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# Determination of the rates of formation and hydrolysis of the Schiff bases formed by 5′-deoxypyridoxal and poly-L-lysine

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**Abstract**

The kinetics of the reaction between 5′-deoxypyridoxal (DPL) and poly-L-lysine in aqueous solutions at a variable pH and a constant ionic strength of 0.1 M was studied spectrophotometrically. The rate constants of formation and hydrolysis of the resulting Schiff base and its stability constant at a variable pH were also determined. A comparison of the formation rate constant for the Schiff base with those for the models of pyridoxal 5′-phosphate (PLP) with poly-L-lysine and DPL with *n*-hexylamine revealed the effect of both the phosphate group of PLP and of the polypeptide chain on intramolecular catalysis in the dehydration of the intermediate carbinolamine formed. The effects of the polypeptide and the phosphate group on the hydrolysis of the Schiff base and hence on the base stability are also demonstrated.

*Keywords:* Schiff base; 5′-deoxypyridoxal; Poly-L-lysine; Hydrolysis

## 1. Introduction

The term vitamin B-6 is used to designate several 3-hydroxy-2-methylpyridine derivatives, foremost among which is pyridoxal 5′-phosphate (PLP). In fact, PLP is a required coenzyme for a number of reactions involved in $\alpha$-aminoacid metabolism including transaminations, decarboxylations, $\alpha,\beta$-eliminations, etc. In all these processes, the coenzyme is an essential part of the active site of the enzyme involved, as shown by the fact that the reactions are catalyzed by PLP without the need for an apoenzyme [1].

As a rule, PLP binds to enzymes to form a Schiff base (imine) by reaction of its aldehyde carbonyl group with the terminal $\varepsilon$-amino group of a lysine residue in the polypeptide chain. This reaction yields a tetrahedral intermediate, a carbinolamine, which is subsequently dehydrated to the corresponding imine [2–4]. Both steps are reversible and subject to general acid–base catalysis [5]. In addition, the dehydration of PLP-related carbinolamines exhibits intramolecular acid catalysis [6–8].

Elucidating the enzymatic process at the molecular level and characterizing the proper-

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ties of the Schiff bases of PLP with aminoacids and proteins are essential with a view to understanding the role of this coenzyme in enzyme-catalyzed process; however, such a comprehensive characterization is elusive and has compelled researchers to develop model systems simulating the biological behaviour of PLP. The most simple possible model for the binding of PLP to proteins is the PLP–hexylamine system, which has been studied in relation to the formation and hydrolysis of the corresponding Schiff base, as well as in terms of stability in both aqueous (water [6–8], ethanol–water [9], water–dioxane [10]) and non-aqueous media (pure or mixed solvents [11,12]). This model cannot be used to account for the enzymatic behaviour of PLP, however.

In search of a more faithful enzymatic model, the kinetics of formation and hydrolysis, and the stability of Schiff bases of PLP with L-lysine homopolymers (the PLP–LYS system [13]) and copolymers of the same aminoacid [14] have been investigated. The ensuing model has shown the Schiff bases formed to be highly stable as the likely result of specific interactions with the polypeptide chain and/or the phosphate group at 5′ [15].

In this work, the formation and hydrolysis of the Schiff bases of 5′-deoxypyridoxal (DPL) and poly-L-lysine (the DPL–LYS system) in an aqueous medium were studied. DPL was chosen because it makes a suitable model for $\alpha$-aminoacid metabolic reactions as it possesses the three allegedly essential chemical groups for catalytic activity (–CHO, –OH and =N–). Also, because it lacks the phosphate group at 5′, it does not allow its Schiff base to be stabilized by the same mechanism as PLP.

## 2. Experimental

5′-deoxypyridoxal was synthesized from pyridoxine hydrochloride (Merck) using the method reported by Iwata [16]. Poly-L-lysine was purchased from Sigma Chemical at a viscome-trically determined molecular weight of 141 000 Da (DP = 675). All other reagents were Merck p.a. grade chemicals.

