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Stability of Schiff bases of amino acids and pyridoxal-5′-phosphate

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Summary. Stability of Schiff bases from Pyridoxal-5′-phosphate and α- and non α-amino acids and amines have been studied in a wide range of pH. Furthermore the transamination process for the PLP-serine Schiff base and the cyclization reaction of PLP-histidine Schiff base have also been studied.

Results show that the α-position on carboxyl group of amino acids plays an important role on the mechanism of hydrolysis of imine bond. Absence of ionic groups in the surroundings of that bond seems to be an important fact of stability.

In the transamination reaction, the rate-determining step is the isomerization of the Schiff base to ketoimine, since the rate constants for disappearance of Schiff base coincide with the rate constants for PMP formation. This process is catalyzed by the OH^-/H_2O system and the monoprotonated amino acid.

Keywords: Amino acids – Schiff bases – Kinetics – Pyridoxal 5′-phosphate – Vitamin B-6 group

Introduction

Pyridoxal-5′-phosphate (PLP) acts as coenzyme of a variety of enzymes which catalyze different reactions in the metabolism of amino acids. For all these enzymes, PLP is present in the form of a Schiff base resulting from the coupling of its carbonyl group to the ε-amino function of a lysine residue from the polypeptide chain (Snell, 1986)

The first step for all these enzymatic catalyzed reactions (transamination, β-elimination, dealdolation, razemization, etc.) seems to be the nucleophilic attack of the amine group of amino acid on the carbon of the imine to form another different Schiff base which later on yields products (Snell, 1986). This mechanism does not occur in the glycogen phosphorylase case, enzyme which

is not involved in the amino acid metabolism but in the glycogen metabolism (Madsen and Withers, 1984; Sansom et al., 1984; Klein et al., 1984).

Once the new Schiff base is formed, it can evolve througout different reactions: dealdolation, razemization, transamination, elimination (Abbot and Bobrick, 1973; Marcello and Martell, 1981; Martell, 1989). Thus, it is indispensable to have a comprehensive knowledge of the properties of the Schiff bases formed between PLP and amino acids and amines to fully understand the role played by this coenzyme in biocatalytic processes.

In this paper we show the results of stability of Schiff bases formed between PLP and different α-amino acids and non α-amino acids. The kinetic constants of formation as a function of pH as well as their hydrolytic kinetic constants are given. The evolution of these Schiff bases is studied for two specific processes: transamination of L-serine and cyclization of L-histidine.

Experimental

The α-amino acids: L-glycine (Gly), L-leucine (Leu), L-isoleucine (Ile), L-serine (Ser), L-histidine (His); the ε-aminocaproic acid (CA) was purchased from Sigma Chemical Co. Pyridoxal-5′-phosphate and all other chemicals were reagent grade and purchased from Merck.

PLP solutions were prepared in a suitable buffer and kept in dark. Their exact concentration was determined by diluting in 0.1 M HCl and subsequent measurement of its absorbance a 295 nm (Peterson and Sober, 1954). Concentration thus found was about 5.10^{-4} M. Amino acids solutions were also prepared by diluting the appropriate amount of the stock solution with a suitable volume of buffer and adjusting pH with HCl or NaOH when needed.

A Uvikon 940 spectrophotometer was used to carry out the measurements of absorbances and a Perkin Elmer MPF 66 for fluorimetric experiments.

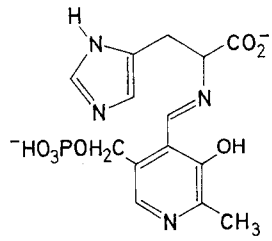
Formation of Schiff base was started by adding a few milliliters of the amino acid solution to the cell which already contained the previously thermostated PLP solution, prepared in the same buffer and at the same pH. Amino acid concentration in the measuring cell was 50–500 times higher than that of PLP. Reactions of formation and hydrolysis were monitored by measuring the increase in absorbance at 420 nm.

The overall reaction between the aldehyde and the amino acid, can be represented by:

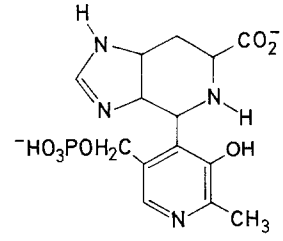
$$R{-}CHO + R'{-}\overset{CO_2^-}{\underset{|}{C}}H{-}NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R{-}CH{=}N{-}\overset{CO_2^-}{\underset{|}{C}}H{-}R' + H_2O$$

The method used to determine k_1 and k_2 are described in detail elsewhere (Garcia del Vado et al., 1987; 1988).

When the Schiff base is formed with histidine a subsequent cyclization reaction occurs and product II is quickly formed depending on pH.



Schiff base (I)



Cyclized product(II)

Ring closure of this Schiff base is regarded to be an irreversible process (Kondo et al., 1982), and complete conversion to the cyclized product II is obtained at any pH. Thus the overall reaction between PLP and histidine can be represented by:

$$PLP + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} BPLP \xrightarrow{k_c} C$$

where L, BPLP, and C stand for histidine, its Schiff base (I) and cyclized product (II).

