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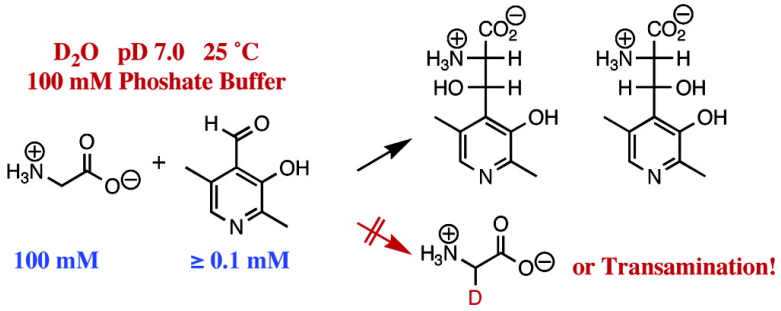

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Claisen-Type Addition of Glycine to Pyridoxal in Water

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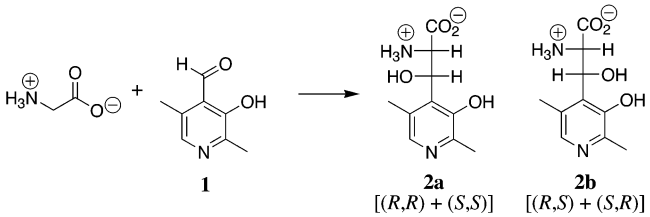
We report that the reaction between the pyridoxal 5′-phosphate (PLP) analogue **1** (10 mM) and glycine (100 mM) in D_2O buffered at neutral pD does not give the expected products of a PLP-catalyzed reaction, but rather gives a *quantitative* yield of the diasterioisomers **2a** and **2b** from the formal Claisen-type addition of glycine to **1** (Scheme 1).

Scheme 1

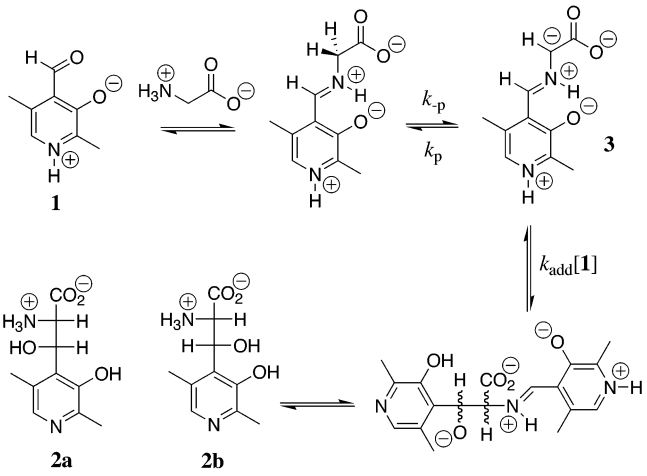


The highly effective catalysis by acetone of deuterium exchange of the α-amino protons of glycine methyl ester in D_2O results from the seven-unit decrease in the pK_a of these protons upon formation of the iminium ion adduct with acetone.[1] This is analogous to the effect of formation of a Schiff's base with PLP on the carbon acidity of α-amino acids, which is thought to be well understood.[2a] We expected that an examination of the deuterium exchange reaction of glycine catalyzed by the PLP analogue **1** would provide the carbon acid pK_a of the α-amino protons of the iminium ion adduct of glycine with **1**.[2b] However, ^{1}H NMR analysis[3a] of the reaction of glycine (100 mM) with **1** (10 mM) in D_2O buffered at pD 7.0 with 100 mM phosphate at 25 °C (I = 1.0, KCl) revealed the first-order *disappearance* of **1** to give an equilibrium mixture containing 3% **1**[4] and 97% of the diastereomeric products **2a** and **2b** in a ratio of 2:1,[5] but *no detectable (<1%) incorporation of deuterium from D_2O into glycine or transamination to give 5′-deoxypyridoxamine*.[6] The sum of the normalized integrated peak areas for the protons of **1**, **2a**, and **2b** was constant during reaction of more than 90% of **1**, and both the disappearance of **1** and the appearance of **2a** and **2b** are governed by the same first-order rate constant, k_{obsd} = 4.3 × 10^{-5} s^{-1}. ^{13}C NMR analysis of the reaction of [2-^{13}C]-labeled glycine under the same conditions revealed a pair of signals for **2a** and **2b**. A value of k_{obsd} = 4 × 10^{-5} s^{-1} was determined by monitoring the disappearance of the signal for the iminium ion adduct of [2-^{13}C]-labeled glycine with **1**.[3b]

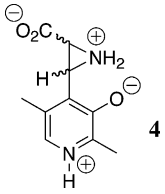
The reaction of glycine with pyridoxal to give **2** was reported 50 years ago in a study that focused on the role of metal cations.[7] However, for many years the literature has emphasized the *similarity* between nonenzymatic and enzymatic reactions promoted by pyridoxal,[2a,8] so that our failure to observe the expected products of the reaction of glycine with the PLP analogue **1** represents a new "wrinkle" in the chemistry of this important cofactor.