The solution pH was kept constant by using 0.01 M acetate, phosphate and carbonate buffers. The ionic strength was adjusted to 0.1 M with KCl in every case. DPL solutions were prepared daily in the required buffer and stored in the dark. Their exact concentrations were determined from absorbance measurements at 390 nm ($\varepsilon = 6300$ dm$^3$ mol$^{-1}$ cm$^{-1}$) following dilution in 0.1 M NaOH [17]. The working concentrations ranged from $3 \cdot 10^{-5}$ to $7 \cdot 10^{-5}$ M. Poly-L-lysine solutions were also made daily at concentrations from $7 \cdot 10^{-4}$ to $1 \cdot 10^{-2}$ M by dissolving an appropriate amount of polymer in the selected buffer.

The kinetics of formation of the Schiff bases was monitored spectrophotometrically via absorbance changes at 280 nm as a function of pH that were recorded on a Perkin-Elmer spectrophotometer equipped with a thermostated cell compartment. The imines were formed by adding 20 $\mu$l of DPL solution to 3 ml of a polypeptide solution of preset concentration and pH (both were previously thermostated at 25 ± 0.05°C). pH measurements were made with a Crison pH-meter equipped with a Metrohm EA 120 electrode that was previously calibrated using aqueous buffers at 25°C. The difference between the initial and final reaction pH was always less than 0.03 pH units.

The reaction between an aldehyde and an amine can be schematized as follows:

$$R_1\text{–CHO} + NH_2\text{–}R_2 \underset{k_2}{\overset{k_1}{\rightleftarrows}} R_1\text{–CH=N–}R_2 + H_2O$$

where $k_1$ and $k_2$ are the macroscopic formation and hydrolysis constant, respectively, for the Schiff base. The values of both constants and that of the equilibrium constant ($K_{pH} = k_1/k_2$) for the Schiff bases of DPL and poly-L-lysine were obtained by using the same procedure as for the reaction with *n*-hexylamine [7]. Table 1 gives the p$K$ values and those of the microscopic rate constants, $k_1^i$ and $k_2^i$, obtained by

Table 1
Best kinetics constants and p$K$ obtained in the fitting experimental $k_1$, $k_2$ and $K_{pH}$ values

| | DPL–NHA [a] | DPL–LYS | PLP–LYS [b] |
|---|---|---|---|
| Log $k_1^0$ | 7.30 | 7.20 | 8.74 |
| Log $k_1^1$ | — | — | 6.13 |
| Log $k_1^2$ | 4.80 | 4.60 | 5.45 |
| Log $k_1^3$ | 3.92 | 4.50 | 3.53 |
| Log $k_2^{-1}$ | −1.12 | 1.05 | — |
| Log $k_2^0$ | −0.48 | −0.75 | −0.17 |
| Log $k_2^1$ | — | — | −2.29 |
| Log $k_2^2$ | −0.90 | −0.57 | −0.42 |
| Log $k_{OH}$ | 1.93 | 0.15 | 1.04 |
| p$K_{1P}$ | 4.12 | 4.14 | 3.46 |
| p$K_{2P}$ | — | — | 6.02 |
| p$K_{3P}$ | 7.98 | 7.98 | 8.22 |
| p$K_N$ | 10.75 | 10.03 | 10.03 |
| p$K_{0B}$ | 3.39 | 3.24 | — |
| p$K_{1B}$ | 6.23 | 6.68 | 6.62 |
| p$K_{2B}$ | — | — | 7.74 |
| p$K_{3B}$ | 11.69 | 9.21 | 10.92 |

