Flow ^{1}H nmr study of the rapid nucleophilic addition of amino acids to 4-formylpyridine

LIANG K. TAN AND MICHAEL COCIVERA[1]

The Guelph–Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ont., Canada N1G 2W1

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Rapid addition of primary amino acids and imidazole to 4-formylpyridine to form carbinolamine intermediates in water has been studied by means of stopped-flow ^{1}H nuclear magnetic resonance spectroscopy. The equilibrium constant K_n for this process was determined using stopped-flow uv spectroscopy. For the amino acid D,L-alanine and its derivatives, D,L-alanine methyl ester and D,L-alanylglycine, K_n does not appear to correlate with K_a, the acid dissociation constant. Values of k_n, the addition rate constant, appear to be independent of pH and buffer concentration for these nucleophiles. The limited data indicate that the variation in rates of nucleophilic addition to 4-formylpyridine cannot be accounted for in terms of pK_a, K_n or related to systematic changes in structure of the nucleophile. As a group, the primary amino acid D,L-alanine and its derivatives have comparable k_n values within a factor of two in spite of their different pK_a values. Imidazole, an aromatic secondary diamine, has a k_n value that is 10 to 20 times larger than these primary amines and has a relatively small K_n value. At the same time, its k_n value is comparable to that found previously for sarcosine, a secondary acyclic amino acid having substantially larger pK_a and K_n values. The nucleophilic addition step of the primary amino acid and derivatives is rapid relative to the subsequent dehydration and prototropic shift steps. It occurs in a pH range at which pyridine nitrogen protonation is negligible.

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Faisant appel à la spectrocopie de rmn du ^{1}H à flux stoppé, on a étudié l'addition rapide des acides aminés primaires et de l'imidazole sur la formyl-4 pyridine en vue d'obtenir les intermédiaires du type carbinolamine dans l'eau. On a déterminé la constante d'équilibre K_n de cette réaction en utilisant la spectroscopie uv découlement à flux stoppé. Dans le cas de D,L-alamine et de ses dérivés, l'ester méthylique de la D,L-alanine et de la D,L-alanylglycine, il n'existe pas de relation en K_n et K_a, la constante de dissociation de l'acide. Les valeurs de K_n, la constante de vitesse d'addition, semblent être indépendantes du pH et de la concentration du tampon de ces nucléophiles. Des données limitées indiquent que la variation de vitesse de l'addition nucléophile sur la formyl-4 pyridine ne peut être reliée à pK_a, à K_n ou à des changements systématiques apparents dans la structure du nucléophile. En tant que groupe, l'acide aminé primaire D,L-alanine et ses dérivés ont des valeurs de k_n qui sont comparables puisqu'elles ne varient pas plus que du simple au double même si les valeurs de leur pK_a sont différentes. L'imidazole, une diamine secondaire aromatique, a une valeur de k_n qui est de 10 à 20 fois plus élevée que celles des amines primaires, alors que son K_n est relativement faible. En même temps, la valeur de k_n est comparable à celle trouvée antérieurement pour la sarcosine, un acide aminé secondaire acyclique ayant des valeurs de pK_a et K_n nettement plus élevées. L'étape d'addition nucléophile de l'acide aminé primaire et des ses dérivés est rapide par rapport aux étapes de déshydratation et de glissement protopiques subséquentes. Elle se produit dans un domaine de pH où la protonation de l'azote de la pyridine est négligeable.

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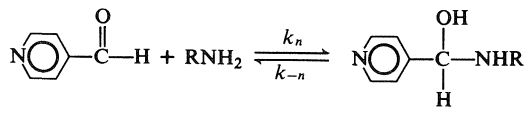
Introduction

Nucleophilic addition of amino acids to 4-formylpyridine (FPY) and its analogues has been used as a model reaction to probe the details of enzymatic reactions involving pyridoxal phosphate (1–3). These studies indicate that imine formation proceeds through a tetrahedral intermediate (1). At high pH equilibration between the aldehyde and this intermediate is rapid relative to the subsequent dehydration step (1, 2) and it has been possible to measure the equilibrium constant for a variety of nucleophiles (2, 4). Measurement of the rate of addition has been reported only for secondary amines for which the reaction with FPY does not proceed beyond the carbinolamine intermediate (2).

