Rates and Equilibria of Aldimine Formation between Pyridoxal 5'-Phosphate and *N*-Hexylamine

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The rate and equilibrium constants of the reaction between n-hexylamine and pyridoxal 5'-phosphate have been studied in aqueous solution as a function of pH at 25 ± 0.1 °C and at an ionic strength of 0.1. The pH profiles of the observed constants have been explained by protonation of the pyridoxal 5'-phosphate and its Schiff's base. This study shows that there is an intramolecular general acid catalysis of the Schiff's base formation, which can explain why the aldimine is formed at a reasonably high rate at neutral pH, in spite of the low amount of unprotonated reacting amine at this pH.

The study of the equilibrium constants of aldimines formed between pyridoxal 5'-phosphate (PLP) and primary amines or amino-acids has been mainly carried out by Metzler and his co-workers.[1] A detailed kinetic study, however, has not yet been made, although there are some data at some pH values [2-5] which could not be analysed in terms of the rate constants for individual reaction. The only available set of data corresponding to the rate constants of individual reactions appeared in a communication on the rate of carbinolamine formation in the reaction between PLP and alanine in the presence of Cu^{2+} ions.[6] Here we describe the results of a study of the reaction of PLP with n-hexylamine as a function of pH, in which we have found evidence for an intramolecular acid catalysis.

Results and Discussion

The results obtained for the rate constants of aldimine formation and hydrolysis, k_1 and k_2, respectively, are given as a function of pH in Figures 1 and 2. The ionic forms existing in solution in the pH range studied (above pH 4) are given in Scheme 1, in which P and B indicate PLP and its aldimine, respectively. The subindices (0—3) indicate the number of the net negative charges on the molecules. The solid lines in Figures 1 and 2 are those obtained from the theoretical model for Scheme 1 (see Experimental section) using the values shown in Table 1.

A progressive increase of k_1^1 can be observed on the successive addition of protons to the PLP molecule (Table 1) which could be due to an intramolecular proton catalysis. This type of catalysis occurs in the reaction of acetone with amines bearing acidic substituents.[7] The intramolecular catalysis in the dehydration of similar carbinolamines (the rate-limiting step, see ref. 6) has been attributed only to the 3-hydroxy-group.[6,8,9] A plot of log k_1^i values *versus* the corresponding pK values of the PLP molecule, however, (Figure 3) appears to indicate that the other protonable groups of the molecule also catalyse the reaction. This ' Brönsted-like ' plot is given in Figure 3 with a slope α of 0.57. The values for rate constants of other imines (obtained under slightly different conditions) of PLP and 3-hydroxypyridine-4-carbaldehyde appear to be fitted in a similar mode (Figure 3). The higher value in these latter cases (α 0.8) might be expected due to the differences in the nucleophilic character of the amine (n-hexylamine in our case, pK 10.7; other amines in Figure 3, pK 9.3—9.6).

Other reasons can be adduced to explain the variation observed in Figure 3. An intermolecular catalysis by H_3O^+ can be ruled out because the rate constants calculated for such a mechanism are greater than 10^{10} l^2 mol^{-2} min^{-1}. Furthermore, neither the buffer nor the amine itself was observed to give rise to general acid catalysis (see Experimental section).

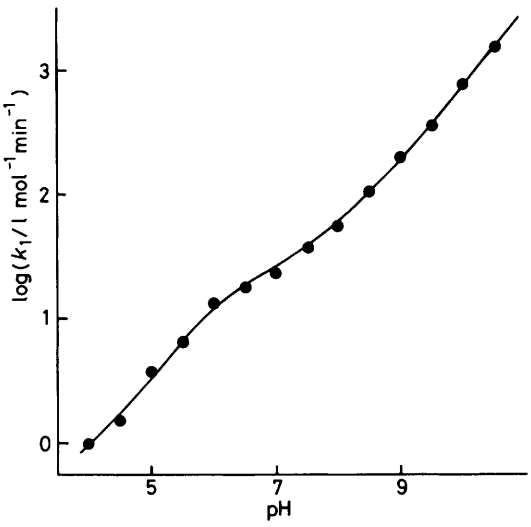


Figure 1. Dependence of logk_1, the apparent second-order rate constants for aldimine formation, on pH: ●, experimental values of log k_1. The line is calculated from equation (4) and the values for constants given in Tables 1 and 2

A similar observation was made by Auld and Bruice [8,9] in the case of aldimine formation from 3-hydroxypyridine-4-carbaldehyde. The variation in k_1^1 is too large (four orders of magnitude) to be explained solely by electrostatic factors. There is no correlation of k_1^1 with the charges on the amine, aldehyde, or carbinolamine. Intramolecular catalysis appears to be the only possible explanation for the effect of the phosphate group protonation. Moreover, as might be expected, the protonation of this group has no influence on the electronic spectra, thus indicating that the possible effects on the electronic distribution are negligible.