The reaction of aminomalonate with **1** has been reported to give CO_2 and **2**.[9] If this proceeds by decarboxylation of the iminium ion adduct of aminomalonate with **1** to give the enolate **3** followed by addition of **3** to a second molecule of **1** (Scheme 2), then deprotonation of the iminium ion adduct of glycine with **1** to give **3** should also result in the formation of **2** (Scheme 2), *although not necessarily as the only product*. ^{1}H NMR analysis[3a] of the reaction of aminomalonate (100 mM) with **1** (10 mM) in D_2O buffered at pD 5.7 with 40 mM acetate at 25 °C (I = 1.0, KCl) showed complete reaction within 10 min and the essentially quantitative formation of **2a** and **2b**.[5]

Scheme 2



This formal Claisen-type addition of glycine to **1** could proceed by cyclization of the enolate **3** to give the aziridinium ion **4**, followed by regiospecific nucleophilic attack of water at the α-pyridyl carbon. However, the following results from kinetic analyses of the reaction of glycine (100 mM) at both low (0.10 mM) and high (10 mM) initial concentrations of **1** in H_2O (pH 6.5) or D_2O (pD 7.0) buffered by 100 mM phosphate ([B]/[BL^+] = 1.0) at 25 °C (I = 1.0, KCl) provide strong support for the reaction mechanism shown in Scheme 2.



(1) The reactions of **1** with glycine at $[\mathbf{1}]_o$ = 10 mM in D_2O monitored by ^{1}H NMR[3a] or spectrophotometrically at 412 nm[10a] (k_{obsd} = 4.3 × 10^{-5} s^{-1}) and in H_2O monitored at 412 nm[10a] (k_{obsd} = 5.0 × 10^{-5} s^{-1}) are all first-order in [**1**] for four reaction half times. This is consistent with rate-determining deprotonation of the iminium ion adduct to give **3** (k_{-p}), because the concentration of **1** at all times in these experiments is sufficient for the effective trapping of **3** by **1** (k_{add}[**1**] ≫ k_p, Scheme 2).

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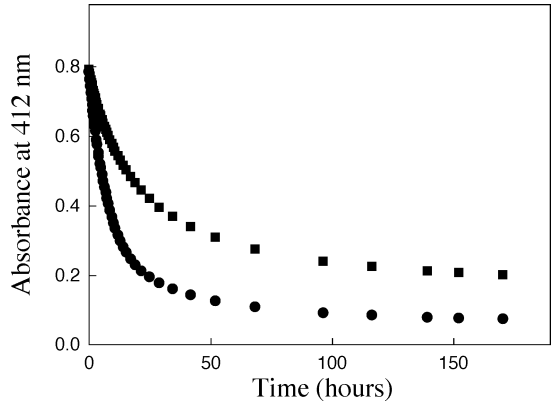

Figure 1. Time courses for reaction of glycine (100 mM) with **1** (0.1 mM) in H_2O at pH 6.5 (■) and in D_2O at pD 7.0 (●) buffered by 100 mM phosphate at 25 °C and $I = 1.0$ (KCl), monitored at 412 nm.

(2) Figure 1 shows the time courses for the reactions of **1** with glycine at $[\mathbf{1}]_o = 0.1$ mM in H_2O and D_2O monitored at 412 nm.[3c,10b] Now, neither reaction is first-order in [**1**], and no stable endpoint is observed after ca. 40 half times calculated for these reactions at $[\mathbf{1}]_o = 10$ mM. This is consistent with a change to a reaction that is second-order in [**1**] when $[\mathbf{1}]_o$ is decreased from 10 mM to 0.1 mM. At $[\mathbf{1}] = 0.1$ mM, deprotonation of the iminium ion is partly reversible so that the addition of **3** to **1** is partly rate-limiting ($k_{add}[\mathbf{1}] \approx k_p$, Scheme 2).[11] The initial velocity, corrected for the estimated difference in the endpoints, is ca. 1.8-fold *larger* for reaction in D_2O than in H_2O. This contrasts the 14% *smaller* value of k_{obsd} for the reaction of **1** in D_2O than that in H_2O at $[\mathbf{1}]_o = 10$ mM. The increase in the velocity for the reaction of **1** in D_2O relative to that in H_2O as the rate-limiting step changes from k_{-p} at high [**1**] to k_{add} at low [**1**] is a consequence of the normal primary deuterium isotope effect on the *protonation* of **3** (k_p, Scheme 2). This is because the slower protonation of **3** in D_2O than in H_2O results in more favorable partitioning of **3** to product in D_2O, and this partitioning controls the overall reaction velocity when k_{add} is partly rate-determining.[12]

