37,38] or the result of the favorable orientation and coplanarity of the donor and acceptor groups.[39]

Table 4 shows that the effect of an *o*-O^- substituent on the pK_a of the iminium ion formed between 4-pyridine-carboxaldehyde and glycine is 1 unit lower than the value reported in Table 3 for the effect of the same substituent on the pK_a of the benzaldehyde iminium ion. The smaller stabilizing interaction provided by this substituent when the phenyl ring is substituted by a pyridine ring does reflect the transfer of negative charge to the pyridine nitrogen, which should reduce the extent of resonance stabilization due to delocalization of negative charge on the iminium ion group of **8**. We do not expect a significant change in the strength of the intramolecular hydrogen bond in the iminium ion **5** upon the introduction of a pyridine ring to give **8**. Table 3 shows that the substitution of C-4 of the ring by N leads to a 1.3 unit decrease in the pK_a of the benzaldehyde iminium ion. This is similar to the observed 1.4 unit decrease in pK_a on going from phenol ($pK_a = 10.0$) to 3-hydroxy-pyridine ($pK_a = 8.6$).[40] This suggests that the introduction of the pyridine nitrogen makes both the hydrogen bond donor and acceptor more acidic by the same amount, so that the strength of the hydrogen bond is not significantly affected by this substitution. We conclude that the stabilizing energy involved in formation of an internal hydrogen bond in PLP iminium ions in water should be similar to the ca. 3 kcal/mol estimated

(35) Hermans, J., Jr.; Leach, S. J.; Scheraga, H. A. *J. Am. Chem. Soc.* **1963**, *85*, 1390–5.
(36) Sobczyk, L.; Grabowski, S. J.; Krygowski, T. M. *Chem. Rev.* **2005**, *105*, 3513–3560.
(37) Gilli, G.; Bellucci, F.; Ferretti, V.; Bertolasi, V. *J. Am. Chem. Soc.* **1989**, *111*, 1023–8.
(38) Gilli, P.; Bertolasi, V.; Ferretti, V.; Gilli, G. *J. Am. Chem. Soc.* **2000**, *122*, 10405–10417.
(39) Sanz, P.; Mo, O.; Yanez, M.; Elguero, J. *J. Phys. Chem. A* **2007**, *111*, 3585–3591.
(40) Metzler, D. E.; Harris, C. M.; Johnson, R. J.; Siano, D. B.; Thomson, J. A. *Biochemistry* **1973**, *12*, 5377–92.

Scheme 4

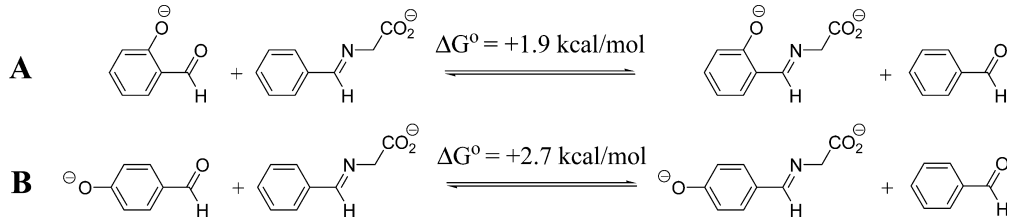


Chart 3

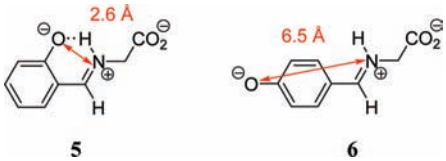
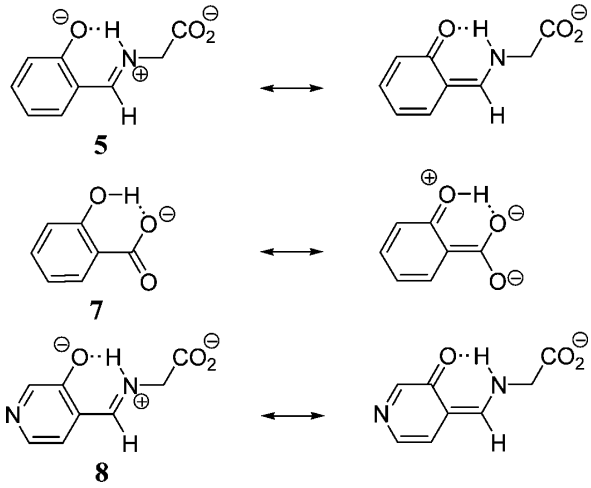