In the present paper, we report rate data for the rapid addition of primary amino acids to FPY to form carbinolamine intermediates (CA), which do, in fact, undergo subsequent dehydration. By means of stopped-flow nmr it has been possible to measure the exchange broadened proton nmr resonance of the aldehyde proton of FPY from which k_n and k_{-n} can be calculated for the equilibrium

[1] 

FPY CA

in which RNH_2 is alanine (ALN), alanine methyl ester (AME), and alanylglycine (AGY). The details of the subsequent steps are to be reported in the following paper.

Experimental

Chemicals

4-Formylpyridine (FPY) was vacuum distilled and stored over molecular sieves at −25°C. Imidazole was recrystallized

[1]To whom all correspondence should be addressed.

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twice from benzene. Diazobicyclooctane (DABCO) was sublimed at reduced pressure. Benzyl alcohol, spectrograde acetonitrile, D,L-alanine (ALN), D,L-alanine methyl ester hydrochloride (AME), and D,L-alanylglycine (AGY) were used without purification. The last two compounds were stored at −25°C. The oxazolidine of 4-acetylpyridine was prepared from 4-acetylpyridine (vacuum distilled) and 2-amino-2-methyl propanol-ol as described previously (5). Deionized water was distilled in an all-glass apparatus.

Solutions

In order to avoid possible heavy metal catalyzed reactions of FPY, 1×10^{-4} *M* sodium ethylenediamine tetraacetate was included in all solutions. To reduce the possibility of decomposition of FPY and AME, which are not stable for long periods at high pH, these compounds were added only after the solutions had been brought close to the desired pH value; then the final minor pH adjustments were made. Solutions were buffered with imidazole, phosphate, or DABCO at an ionic strength of 1.3 *M* (KCl). A Radiometer PHM 63 digital pH-meter was used. The pD value was obtained by adding 0.4 to the pH meter reading of the D_2O solution (6).

Exchange reaction

Proton exchange data were obtained at 30°C by measuring the line width of the broadened signal of the aldehyde proton of FPY relative to that for the signal due to the aromatic protons of benzyl alcohol, which was used as an internal reference to monitor changes in chemical shift and magnetic field homogeneity. Stopped-flow was employed to measure the nmr spectra (7, 8) because CA undergoes dehydration at a substantial rate as described in the following paper (8*c*). To conserve material, the line width was measured as a function of time during this dehydration reaction. Consequently, the exchange rate and, therefore, the line width was time-dependent since it is related to the concentration of FPY and amino acid as described below. The concentration of these reactants at the time of each line width measurement was simulated using rate constants and rate equations presented in the following paper (8*c*).

Equilibria

The acid dissociation equilibrium constant K_a for the amino acids in H_2O were measured at 30°C and an ionic strength of 1.3 *M* (KCl) by titration to half neutralization with standardized NaOH. In the case of AME, nmr was used to verify that no significant hydrolysis had occurred during the measurement.

The equilibrium constant for hydration of FPY, K_h ($\mu = 1.3$ *M* KCl) in H_2O and K_h' in D_2O, was determined by measuring the areas of the proton nmr signals due to the aldehyde proton of FPY and the corresponding proton of the hydrate of FPY. For these measurements solutions were buffered with phosphate and DABCO in the pH range 6.5 to 8.7 and in the pD range 8.0 to 9.7.

The equilibrium constant K for the addition step to form carbinolamine was determined at 30 ± 1°C as described previously (4) using a D-110 Durrum stopped-flow spectrophotometer equipped with an oscilloscope. The reaction was followed as a function of time over a period of 20 ms at a wavelength of 280 nm. Extrapolation to zero time gave the absorbance of aldehyde remaining at equilibrium. The absorbances of nucleophiles and CA at this wavelength were also taken into account in this determination.[2] The molar absorptivity of CA is assumed to be the same as that for the structurally similar oxazolidine of 4-acetylpyridine.[3] The pK_a of CA for each nucleophile was estimated according to the previous method (5, 9) by adjusting the pK_a value for the corresponding nucleophile for substituent effects using σ^* values.[4] The pK_a values obtained in H_2O and 5.4, 3.4, and 3.7 for CA derived from ALN, AME, and AGY, respectively. The rate of addition of each amino acid is too fast to be measured by stopped-flow uv. For ALN and AGY, K was determined at pH 10.2 (0.200 *M* DABCO). For AME K was determined at pH 7.5 (0.100 *M* imidazole); at this pH ester hydrolysis is not an important factor during the period of the determination. For each nucleophile four concentrations were employed. The value for K does not take hydration of the aldehyde into account, i.e., K = [CA]/[N] ([aldehyde] + [hydrate]) in which [N] is the nucleophile free base concentration. The equilibrium constant K_n based on eq. [1] is calculated using the expression $K_n = K(1 + K_h)$.