The formal Claisen-type addition of glycine to **1** has escaped characterization and it *appears* very unlikely to occur in water, a moderately acidic solvent that rapidly protonates the highly basic enolates of simple carboxylic acid derivatives, including amino acids.[13] The protonation of **3** would result in the "normal" product of a PLP-catalyzed reaction, so that the extensive formation of **2a** and **2b** from addition of **3** to **1** that is present at ≥0.1 mM in water is a consequence of an unprecedented large *selectivity* of **3** toward addition to **1** in a protic solvent buffered at neutral pH. By comparison, the protonation of an acetone-like enolate by buffer acids is significantly faster than its intramolecular addition to a benzaldehyde-type carbonyl group.[14] Apparently, the extensive resonance stabilization of **3** favors carbonyl addition in water because it results in a larger increase in the intrinsic barrier to its protonation than in that for carbonyl addition.[15]

The 5′-deoxypyridoxal-stabilized enolate of alanine generated by loss of CO_2 from the iminium ion adduct of α-methylamino-malonate with **1** has been reported to undergo a reaction similar to that reported here.[16] However, the reaction of dilute 3-hydroxy-4-pyridinecarboxaldehyde (0.5 mM) with a large excess of alanine in strongly buffered solution reportedly yields only pyruvate from a transamination reaction,[17] which suggests that the glycine enolate **3** is unusually reactive toward carbonyl electrophiles. Finally, although we are not aware of the biological relevance of the reaction shown in Scheme 2, we are reluctant to conclude that it has no biochemical implications.

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(2) (a) Jencks, W. P. *Catalysis in Chemistry and Enzymology*, 2nd ed.; Dover: New York, 1987; pp 133−146. (b) There are several published studies on the exchange of α-amino protons catalyzed by PLP-dependent enzymes, including: Malthouse, J. P. G. *Biochim. Biophys. Acta* **2003**, *1647*, 138−142.
(3) (a) ^{1}H NMR spectra (64 transients) at 500 MHz in D_2O at 25 °C were obtained using a Varian Unity Inova 500 spectrometer with a 6000 Hz sweep width, a 6 s acquisition time, a 90° pulse angle, and a 70 s relaxation delay, which was at least 7-fold greater than T_1 for the protons of interest. Integrated peak areas were normalized using that for the methyl groups of added $Me_4N^+ \cdot HSO_3^-$ (1 mM). (b) [2-^{13}C]-Glycine (99 atom %) was from Cambridge Isotope Laboratories. ^{13}C spectra were obtained using a Bruker Avance DRX 500 standard-bore spectrometer operating at 125.8 MHz for ^{13}C with a 0.54 s acquisition time, a 90° pulse angle, and a 7.8 s relaxation delay. The chemical shifts for the enriched carbons of the iminium ion adduct of glycine with **1** and the major and minor diastereomers of **2** are 55.6, 59.7, and 59.2 ppm, respectively. (c) ^{1}H NMR analysis showed that **2a** and **2b** are the major products (>90%) of the reaction of glycine (100 mM) with **1** (0.1 mM) in D_2O at pD 7.0.[5]
(4) ^{1}H NMR analysis[3a] showed that, under these conditions, **1** exists as a mixture consisting of 23.4% of the free aldehyde, 11.6% of the hydrated aldehyde, and 65.0% of the glycine iminium ion adduct.
(5) The diastereomeric products **2a** and **2b** were identified by ^{1}H NMR spectroscopy by spiking the reaction mixture with an authentic mixture of these diastereoisomers prepared as the dihydrochloride salt using a published procedure.[9] ^{1}H NMR (D_2O, pD 7.0, chemical shifts reported relative to HOD at 4.67 ppm): Major diastereoisomer: δ 2.24, 2.40 (3H, s, CH_3), 3.95 (1H, d, $J = 5$ Hz, $CH(ND_3)^+$), 5.29 (1H, d, $J = 5$ Hz, CH(OD)), 7.45 (1H, s, ArH); minor diastereoisomer: δ 2.29, 2.37 (3H, s, CH_3), 4.21 (1H, d, $J = 5$ Hz, $CH(ND_3)^+$), 5.50 (1H, d, $J = 5$ Hz, CH(OD)), 7.41 (1H, s, 1H, ArH). Anal. ($C_{10}H_{16}Cl_2N_2O_4$) C, H, N. We have not determined the relative stereochemistry of the major and minor diastereomeric products so that either **2a** or **2b** could be the major product of the reaction of glycine with **1**.
(6) The products of these reactions were not detectable during the first four reaction halftimes and were barely detectable at longer reaction times.
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(10) (a) The reaction of **1** with glycine at $[\mathbf{1}]_o = 10$ mM was followed by monitoring the decrease in absorbance at 412 nm that was determined after making a 100-fold dilution of an aliquot of the reaction mixture into 100 mM phosphate buffer in H_2O at pH 6.5. (b) The reaction of **1** with glycine at $[\mathbf{1}]_o = 0.10$ mM was followed directly by monitoring the decrease in absorbance at 412 nm (Figure 1).
(11) We have not attempted to fit these data to an integrated rate equation for the mechanism shown in Scheme 2 under conditions where there are, formally, two molecules of **1** in the transition state for rate-determining addition of **3** to **1**. However, the observation that the effective halftime for this reaction increases with increasing reaction time (Figure 1) provides good qualitative evidence for a reaction that is second-order in [**1**].
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