The spectroscopic properties of II are very different from those of PLP and the Schiff base. The reaction was started as explained before but now it was monitored at two different wavelengths, 400 and 450 nm where the absorbances of aldehyde and imine differ considerably and the histidine and the product II do not absorb. This procedure allow us to calculate the value of the constants k_1, k_2 and k_c at each pH. The method to obtain these values is described in detail by Coll et al., (1991).

Once the Schiff base is formed, a transamination reaction between the aldehyde part and the aminoacid part is posible to occur. Such reaction can be represented by:

$$R{-}CH{=}N{-}\underset{\displaystyle CO_2^-}{\underset{|}{CH}}{-}R' + H_2O \underset{k_{2t}}{\overset{k_{1t}}{\rightleftharpoons}} R{-}CH_2{-}NH_2 + R'{-}CO{-}COO^-$$

The rate measurement were based on the disappearance of Schiff base, as well as the appearance of pyridoxamine 5′-phosphate (PMP). For the quick and complete conversion of PLP into the aldimine as a first step to study that reaction, a great excess of amino acid (10^4–10^5 fold) over PLP was needed.

The Schiff base and PMP concentrations were monitored by UV absorption spectroscopy and fluorimetry. In all fluorescence and absorption experiments the concentration of amino acid remained constant in the reaction and pseudo-first order kinetic were obtained. The exact method to determine the kinetic constants was described by Vázquez et al. (1991).

Results and discussion

The formation of Schiff bases

Nucleophilic atack of $-NH_2$ group of amino acids on the aldehyde group of PLP rend a intermediate calbinolamine that deshydrates to give the Schiff base. The deshydration process is catalyzed by acids (Jones, 1987).

Fig. 1 shows variation of log k_1 (the overall kinetic constant of Schiff base formation) with pH for the Schiff base of PLP and L-serine. All the other amino acids studied here show the same kind of behaviour than serine.

A progresive increase of k_1 is observed as a result of the increase of the amount of deprotonated (R-NH_2) amine present in the solution. In spite of this, it has to be considered that PLP presents four different ionic species and three ionization constants in the pH range from 3 to 11, corresponding to ionization of pyridine nitrogen, phosphate and phenolic group. In scheme I these species are represented by PLP_3, PLP_2, PLP_1 and PLP_0. The subcript means the net negative charge on the molecule. These species have different reactivity againts the nucleophile and thus different rate constant $k_1{}^i$. It is possible to determine the value of these constants from experimental values of the overall kinetic constant k_1, since the overall formation rate of the imine may be represented by:

$$v_1 = [L_1] \sum k_1{}^i[PLP_i] = k_1[L]_t[PLP]_t$$

t refers to the total concentration of all species.

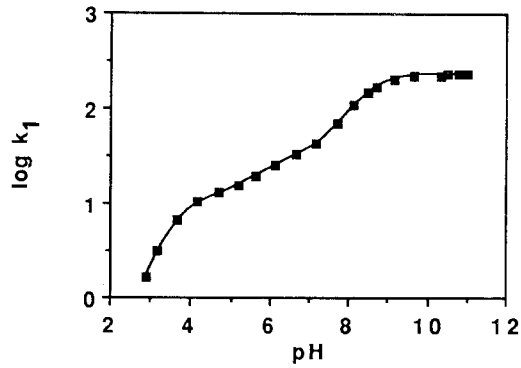
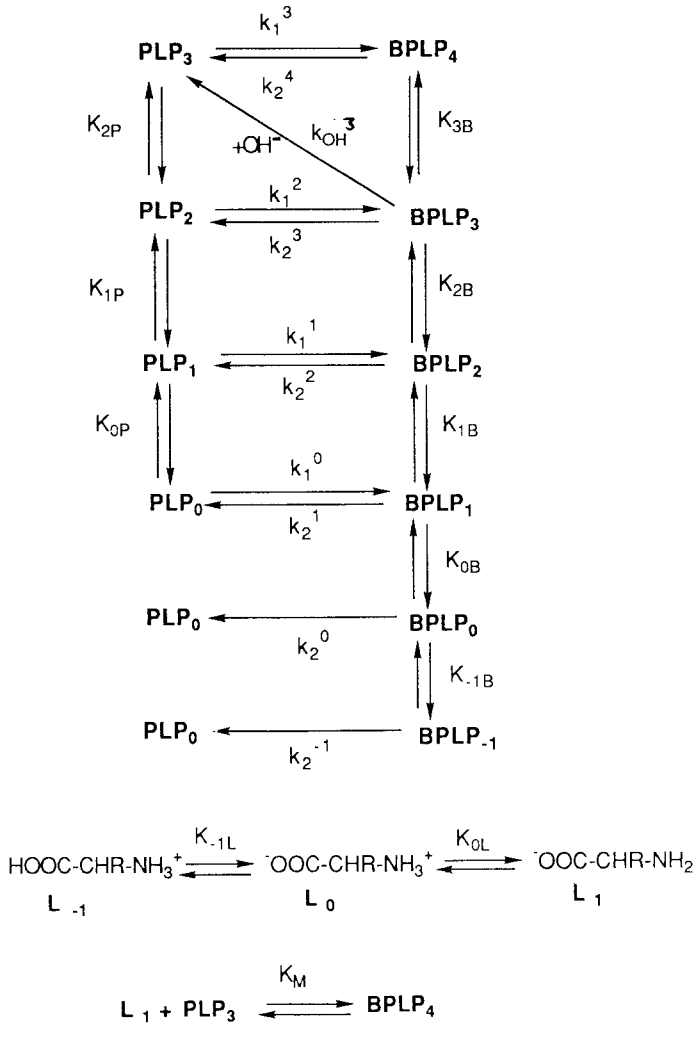