Nevertheless, the fact that 3-*O*-methylpyridoxal phosphate reacts with amines at a slower rate [10] indicates that the 3-hydroxy-group is very important for intramolecular catalysis. Arhens *et al.* have shown the existence of very fast intramolecular proton transfers among the three protonable groups in PLP.[11] This situation could also be expected for its Schiff's bases and for the carbinolamine. In this case, each new proton bound to the molecule would be proportionally shared by all the protonable groups according to the tautomeric equilibria. This pool of protons could then catalyse the reaction by a protonation of the hydroxy-group in the 4'-position of the carbinolamine through the intermediate protonation of the 3-hydroxy-group. The result is a proton

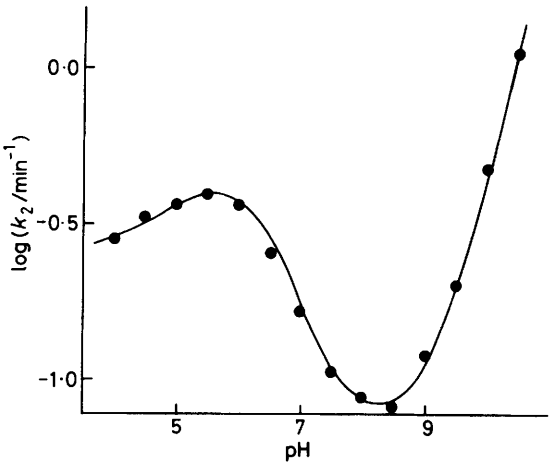
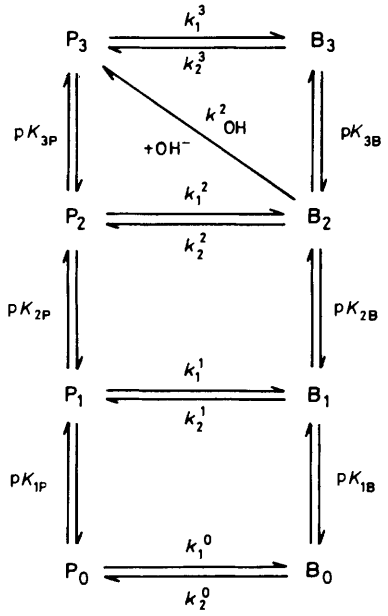


Figure 2. Dependence of logk_2, the apparent first-order rate constants for aldimine hydrolysis, on pH: ●, experimental values of log k_2. The line is calculated from equation (5) and the values for the constants given in Tables 1 and 2



Scheme 1.

transfer from the pool of protons to the reaction centre. Auld and Bruice discussed three probable mechanisms for the catalysis of the 3-hydroxy-group,[8] and thus it appears to us that the mechanism given in Scheme 2 is most probable.

The equilibrium constants, K_{pH}, have also been determined and are shown in Figure 4 with the theoretical lines using the pK values given in Table 2 (see Experimental section). These pK values agree reasonably well with those previously published, considering the slightly different conditions and methods of determination.[1,11-17] The only apparent discrepancy is the low value obtained for pK_{1B}, 5.0, compared with those published by other authors for other PLP aldimines formed with amino-acids (5.6—6.0 [1,12]). A potentiometric titration at higher ionic strength (1.0) gave a similar value, 5.1. Although the reason for this low value is unknown the local electrostatic effects of the carboxylate group of the amino-acid may explain it.

Table 1. Best kinetic constants obtained in the fitting of k_1 and k_2 experimental values to equations (4) and (5)

log k_1^0 7.00	log k_2^0 −0.57
log k_1^1 6.10	log k_2^1 −0.33
log k_1^2 4.60	log k_2^2 −1.18
log k_1^3 3.62	log k_{OH} +0.84