Results and discussion

Figure 1 illustrates the 100 MHz proton nmr spectra in D_2O at pD 9.7 (0.400 *M* DABCO, $\mu = 1.3$ *M* KCl) at 30°C: (*A*) 0.604 *M* FPY; and (*B*) mixture of 0.302 *M* FPY and 0.100 *M* AGY. Only the portion of the spectrum downfield from the HDO signal is illustrated. In Fig. 1*A* the signals labeled *a* and *h* are due to the aldehyde proton of FPY and the corresponding proton of the hydrate of FPY, respectively. The reference signal is labeled *r*. The remaining signals are due to the ring protons of the aldehyde and its hydrate. Note in Fig. 1*B* that signal *a* is broadened but has not shifted. Furthermore, the position and width of signal *h* appears unaffected by the presence of AGY. These results permit two conclusions. First, the equilibrium given by eq. [1] is fast enough to affect the nmr line shape and is in the slow exchange category since the position of signal *a* does not change (10). If the exchange were fast relative to δ, the chemical shift between the two exchanging sites, the signals of the two sites would coalesce to form one signal having a weighted chemical shift (10). Thus in contrast with Fig. 1*B*, the broadened signal should have been observed upfield from signal *a* by about 0.64 ppm,[5] for fast exchange. Second, the hydration equilibrium is slow on the nmr time scale since both the aldehyde and hydrate signals are observed in Fig. 1*A*. Furthermore, the presence of AGY does not increase the line width of the hydrate signal

[2]Molar absorptivities of AME and AGY are found to be 0.04 and 0.19 M^{-1} cm^{-1} respectively at λ 280 nm, μ 1.3 *M* (KCl), and 30°C; molar absorptivity of ALN is negligible under these conditions.

[3]Molar absorptivity of oxazolidine of 4-acetylpyridine is found to be 11.6 ± 1.0 M^{-1} cm^{-1} at λ 280 nm, μ 1.3 *M* (KCl), and 30°C.

[4]Since σ^* for $-CH_2Py$ is not available, it is obtained from pK_a values for 4-(dimethyl amino methyl)pyridine ($pK_a = 7.66$) and trimethylamine ($pK_a = 9.81$), giving a value of 0.65.

[5]This value is calculated assuming the chemical shift for the carbinolamine is about the same as that for the hydrate, as has been found previously (see ref. 8*a*). A comparable value is estimated by the method of Shoolery (see ref. 11).