Fig. 1. Variation of log k_1 as a function of pH for the Schiff base of PLP and serine. Points are experimental values and the line is the theoretical function from Scheme 1



Scheme 1

Table 1 shows the values of these constants as well as the best values of ionization constant of PLP obtained from fitting of the experimental values of k_1 to equations from Scheme 1.

A progresive increase of k_1^i can be observed in any case on the sucessive addition of protons to the PLP molecule, which has been interpreted in terms of a intramolecular proton catalysis for the deshidratation of intermediate carbinolamine (Sancher-Ruiz et al., 1982; Garcia del Vado et al., 1987). For each Schiff base, the plot of log k_1^i values versus the corresponding pK values of the PLP molecule yields straight lines. These "Bronsted-like" plots give slope values (α) very similar (0.74–0.80) for the amino acids here studied, except for *n*-hexylamine (0.66) (Garcia del Vado et al., 1987), and could indicate that for the acid catalysis of the dehydration process the amino acid residue of Schiff base is not relevant.

On the other hand straight lines are also obtained when for a specific ionic specie the log k_1^i is represented versus pK of the amine. The slope values of these Bronsted plot (β) are different for each ionic species and very high in some case: 0.91, 0.95, 0.80 and 0.72 for the PLP_3, PLP_2, PLP_1 and PLP_0 species respectively, which could be indicative of the nucleophilic charater of the reaction.

The hydrolysis of Schiff bases

The reverse reaction of imine formation can happen and its rate constant is a function of pH. Fig. 2 represents such variation for the Schiff base of L-serine and PLP. As for the formation kinetic, the overall pseudo-first order constant k_2 can be expresed as a function of concentration and reactivities of different ionic species present in the solution. For the Schiff base of amino acids six ionic species can be considered in aqueous solutions from pH 3 to 11, except for histidine which bears another more ionizable group: the imidazol nitrogen ($pK = 6$). In water solution, scheme II seems to be the most realistic for the structural formulae of the ionic species of Schiff bases of amino acids that have been represented in kinetic Scheme 1 as $BPLP_4$, $BPLP_3$, $BPLP_2$, $BPLP_1$, $BPLP_0$ and $BPLP_{-1}$. Subscripts mean net negative charge on the molecule.

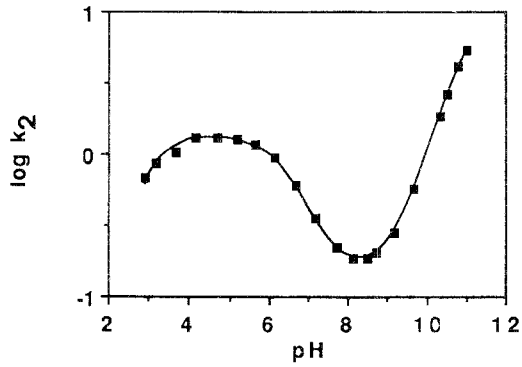


Fig. 2. Variation of log k_2 as a function of pH for the Schiff base of PLP and serine. Points are experimental values and the line is the theoretical function from Scheme 1

As in the formation mechanism and according to the Scheme 1, the hydrolysis rate of the Schiff base may be represented by:

$$v_2 = k_{OH}{}^3[BPLP_3][OH^-] + \sum k_2{}^i[BPLP_i] = k_2[BPLP]_t$$

$[BPLP]_t$ stands for the total imine concentration.

Table 1 displays values of microscopic rate constants $k_2{}^i$ calculated by fitting the overall experimental values of k_2 as a function of proton concentration to theoretical equations obtained from Scheme 1.

Succesive additions of protons to Schiff bases do not have the same effect upon the hydrolysis process than upon formation: no straigth line is found when $\log k_2{}^i$ is represented versus pK_{iB}.