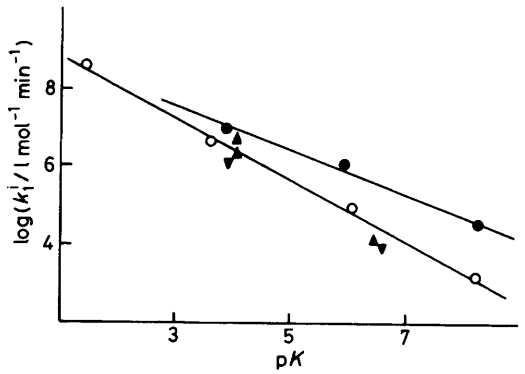
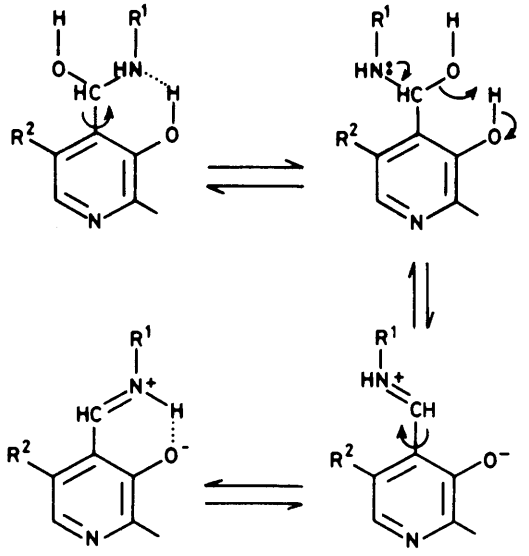


Figure 3. Plot of log k_1^1, the second-order rate constants for the imine formation from several species of PLP and 3-hydroxypyridine-4-carbaldehyde, *versus* pK of the species: ●, PLP + n-hexylamine, I 0.1, T 25 °C (this work); ○, PLP + alanine, I 0.5, T 25 °C.[6] pK Values of PLP at these conditions were taken from ref. 16. ▼, 3-hydroxypyridine + alanine, I 1, T 30 °C (ref. 8); ▲, 3-hydroxypyridine + other amino-acids, I 1, T 30 °C (ref. 9)



Scheme 2.

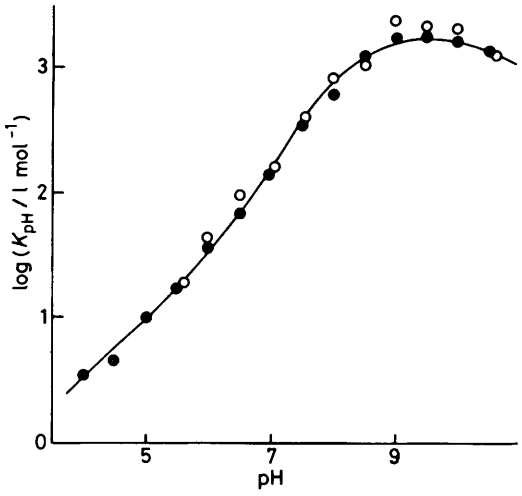


Figure 4. Equilibrium constants *versus* pH profile for aldimine formation from PLP and n-hexylamine. ●, Values calculated as k_1/k_2; ○, values obtained from $A_0/(A_\infty - A_0)$ *versus* $1/b_e$ plots. The line is calculated from equation (6) and the parameters given in Table 2

Table 2. pK Values used in the curve fitting, range of variability allowed, and the final values obtained

	Initial	Range	Final
pK_{1P}	3.6	3.4—3.9	3.86
pK_{2P}	6.0	5.9—6.2	5.95
pK_{3P}	8.3	8.1—8.7	8.21
pK_{1B}	5.1	4.9—5.3	5.00
pK_{2B}	6.3	6.1—6.7	6.60
pK_{3B}	11.7	11—12.5	11.21
pK_N	10.5	10.3—10.8	10.70
log K_M	1	0—5	2.78

Finally we should mention that the large change (from pK_{3P} 8.2 to pK_{3B} 11.2) in the highest pK has been explained by the existence of an intramolecular hydrogen bond in the case of aldimine (1). This means that this aldimine can be formed in high proportion at neutral pH [see equation (6)]. Those aldimines of analogous compounds without the 3-hydroxy-group should be formed at a lower proportion due to the fact that significant changes in this pK could not be expected. The existence of intramolecular catalysis in PLP allows the Schiff's bases to be formed at a relatively high rate at pH values 3 or 4 units lower than the pK of the corresponding amine. Many enzymes in which PLP is an essential co-factor would then make use of both the high proportion of aldimine and high rate of aldimine formation at neutral pH, characteristics that are rarely found in the aldimines of other aldehydes. These findings, together with the possible occurrence of subsequent prototropies, might explain why, during evolutionary processes, this vitamin has seldom been replaced in metabolic pathways.

Experimental

The material and methods are described in detail in ref. 18. The experiments were carried out in buffer systems at a concentration of 0.010M, maintaining a constant ionic strength of 0.1 by addition of KCl. Preliminary experiments were carried out to exclude the possible influence of general acid–base catalysis on this reaction by the buffer systems.[18]

$$R^1CHO + R^2NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R^1CH{=}NR^2 + H_2O \qquad (1)$$

We followed the reaction by observing the increase of the absorbance at the maximum of the absorption band of the aldimine (415 nm). The temperature was kept at 25 ± 0.1 °C.