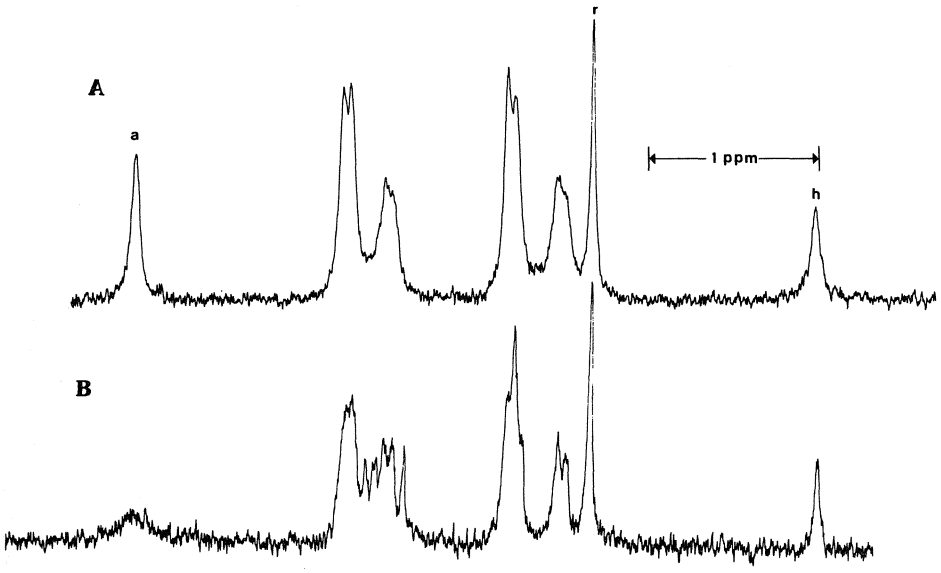

FIG. 1. Partial ^1H nmr spectra at 100 MHz in D_2O at pD 9.7 buffered by 0.400 *M* DABCO at 30°C with 1.3 *M* ionic strength. Magnetic field increases from left to right. About 20% acetonitrile was used as an internal lock signal. *A*. portion of the spectrum downfield from the lock signal for a solution containing 0.602 *M* FPY; *B*. same region for a reaction mixture containing 0.301 *M* FPY and 0.100 *M* AGY initially, scan starts immediately after the flow of the liquid is stopped and takes about 4 min to complete. Assignment of signals: *a*, aldehyde proton of FPY; *h*, corresponding proton for hydrated FPY; *r*, ring protons of benzyl alcohol used as an internal line width and chemical shift reference; unlabeled signals are due to the ring protons of the aldehyde and hydrate forms of FPY in *A*, plus product in *B*. During the measurement of the aldehyde proton resonance very little dehydration has occurred.

and, therefore, does not increase the rates for hydration/dehydration. Consequently, the excess line width of the aldehyde signal has no contribution from the hydration reaction.[6]

As a result, the excess line width observed for the aldehyde signal in D_2O in the presence of AGY appears to be due solely to the addition reaction [1], and k_n and k_{-n} are calculated using the analysis for slow exchange between two sites (5, 8*b*, 10). In this case the excess line width in rad/s is equal to $1/\tau_{FPY}$, and $1/\tau_{FPY} = k_n[AGY]$ since τ_{FPY} is the average lifetime for the aldehyde proton. [AGY] is the concentration of AGY free base. For these calculations, the excess line width has been corrected for variations in magnetic field homogeneity by means of an internal reference line width (benzyl alcohol) as described previously (5). The corresponding signal due to CA is not observed because it is small and broad (about 1/5 the area for the signal due to the aldehyde proton of FPY). Consequently the value for k_{-n} is calculated using the value for K_n and k_n. Consistent with slow exchange is the fact that $\delta\tau$ is 5, in agreement with the requirement that $\delta\tau$ should be about 4 or larger (10).[7]

When H_2O is the solvent, the excess line width again appears to be due to slow exchange for AGY, ALN, and AME addition to FPY. However, at low pH the signal shift calculated on the basis of fast exchange is small (around 0.1 ppm or less) because the concentration of nucleophile free base is low under the conditions employed. Consequently the chemical shift measurements do not provide conclusive information about the exchange rate. As a result, the solvent deuterium isotope effect is used to decide between slow and fast exchange at low pH.

For fast exchange in H_2O, the ratio $(k_n^{H_2O}/k_n^{D_2O})$ is 31 and 25 for AGY at pH 7.4 and 7.5, respectively.[8] There appears to be no precedent for such large

[6]On the other hand, this hydration reaction is fast relative to the dehydration of CA, i.e., both the addition and hydration equilibria for FPY are maintained during the dehydration of CA.

[7]For this calculation, the value for δ is 2520 rad/s (see footnote 5) and τ is calculated using $1/\tau = k_n[AGY] + k_{-n}$ (see refs. 5 and 10).

[8]For this calculation, the chemical shift δ between the signals of the two sites is estimated as described above. The uncertainty in the chemical shift is unlikely to exceed 0.5 ppm. Consequently, the error in k_n for H_2O solvent is unlikely to exceed 25%. The line width for CA in the absence of exchange is assumed to be equal to the one for the hydrate of FPY since aldehyde–hydrate exchange is slow at this pH.

solvent deuterium isotope effects. For example, for the analogous reaction involving hydrolysis of *meta*- and *para*-substituted 2,2,2-trifluoro-*N*-methylacetanilides via a carbinolamine intermediate none of the various steps in the mechanism is found to have a solvent isotope effect that exceeds 3.0 (12). Similar results are found for hydrolysis of *para*-substituted benzoylimidazoles (12). Consequently, when H_2O is the solvent it appears that the excess line width in the presence of AGY is due to slow exchange rather than fast exchange. In view of the close similarity in structure between the three nucleophiles, we conclude that the slow exchange interpretation also applies at low pH for ALN and AME.