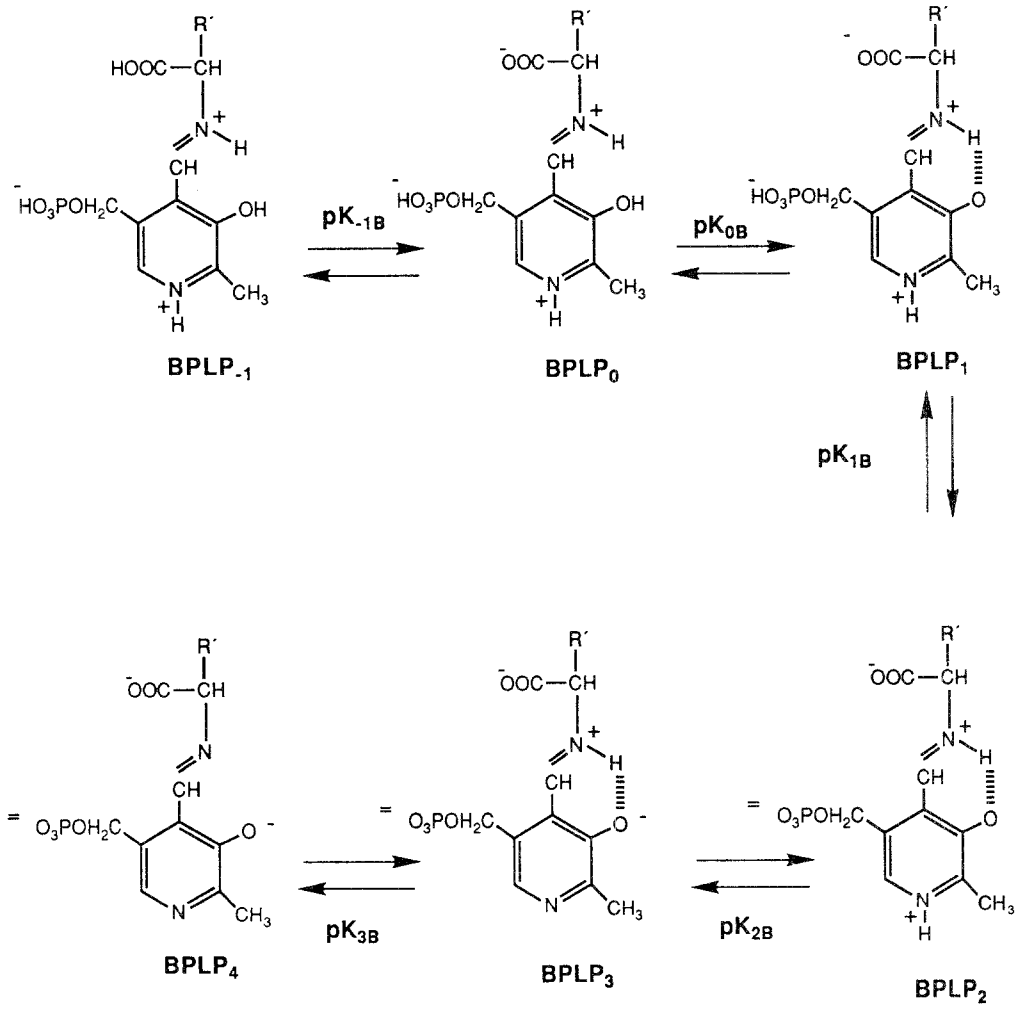
Comparison of the values of hydrolysis rate contants of these compounds shows that at any pH, glycine Schiff base has the higest constants, whereas ε-aminocaproic acid has the lowest ones. When comparing the histidine it has to be beared in mind that it has the imidazole ring and thus it has got three ionizable groups in a very narrow range of pH (7.28–5.84) pyridine nitrogen, imidazole nitrogen and phosphate. Comparing these pK_{iB} with those in Table 1, it seems to be clear that 5.84 must be attributed to phosphate ionization. pK of the pyridine nitrogen of Schiff bases of 3-hydroxypyridine-4-aldehydes is little affected by the structure and for most of them such pK is around 6.4 (Metzler

Table 1. Best kinetic constants, pK and K_M obtained from fitting of experimental values of k_1 ($L \cdot mol^{-1} \cdot min^{-1}$), k_2 (min^{-1}) and K_{pH} ($L \cdot mol^{-1}$) for PLP and ε-aminecaproic (*CA*), L-leucine (*LEU*), L-isoleucine (*ILE*), glycine (*GLY*) and L-histidine (*HIS*)

	CA	LEU	ILE	GLY	HIS
pK_{2P}	8.37	8.34	8.33	8.33	8.21
pK_{1P}	5.90	5.90	5.86	5.90	6.01
pK_{0P}	3.58	3.61	3.61	3.58	—
pK_{0L}	10.45	9.76	9.77	9.76	10.00
pK_{-1L}	4.07	2.31	2.31	2.37	6.00
pK_{3B}	11.70	11.61	11.57	11.35	10.30
pK_{2B}	6.42	6.65	6.66	6.36	7.28
pK_{1B}	5.69	5.68	5.67	5.46	6.88
pK_{0B}	3.93	2.83	2.83	2.84	5.84
pK_{-1B}	2.99	2.25	2.18	2.16	—
$\log k_1{}^3$	3.16	2.25	2.34	2.50	2.76
$\log k_1{}^2$	4.15	3.36	3.54	3.79	3.32
$\log k_1{}^1$	5.52	5.05	5.20	5.52	4.84
$\log k_1{}^0$	7.78	7.16	7.31	7.30	—
$\log k_2{}^4$	1.11	1.08	1.02	1.16	0.70
$\log k_2{}^3$	−1.28	−0.90	−1.00	−0.55	−0.72
$\log k_2{}^2$	−0.41	0.08	−0.17	0.68	−0.46
$\log k_2{}^1$	−0.27	0.17	−0.01	0.58	−0.29
$\log k_2{}^0$	−0.63	−0.06	−0.31	0.26	−0.71
$\log k_2{}^{-1}$	−0.75	−0.58	−0.55	−0.36	—
$\log K_M$	2.07	1.19	1.31	1.33	2.04