Chart 4



above for **5**. Our data are therefore consistent with the existence in aqueous solution of an intramolecular hydrogen bond in iminium ions derived from *o*-hydroxyaryl aldehydes and amino acids.

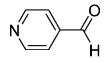
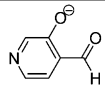
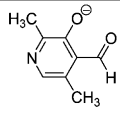
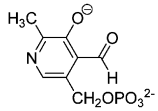
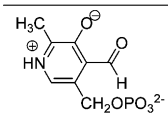
Limbach and co-workers have observed that the ^{15}N chemical shift for the protonated imine nitrogen of pyridoxal in CD_3OH decreases from 238 ppm for the aldimine to 154 ppm for its hydrogen-bonded complex with perfluorobutyric acid (Chart 5A).[41] This shows that partial protonation of the pyridine nitrogen is coupled to a shift of the proton in the strongly covalent intramolecular $-O^{-}\cdot{}^{+}HN-$ hydrogen bond, away from the phenoxide oxygen and toward the imine nitrogen. By comparison, in water, the low chemical shift of $\delta = 146$ ppm for the iminium nitrogen observed at pH 10, where the pyridine ring is deprotonated, is consistent with nearly full protonation of this nitrogen.[20] The ^{15}N chemical shift for this nitrogen shows no detectable change from $\delta = 146$ ppm as the pH is decreased in water from 10 [$\ll$1% protonation of the pyridine nitrogen] to 6 [60% protonation of the pyridine nitrogen],[20] so that the proton at the iminium ion remains essentially stationary upon protonation of the pyridine nitrogen. These results show either that there is no direct hydrogen-bonding interaction between the cationic iminium ion and the phenoxide anion in water or that the intramolecular hydrogen bond between these atoms in water behaves as an ion-pair, with minimal proton transfer from nitrogen to oxygen.

We estimate that the magnitude of the stabilizing interaction between the iminium cation and phenoxide anion is ca. 3 kcal/mol stronger than for a simple through-space electrostatic interaction between the corresponding free ions. This provides strong evidence for the existence of an intramolecular $-O^{-}\cdot{}^{+}HN-$ hydrogen bond in water. The 3 kcal/mol stabilization no doubt reflects a small degree of proton transfer from nitrogen to oxygen in a double-potential energy minimum hydrogen bond. The ^{15}N chemical shift data show that the position of the proton in this hydrogen bond does not change detectably upon protonation of the pyridine ring. However, a large shift in this proton toward nitrogen is not possible, because the change in solvent from methanol to water has caused in a change in the relative basicities of the phenoxide oxygen and the imine nitrogen and a shift in the position of the proton from oxygen to nitrogen (Chart 5).