Imidazole also undergoes nucleophilic addition to FPY; however, the rate is fast relative to the chemical shift δ between the signals due to the two exchanging sites because the line width and the position of the broadened signal depend on the concentration of imidazole at pH 8.2. Because the equilibrium constant for the addition appears to be small and the concentration of imidazole could not be increased conveniently above 0.8 *M*, only a semiquantitative value for K_n and δ were obtained by successive approximation using eq. [1] and $\delta_C - \delta_{FPY} = p\delta$ in which p is the proton fraction for CA and δ_C is the chemical shift of the coalescence signal. As a result, the values for k_n must also be considered to be semiquantitative.

To determine k_n for the amino acid nucleophiles when imidazole is used to buffer the solutions, it is necessary to include the effect of the imidazole equilibrium on the line width of the aldehyde signal as well as on the concentration of the amino acid free base. Because nucleophilic substitution at the tetrahedral carbon atom of CA is expected to be substantially slower than nucleophilic addition to the aldehyde carbon, addition by amino acid and imidazole can be treated as separate competitive exchange processes. Consequently, the contribution to the line width made by each exchange process is additive (10). This consideration also applies to the aldehyde–hydrate exchange, whose contribution increases as the pH increases. As discussed above, to calculate k_n from this excess line width, it is necessary to know the concentration of amino acid free base in equilibrium with its corresponding CA. In the presence of imidazole, this concentration was calculated by including the imidazole addition equilibrium as well as the two acid–base equilibria. If the concentration of amino acid free base is not adjusted in this manner, the k_n value calculated from the excess line width is about 18 and 26% larger than the ones listed in Table 1 for AGY and AME, respectively.

Values for K_n, k_n, and k_{-n} are listed in Table 1 along with the pK_a for each nucleophile. As has been found previously, there appears to be no direct correlation between K_n and K_a (4, 5). In agreement with our $K_n/(1 + K_h)$ values of 2.3[9] and 0.3 M^{-1} for ALN and imidazole, respectively,[10] Sander and Jencks measured values of 2.1[9] and 0.3 M^{-1}, respectively (4).

Values of k_n listed in Table 1 are calculated using a linear dependence between $1/\tau$ and the concentration of nucleophile. Temperature jump studies of the addition of sarcosine and piperazine to FPY indicate both a linear and nonlinear term for $1/\tau$ (2); however, a ten-fold variation in nucleophile concentration was possible in these studies, and the nonlinearity becomes apparent at the high concentrations. In the present study a maximum of only about a four-fold variation in concentration was possible because of the limitations in accuracy of nmr line width measurements for this system. Within these limitations, a nonlinear term was not observed. In addition, the nature and concentration of the buffer appear to have no effect on the rate of addition. Thus, varying the concentration of imidazole or DABCO from 0.4 *M* to 0.8 *M* had no effect on the value of k_n for the amino acid nucleophiles. Likewise comparison of the results for phosphate vs. imidazole or imidazole vs. DABCO in Table 1 indicates no dependence on the nature of the buffer. Similar results have been found previously for the addition of various α-nucleophiles to a variety of ketones (5, 7, 8*b*).

Correlation between k_n or k_{-n} and K_a or K_n for the various nucleophiles is not apparent in Table 1. Considering only the amino acids including sarcosine for which the pK_a is 10.03 and K_n is 5.0 M^{-1} (2*b*), the rate constants again do not appear to correlate with either K_a or K_n. Thus a plot of log k_n or log k_{-n} vs. pK_a or log K_n is not linear. For this consideration, we calculate an average k_n value of about 5×10^4 M^{-1} s^{-1} for sarcosine assuming $1/\tau$ has a linear dependence on the nucleophile concentration for data at pH 10.65 (2*b*).

This value of k_n for sarcosine is about 10 times the one for ALN despite the fact that both amino

[9]These values differ from the one obtained kinetically (see ref. 1*a*).

[10]Although there are several reports concerning K_h none refers to the conditions employed in our study. Furthermore, there is quite a spread in values, ranging from 1.28 to 0.67 (see refs. 2*a*, 4, and 14). For these reasons, we have determined the value listed in Table 1 as described above.