et al., 1980). Furthermore Bruice and Lombardo (1969) studied the cyclization of histamine-pyridoxal Schiff base and they found a value of 7.15 for the protonation of imidazole ring in this Schiff base. Hence we think that $BPLP_2$ form corresponds to the Schiff base, in which the imidazole ring is protonated but not the pyridine nitrogen and the phosphate group, which means that the ionic species BPLPi for the histidine Schiff base do not exactly correlate with those of glycine, *n*-hexylamine, etc and that their reactivities can not be compared straightforward.

Between the glycine and the ε-aminocaproic Schiff bases, two clear differences can be pointed out: their molecular weight and the position of carboxyl group. Molecular weight can not be an important factor, since leucine and isoleucine have the same molecular weight than ε-aminocaproic acid and they have different values of. So it must be the α-position of carboxyl group that plays an important role on the mechanism of water attack. The absence of ionic group in the surroundings of imine bond must be an important factor of stability.



Scheme 2

Values of *n*-hexylamine Schiff base confirm this point (Garcia del Vado et al., 1987).

The type of amino acid residue also affects the hydrolysis constant. When the residues are bulky nonpolar groups as in the leucine and isoleucine cases, their k_2^i values are lower than when substituens are just hydrogen. Differences can be even distinguished between leucine and isoleucine which have different branched nonpolar residues. Isoleucine has a more branched residue than leucine and it has a lower hydrolysis rate constants too. Presence of hydrophobic groups near the imine bond protect it against water attack. Our results about stability of Schiff bases of PLP and dodecylamine also confirm this extrem (Vázquez et al., umpublished data).

Transamination

However the Schiff bases formed between PLP and α-amino acids are quite stables, spectra of these compounds are slightly different from spectra of non α-amino acid Schiff bases or amine. Such differences has been interpreted as caused by the presence of PMP in the former cases (Vázquez et al., 1991). PMP is produced via a transamination reaction favoured by the higher acid character of the α-proton.

The activation of an α-proton of the amino acid residue can lead to different reactions. Once the Schiff base between the serine and PLP is formed, it is unstable and decomposes slowly, which is reflected in a decrease of the characteristic fluorescence bands at 520 nm and 490–500 nm when the excitation wavelength are 420 and 325 nm respectively. Likewise, an intense band appears at 420 nm, which strongly suggest the presence of PMP (Vázquez et al., 1991).

There are three different reactions that can explain that behaviour: dealdolation, elimination and transamination (Scheme 3). The dealdolation process would yield PLP-glycine Schiff base, which could undergo further rearrangement to give more stable species. The elimination reaction would yield the corresponding ketoacid and PLP with the consequent increase in the absorption at 388 nm, characteristic band of free PLP (Harris et al., 1976). The transamination would give PMP and the corresponding ketoacidate ion.

Because the decomposition of the PLP-serine Schiff base does not increase the absorption at 388 nm, the elimination reaction must be rouled out. On the other hand, the absence of aromatic rings in the serine does not stabilize the activated complex of the dealdolation process and transamination must be considered the most important process (Vázquez et al., 1991; Leussing, 1986).