Other Substituent Effects on the Stability of Iminium Ions. An analysis of the data in Table 4 leads to some interesting conclusions concerning the effect of ring substituents on the stability of pyridoxal iminium ions: (a) The 2.1 unit smaller acidity of the iminium ion of glycine to DPL than of the iminium ion **8** reflects the 2.9 kcal/mol greater stabilization of the iminium ion relative to the neutral imine by the two methyl groups introduced in the pyridine ring. The effect of these electron-donating substituents offsets the acidifying effect of the neutral pyridine nitrogen, so that the pK_a for the DPL iminium ion in water is similar to that for the simple iminium ion **5**. (b) The pK_a values for the iminium ions formed between glycine and DPL (11.2) or between glycine and PLP (11.3) are similar. This shows that the phosphate group does not provide additional stabilization of the iminium ion relative to the neutral imine and suggests that this group does not play a significant role in the high stability of imines to PLP. This is consistent with the assumption that the phosphate group in PLP serves mainly as an attachment of the coenzyme to the protein. The iminium ion formed by addition of glycine to pyridoxal is not a good reference system to evaluate the effect of the phosphate group because the somewhat lower pK_a measured for this compound (10.8) has been suggested to be due to stabilization of the neutral imine by hydrogen bonding between the imine nitrogen and the 5′-hydroxy group.[16,18] (c) There is a ca. 6 kcal/mol increase in the thermodynamic driving force for formation of an iminium ion from glycine zwitterion and benzaldehyde upon addition of an *o*-O^{-} substituent to the phenyl ring (Table 3). This represents a large fraction of the 7.4 kcal/mol greater driving force for addition of glycine zwitterion to the most basic form of PLP than to benzaldehyde and suggests that the phenoxide anion present in the cofactor molecule provides most of the extra stabilization observed in PLP iminium ions relative to the simpler benzaldehyde adduct.

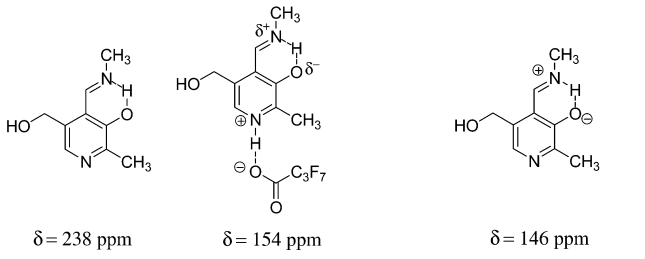
(41) Sharif, S.; Denisov, G. S.; Toney, M. D.; Limbach, H.-H. *J. Am. Chem. Soc.* **2007**, *129*, 6313–6327.

Table 4. Effect of Changing Ring Substituents on the Equilibrium Constant for Addition of Glycine to Pyridine Aldehyde To Form the Corresponding Imine[a]

aldehyde	$(K_{add})_X$ [b]	$\frac{(K_{add})_X}{(K_{add})_H}$	$\Delta\Delta G_X$ [c] (kcal/mol)	$(K_{add})^+_X$ [d]	$\frac{(K_{add})^+_X}{(K_{add})^+_H}$	$\Delta\Delta G_X$ [e] (kcal/mol)	$p(K_a)_{X\text{-}IMD}$ [f]	$\Delta(pK_a)_X$ [g]
	500[h]	1.0	0	0.002[h]	1.0	0	4.95 (D_2O)[h] 4.35 (H_2O)[i]	0
	49[j]	0.10	1.4	10[j]	5,000	-5.0	9.12[j]	4.8
	12.3[k] (13.2)[l]	0.025	2.2	339[k] (437)[l]	1.7×10^5	-7.1	11.22[k] (11.30)[l]	6.9
	21.4[k]	0.043	1.9	833[k]	4.2×10^5	-7.7	11.35[k]	7.0
				8.9[j]	4450	-5.0		