The k_{obs} experimental data for reaction (1) were obtained from the slopes of the plots of ln $(A_\infty - A)$ *versus* time. The correlation coefficients r of these plots were always >0.999. The values of k_1 and k_2 were calculated from the k_{obs} values obtained at several amine concentrations by using the equation (2) [18] (r >0.99), which was deduced assuming that

$$k_{obs} = \{[k_2 + k_1(a + b)]^2 - 4abk_1^2\}^{\frac{1}{2}} \qquad (2)$$

$ab \gg xx_e$ where a and b are the initial concentrations of aldehyde and amine respectively and x and x_e are the aldimine concentration at times t and ∞, respectively. That equation includes the pseudo-first-order one ($k_{obs} = k_2 + k_1 b$) for the case in which $b \gg a$.

The equilibrium constants at each pH, K_{pH}, were determined in two ways: (a) as the ratio k_1/k_2 and (b) using equation (3) [8] where ε_p and ε_b are the molar absorptivities of the

$$\frac{A_0}{A_\infty - A_0} = \frac{\varepsilon_p}{\varepsilon_b - \varepsilon_p} + \frac{\varepsilon_p}{(\varepsilon_b - \varepsilon_p)K_{pH}} \frac{1}{b_e} \qquad (3)$$

aldehyde and Schiff's base, respectively, at this pH and wavelength, and b_e the amine concentrations at equilibrium.

The experimental rate constants of formation and hydrolysis of the aldimine can be described as a function of the rate constants for the individual ionic species. We have followed the approach of ref. 8 for aldimine rate formation from 3-hydroxypyridine-4-carbaldehyde. From this model (see Scheme 1) equations (4) and (5) can be easily obtained [18]

$$k_1 = \frac{k_1^3 + k_1^2 \frac{a_H}{K_{3P}} + k_1^1 \frac{a_H^2}{K_{2P}K_{3P}} + k_1^0 \frac{a_H^3}{K_{1P}K_{2P}K_{3P}}}{\left(1 + \frac{a_H}{K_N}\right)\left(1 + \frac{a_H}{K_{3P}} + \frac{a_H^2}{K_{2P}K_{3P}} + \frac{a_H^3}{K_{1P}K_{2P}K_{3P}}\right)} \qquad (4)$$

$$k_2 = \frac{k_{OH} + k_2^2 \frac{a_H}{K_{3B}} + k_2^1 \frac{a_H^2}{K_{2B}K_{3B}} + k_2^0 \frac{a_H^3}{K_{1B}K_{2B}K_{3B}}}{1 + \frac{a_H}{K_{3B}} + \frac{a_H^2}{K_{2B}K_{3B}} + \frac{a_H^3}{K_{1B}K_{2B}K_{3B}}} \qquad (5)$$

where $a_H = 10^{-pH}$, $k_{OH} = k_2^3 + k^2{}_{OH} \frac{P_w}{K_{3B}}$, and P_w is the ionic product of water.

Finally equation (6) was used to fit the K_{pH} values.[19] The

$$K_{pH} = \frac{\left(1 + \frac{a_H}{K_{3B}} + \frac{a_H^2}{K_{2B}K_{3B}} + \frac{a_H^3}{K_{1B}K_{2B}K_{3B}}\right) K_M}{\left(1 + \frac{a_H}{K_N}\right)\left(1 + \frac{a_H}{K_{3P}} + \frac{a_H^2}{K_{2P}K_{3P}} + \frac{a_H^3}{K_{1P}K_{2P}K_{3P}}\right)} \qquad (6)$$

experimental data were fitted to equations (4)—(6) with the aid of a desk microcomputer. The procedure for curve fitting was analogous to that described by Auld and Bruice.[8] We started adjusting the K_{pH} experimental data to equation (6). The three pK values of PLP given in the literature vary to some extent.[11,13-16] We started with the initial values given in Table 2, allowing a variation within the range given in Table 2.

A rough estimation of the Schiff's base pK values was made by direct potentiometric titration for pK_{1B} and pK_{2B} and by a spectrophotometric one for pK_{3B}. The final values obtained in the adjustment are also given in Table 2. The value obtained for log K_M was 2.78.

With these values we started fitting simultaneously the experimental data for k_1 and k_2 to equations (4) and (5), respectively, because there are only four (among eight) independent rate constants. The kinetic constants obtained are given in Table 1. These values and the final values shown in Table 1 were used to compute the theoretical lines given in Figures 1, 2, and 4.

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