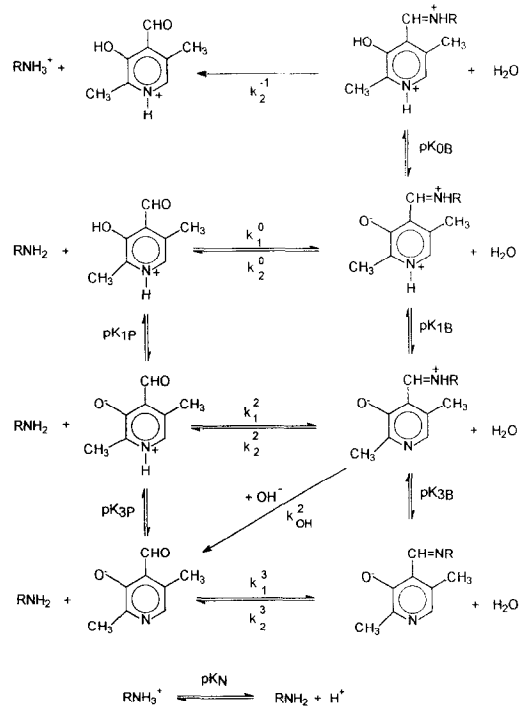
[a] Taken from Ref. [8].
[b] Taken from Ref. [14].

fitting the experimental $k_1$ and $k_2$ values to the equations derived from Scheme 1. The table also shows the p$K$ values and those of the microscopic rate constants for the DPL–*n*-hexylamine (DPL–NHA) system, which conforms to Scheme 1, and for the PLP–poly-L-lysine system, which conforms to Scheme 2. In the table $k_{OH} = k_2^3 + k_{OH}^2 (K_W/K_{3B})$ and $K_W$ is the ionic product of water.

## 3. Results and discussion

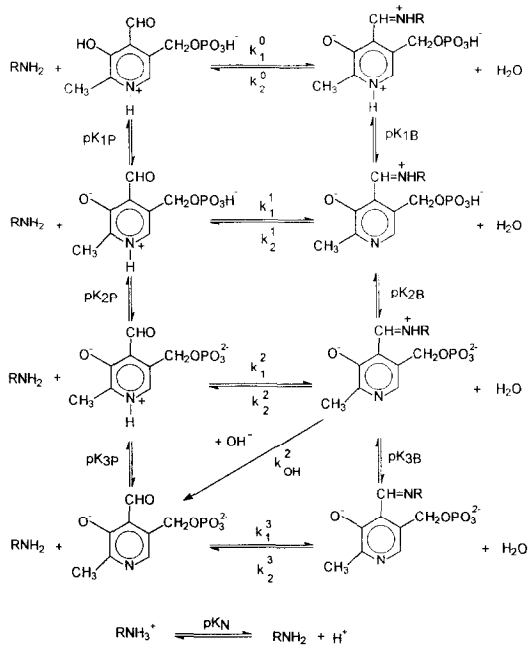
Figs. 1–3 show the values of the rate constants of formation ($k_1$) and hydrolysis ($k_2$), as well as the equilibrium constant ($K_{pH}$), as a function of pH for the reaction between 5′-deoxypyridoxal and poly-L-lysine. They include the results obtained for the reactions of PLP with poly-L-lysine [13] and of DPL with *n*-hexylamine [8], both at the same temperature and ionic strength.

As can be seen in Fig. 1, $k_1$ increased with increasing pH, with a sharp break at about pH 8.5 that can be ascribed to the change in poly-




Scheme 1.

L-lysine from the statistical conformation to the $\alpha$-helix chain [18]. Also, $k_2$ was minimal at pH 6–7 (Fig. 2). These results, similar to those




Scheme 2.