[a] In H_2O or in D_2O. [b] Equilibrium constants for addition of glycine anion to the aldehyde in the first table row to form the corresponding unprotonated imine. [c] The effect of the substituent X on the change in Gibbs energy for formation of the imine from the free aldehyde and amino acid. [d] Equilibrium constants for addition of glycine zwitterion to the aldehyde in the first table row to form the corresponding iminium ion. [e] The effect of the substituent X on the change in Gibbs energy for formation of the iminium ion from the free aldehyde and amino acid. [f] Apparent acidity constants for the substituted iminium ions. [g] The effect of the substituent X on the pK_a for ionization of the iminium ion. [h] Equilibrium constants from Table 1 for reactions in D_2O. [i] Estimated assuming $(K_a)_{H2O}/(K_a)_{D2O} = 4$ (ref 26) for the solvent deuterium isotope effect on the acidity constant of the iminium ion of glycine and pyridine carboxaldehyde. [j] Data in H_2O at 30 °C and $I = 1.0$ (KCl) from ref 13. [k] Data in H_2O at 25 °C and $I = 0.1$ (KCl) from ref 18. [l] Data in H_2O at 25 °C and $I = 0.2$ from ref 16.

Chart 5



We have previously reported that the equilibrium constant for formation of an iminium ion to phenylglyoxylate, a model for the pyruvoyl prosthetic group of some amino acid decarboxylases, is less favorable than that for formation of PLP iminium ions. The stabilizing electrostatic interaction between the charged carboxylate at phenylglyoxylate and the cationic iminium ion might have been expected to cause a decrease in the acidity of the phenylglyoxylate as compared to the benzaldehyde iminium ion. However, Table 3 shows that these two iminium ions have nearly the same pK_a. These data show that the α-CO_2^- substituent provides little or no stabilization of neighboring positive charge, because the formal polar electron-withdrawing effect of the carbonyl functional group is roughly balanced by the field effect of this anionic substituent. By comparison, the introduction of a $-CO_2^-$ group at carbon in methylamine results in only a 1.1 unit decrease on its pK_a from 10.8 to 9.7 for glycine zwitterion.[42]

Enzymatic Catalysis. We find that the 2-O^- substituent at PLP provides 8.0 kcal/mol larger stabilization of the glycine iminium ion relative to the corresponding neutral imine. This stabilization will contribute directly to PLP catalysis of deprotonation of amino acids in solution and at the enzyme active sites for enzyme catalysts. This stabilizing interaction is partly offset by the unfavorable interaction between the anionic 2-O^- substituent and the developing negative charge at the pyridoxal-stabilized α-amino acid carbanion. For example, protonation of the 2-O^- group at DPL results in a 100-fold decrease in the second-order rate constant for deprotonation of the glycine iminium ion by acetate anion.[10]

The small 0.6-fold difference in the stability of iminium ions to 4-pyridine carboxaldehyde as compared to benzaldehyde (Table 3) and the similar activity of the aromatic ketone phenylglyoxylate and DPL toward catalysis of deprotonation of the glycine zwitterion[9] suggest that the pyridine ring nitrogen does not make a large contribution toward the activity of PLP as a catalyst of deprotonation of amino acids. However, there is good evidence that PLP enzymes control the protonation state of the bound cofactor at pyridine ring, to direct the partitioning of enzyme-bound carbanion intermediates.[43] The neutral pyridine ring favors protonation of the delocalized carbanion at the α-amino acid carbon, while nitrogen protonation favors delocalization of negative charge across the α-imino and α-pyridyl carbons required for catalysis of 1,3-isomerization reaction by transaminases. Finally, we suggest that an important, but

(42) Heo, C. K. M.; Bunting, J. W. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2279–2290.
(43) Toney, M. D. *Arch. Biochem. Biophys.* **2005**, *433*, 279–287.

underappreciated, role of the phosphate group of the PLP cofactor is to provide binding energy[44] that may be utilized for stabilization of the α-imino carbanion (quinonoid) intermediates of a host of PLP-dependent enzyme-catalyzed reactions.

(44) Morrow, J. R.; Amyes, T. L.; Richard, J. P. *Acc. Chem. Res.* **2008**, *41*, 539–548.

Acknowledgment. We acknowledge the Ministerio de Educación y Ciencia and the European Regional Development Fund (ERDF) (Grant CTQ2004-06594) and the U.S. National Institutes of Health (GM39754) for generous support of this work.

JA906230N