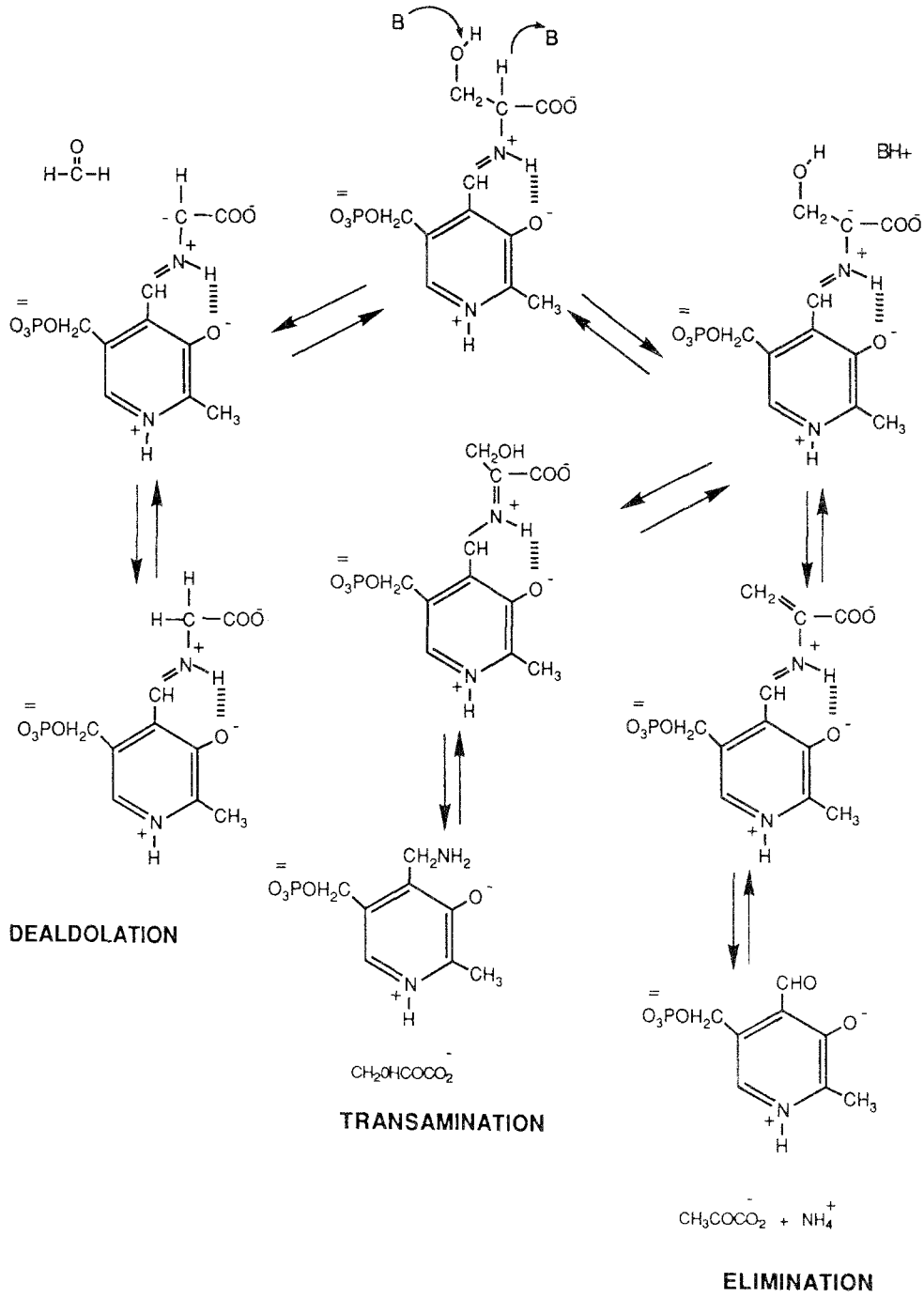
The transamination reaction of PLP-serine is a reversible process in all the pH range studied. Activation of an α-proton leads to tautomerization to give ketimine isomer, subsequent hydrolysis yields the ketoacidate and PMP (Scheme 3) Because the rate constants for disappearance of Schiff base coincides with the rate constants for PMP formation, the formation of the ketimine isomer must be the rate-determining step.

This reaction can be catalized by the amino acid (Scheme 4). Thus the k_{1t} and k_{2t} are given by:

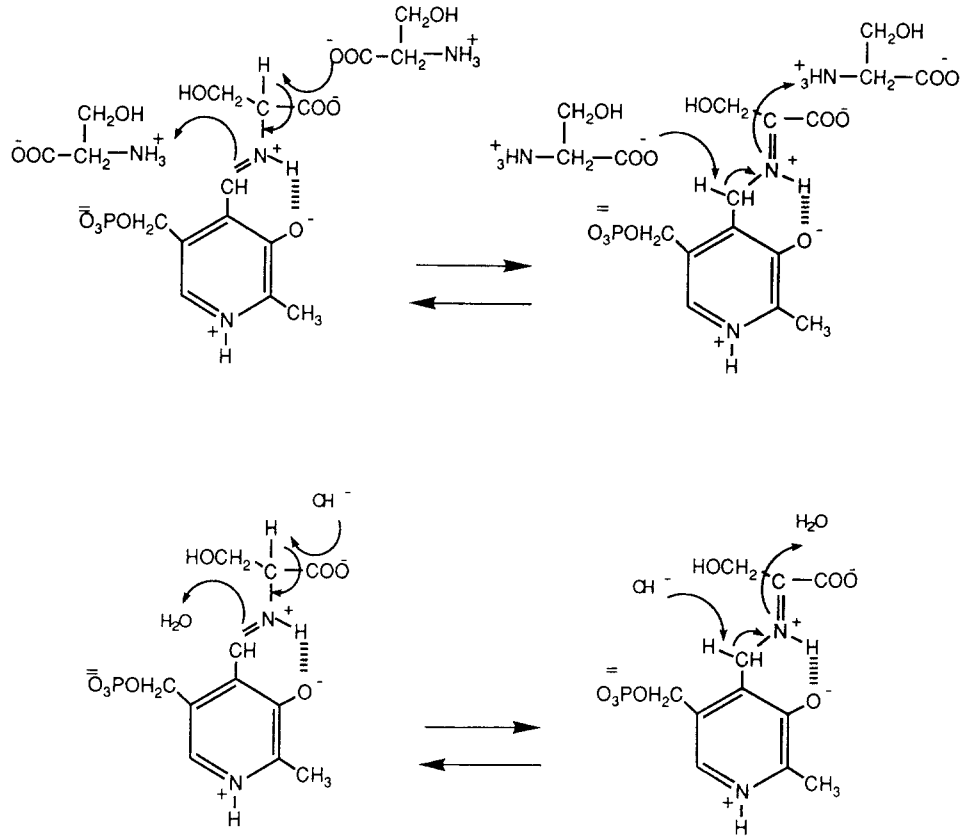
$$k_{1t} = k_{1H} + k_{1L}[L]_t$$

$$k_{2t} = k_{2H} + k_{2L}[L]_t$$

where k_{1H} and k_{2H} are the corresponding rate constants for the non catalized reaction and k_{1L} and k_{2L} are the rate constants for the amino acid catalized



Scheme 3



Scheme 4

reaction. $[L]_t$ stand for the total concentration of amino acid. At any pH between 4 and 12, the plott of the experimental values of k_{1t} and k_{2t} versus $[L]_t$ shows good linear relationships, which means that the rate-determining step is both water and amino acid catalyzed.

Fig. 3 shows the water and serine catalysis rate constant versus pH. In acidic medium, the rate constant is mainly due to the catalysis of the monoprotonated amino acid. The fall in k_{1L} values at pH higher than 6.0 must be attributed to the

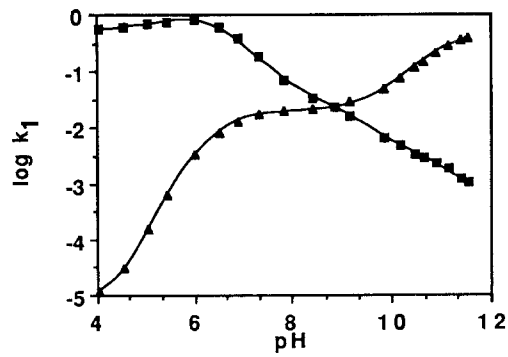


Fig. 3. Variation of log k_{1L} (■) and log k_{1H} (▲) as a function of pH for the Schiff base of PLP and serine . Points are experimental values and the lines are the theoretical functions

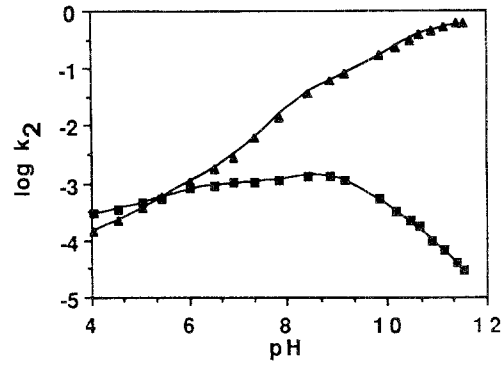


Fig. 4. Variation of log k_{2L} (■) and log k_{2H} (▲) as a function of pH for the Schiff base of PLP and serine. Points are experimental values and the lines are the theoretical functions

deprotonation of the pyridine nitrogen ($pK = 6.5$) and the subsequent pyridine ring deactivation. The k_{1L} values disminishes with pH because at high pH the nonprotonated form, which does not catalyze the reaction, is the majoritary one in solution.

At basic pH, the global rate constant is mainly due to the water catalysis term. The low k_{1H} values at acid pH show that, due to the weak basic character of the water, it is not able to activate the α-proton of the amino acid residue.

The rate constants of Schiff base formation from PMP and ketoacid are plotted in Fig. 4. As can be seen, the global rate constant is due to the contribution of both water and serine catalytic system at acid pH, and only to the water catalytic system at basic pH. The values of the serine catalysis rate constants are practically invariable in a wide pH range, from 4 to 9, and from that pH they disminish slowly due to the appearance of the nonprotonated amino acid form.

Cyclization of histidine-PLP Schiff base

The histidine-PLP Schiff base is a very special imine of PLP and this is because once it is formed a subsequent cyclization reaction produce the compound named II at the Experimental section. This reaction has been proved to be