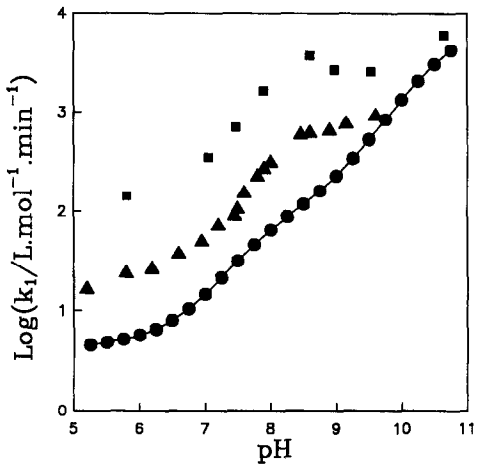



Fig. 1. Plot of $\log k_1$ versus pH for the Schiff bases of 5′-deoxypyridoxal with poly-L-lysine (▲), 5′-deoxypyridoxal with *n*-hexylamine (●) [8], and pyridoxal 5′-phosphate with poly-L-lysine (■) [13].

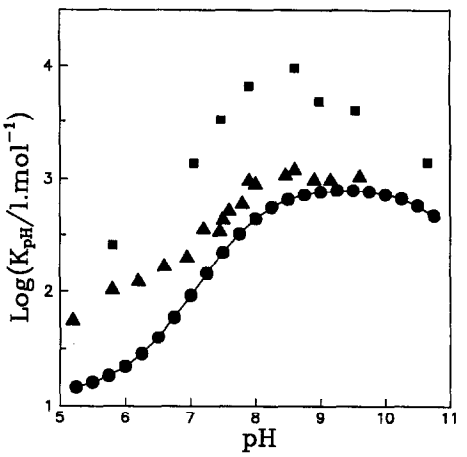



Fig. 3. Plot of $\log K_{pH}$ versus pH for the Schiff bases of 5′-deoxypyridoxal with poly-L-lysine (▲), 5′-deoxypyridoxal with *n*-hexylamine (●) [9], and pyridoxal 5′-phosphate with poly-L-lysine (■) [13].

previously reported for other polymer systems [13–15], arise from the distribution of molecular species in solution, which in turn is dictated by the protonation of the different groups of 5′-deoxypyridoxal (see Scheme 1).

As can also be seen (Fig. 1), the $k_1$ values obtained in this work always exceeded those for the DPL–NHA system. The rate-determining step is the dehydration of the intermediate carbinolamine, so the observed differences in $k_1$ must have originated from differences in the rate of dehydration to the corresponding carbinolamines. Because the dehydration is subject to intramolecular acid catalysis, the presence of protonated groups in the polypeptide skeleton and/or side chains (e.g. groups in the side chains of poly-L-lysine or potential hydrogen bonding between the peptide bond and the solvent) may favor catalysis.

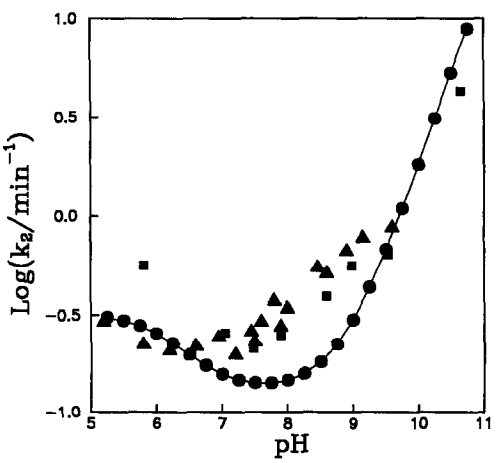



Fig. 2. Plot of $\log k_2$ versus pH for the Schiff bases of 5′-deoxypyridoxal with poly-L-lysine (▲), 5′-deoxypyridoxal with *n*-hexylamine (●) [8], and pyridoxal 5′-phosphate with poly-L-lysine (■) [13].

The similarity between $k_1$ values above pH 10 can be ascribed to the disappearance of the intramolecular catalytic groups ($-NH_3^+$) and hydrogen bonds potentially formed; the effect of the polypeptide skeleton and its side chains loses significance and the model approaches that for the DPL–NHA system. On the other hand, a comparison of our $k_1$ values with those for the PLP–LYS system reveals the effect of the phosphate group on the formation of the Schiff base: that of PLP with poly-L-lysine has a rate constant roughly one order of magnitude greater at any pH. This effect, which could not be observed in comparing the same two aldehydes with *n*-hexylamine [8], suggests an interaction between the phosphate group and the polypeptide by which the dehydration of the intermediate carbinolamine is catalyzed.