TABLE 1. Equilibrium and kinetic data for nucleophilic addition to 4-formylpyridine at 30°C and $\mu = 1.3\ M$ (KCl)

Nucleophile[a]	pK_a	K_n (M^{-1})	pH[b]	Buffer	$k_n \times 10^{-3}$ [c] ($M^{-1}\ s^{-1}$)	$k_{-n} \times 10^{-3}$ (s^{-1})
		Solvent H_2O				
Alanylglycine	8.28	4.7±0.7[d]	7.5	Phosphate	2.1±0.3	0.45
			7.7	Imidazole	2.0±0.2	0.43
			9.2	DABCO	2.3±0.4	0.49
Alanine methyl ester	8.05	1.8±0.1[e]	6.7	Imidazole	2.0±0.4	1.1
			7.0	Imidazole	1.5±0.3	0.83
			7.3	Imidazole	1.5±0.3	0.83
			8.0	Imidazole	2.0±0.4	1.1
Alanine	10.02	4.4±0.7[d]	8.1	Imidazole	5.7±0.8	1.3
			8.2	DABCO	4.9±0.9	1.1
			9.2	DABCO	4.1±0.7	0.93
Imidazole	7.24	0.6[f]	7.0		40	69
			7.8		35	61
			8.2		31±7	52
H_2O		0.93±0.1[g]				
		Solvent D_2O				
Alanylglycine	8.84[h]	3.94[i]	7.5[j]	Phosphate	2.4±0.4	0.54
			9.7	DABCO	1.4±0.2	0.35
D_2O		0.63±0.1[k]				

[a]FPY aldehyde concentration, 0.07 to 0.18 *M*. Nucleophile total concentration after mixing, including all degrees of protonation had values between 0.02 and 0.09 *M* for k_n measurement. Total buffer concentrations: 0.20 *M* phosphate; 0.4 to 0.8 *M* DABCO and imidazole includes all degrees of protonation.
[b]pH after mixing. Value varied only by 0.02 unit during dehydration.
[c]Average of 5 to 12 determinations over various buffer concentrations as indicated in footnote *a*.
[d]Average of four determinations in the concentration range 0.1 to 0.3 *M* nucleophile including all degrees of protonation.
[e]Average of four determinations in concentration range 0.1 to 0.4 *M* nucleophile including all degrees of protonation.
[f]Estimated from the effect of imidazole concentration on chemical shift of the coalescence signal, see text. The value of δ used to calculate k_n is estimated to be 2.90 ppm.
[g]K_h = [hydrate]/[aldehyde], average of 18 determinations in the pH range 6.5 to 8.7.
[h]Calculated using pK_a in H_2O and the relation $pK_a(D_2O) = pK_a(H_2O) + 0.56$ found for the anilinium ion (see refs. 6 and 13).
[i]Calculated as $K(1 + K_h')$ in which K_h' is the hydration constant in D_2O solvent.
[j]pD. Average of pD 7.4 and 7.5 data.
[k]Average of five determinations in the pD range 8.0 to 9.7.

acids have nearly identical values for pK_a and K_n. On the other hand, piperazine, whose second pK_a is about the same as the one for sarcosine, has been found to add to FPY 20 to 40 times faster than sarcosine with a K_n of about 10 times the value for sarcosine (2). Comparison of the data for AGY indicates that there is little or no solvent deuterium isotope effect. This result is similar to that for the analogous reaction in which hydroxide ion adds to substituted *N*-methyltrifluoroacetanilides to form a tetrahedral intermediate. For this system the solvent (H_2O/D_2O) deuterium isotope effect is about 1.3 for the addition step (12).

In summary the limited data for the rates of nucleophilic addition to FPY indicate that the variations cannot be accounted for in terms of pK_a, K_n, or related to systematic changes in structure of the nucleophile. As a group, the primary amino acid and its derivatives, ALN, AME, and AGY, have comparable k_n values within a factor of 2 in spite of their different pK_a values. Imidazole, an aromatic secondary diamine, has a k_n value that is 10 to 20 times larger than these primary amines and has a relatively small K_n value. At the same time, its value is comparable to that for sarcosine, a secondary acyclic amino acid having substantially larger pK_a and K_n values. But the sarcosine rates are 20 to 40 times smaller than those for piperazine, a cyclic, nonaromatic diamine (2). In view of these variations in rates with structure of the nucleophile, it seems that primary amino acids are more suitable for model studies of the details of the addition step involving pyridoxal phosphate. Since the pK_a of FPY is 4.77 (15), the rapid rate of addition of the primary amino acid and derivatives to FPY at high pH could mean that protonation of the ring nitrogen of pyridoxal in the apoenzyme of transaminase need not be a requirement for the addition step. Subsequent protonation may occur prior to the prototropic shift as has been suggested earlier (1*d*). The details of the subsequent steps involving these amino acids and FPY are to be reported in the following paper.

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