The increase in the values of the microscopic rate constants $k_1^i$ for the DPL–LYS system with increase in the medium acidity (Table 1) sup-

ports the occurrence of an intramolecular acid catalysis process that involves the protonating groups of DPL. A Brönsted graph for the process has a slope of $\alpha = 0.69$, which is similar to those for related systems, viz. 0.68 for DPL–NHA [8], 0.57–0.68 for PLP–*n*-hexylamine [10,11], and 0.77 for PLP–LYS [13]. The $k_1^0$ and $k_1^2$ values for the DPL–NHA and DPL–LYS systems are very similar, so the differences observed between the overall constants shown in Fig. 1 must have arisen from the different p$K$ for the amino groups in *n*-hexylamine and poly-L-lysine. A similar comparison for the PLP–LYS system leads to the conclusion that $k_1^0$ and $k_1^2$ are at least one order of magnitude greater, which confirms the above-described effect of the phosphate group.

As regards the hydrolysis of the Schiff base (Fig. 2), the DPL–NHA and DPL–LYS systems have similar $k_2$ values below pH 7 as a result of the protonation of the terminal amino groups in the side chains of poly-L-lysine in acid media. Under these conditions, the polypeptide acquires a highly stretched conformation in order to lessen electrostatic interactions and each side chain behaves as a small, individual molecule. However, between pH 7.0 and 9.0, the DPL–LYS systems exhibits greater $k_2$ values. This can be ascribed to the fact that, because the macromolecule provides a more hydrophobic environment, the p$K$ of the imino group decreases from 11.7 in the DPL–NHA system [8] to 9.3 in the DPL–LYS system (Table 1), thereby increasing $k_2$. On the other hand, a comparison of the $k_2$ values obtained in this work with those for the PLP–LYS system reveals that, above pH 7.5, the Schiff base of DPL is more readily hydrolyzed, which can be ascribed to a stabilizing effect of the interaction of the phosphate group in PLP. This is confirmed by a comparison of the hydrolysis of the Schiff bases of DPL and PLP with *n*-hexylamine [8].

As can also be seen in Fig. 2, the minimum of the log $k_2$ curve lies at pH 6–7, which is lower than that for the reaction with *n*-hexylamine (pH 8). Such a shift to a lower pH was also observed in the Schiff bases of pyridoxal and PLP in comparing the hydrolysis of the corresponding Schiff base with *n*-hexylamine and poly-L-lysine. This behaviour can be attributed to the change in the p$K$ of the Schiff base stemming from that in the medium polarity caused by the polypeptide chain.

The first protonation of the Schiff base always stabilizes the molecule, thereby hindering its hydrolysis, since $k_{OH} \gg k_2^2$. The subsequent protonation restabilizes the Schiff bases of poly-L-lysine. The p$K$ for the imine nitrogen (p$K_{3B}$) of the DPL–LYS Schiff base is considerably lower (about 2.5 pH units) than those for the other Schiff bases. This, together with the small relative value of $k_{OH}$, suggests the presence of a hydrophobic environment for the Schiff base of the DPL–LYS system.

By way of summary of the above discussion as regards $k_1$ and $k_2$ for the systems compared, Fig. 3 shows the $K_{pH}$ values obtained at a variable pH. As can be seen, the stability constant for the DPL–LYS system exceeds that for the system where the bearer of the amino group is *n*-hexylamine (at high pH values, the values for the two systems tend to converge, however). This may be the result of the effect of the polypeptide $\alpha$-helix on the formation rate of the Schiff base of poly-L-lysine.

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