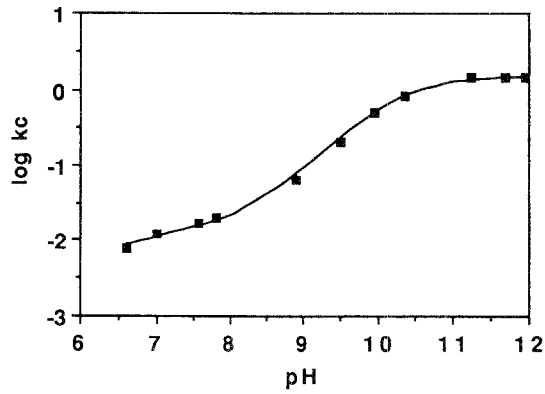
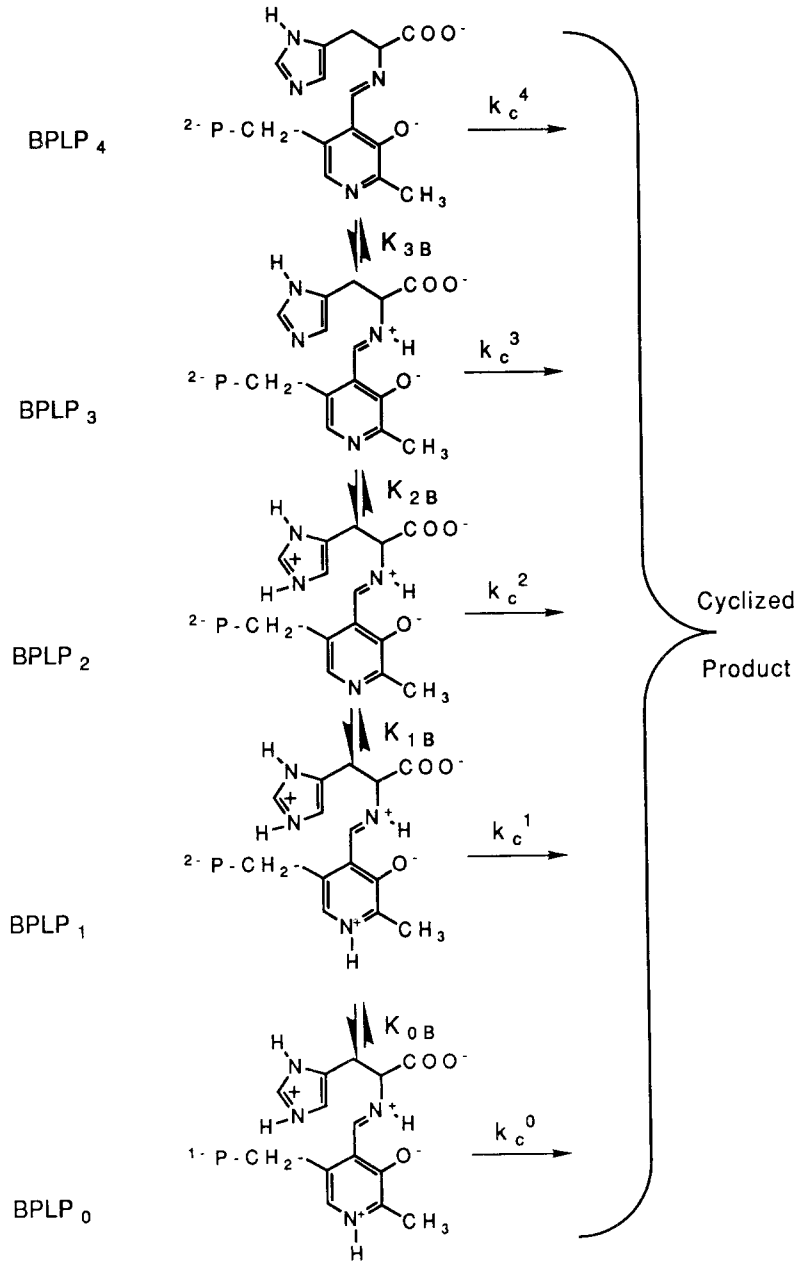


Fig. 5. Variation of log k_c as a function of pH for the Schiff base of PLP and histidine. Points are experimental values and the line is the theoretical function from Scheme 5

irreversible and it does not happen in enzymic conditions and can be prevent in part by bivalent cations (Kondo, 1982) or in hydrophobic conditions (Sunamoto, 1983).

The overall cyclization kinetic constan k_c, is very dependent on the proton concentration. Fig. 5 displays such dependence. As can be seen, reactivity strikinly falls down throug a pK of about 10.

In the same way that the formation, hydrolysis and transamination processes, the overall cyclization constant can be expressed in terms of microscopic



Scheme 5

kinetic constants. So, the cyclization rate is given by:

$$v_c = \sum k_c^i[\mathrm{BPLP}_i] = k_c[\mathrm{BPLP}]_t$$

Scheme 5, has been taken as kinetic scheme to fit data of overall k_c. The sub and superscripts have the same meaning than in formers equations, and Table 2 gives the best values for the kinetic constants obtained from fitting of experimental results to equations from scheme 5. Values of K_{iB} obtained from this last fitting are consistents with those obtainen from the hydrolysis kinetics (Table 1).

Table 2. Kinetic constants and ionization constants obtained from the fitting of k_c experimental values

$\log k_c^4 = 0.16$	$pK_{3B} = 10.30$
$\log k_c^3 = -1.82$	$pK_{2B} = 7.28$
$\log k_c^2 = -1.82$	$pK_{1B} = 6.88$
$\log k_c^1 = -2.22$	

Cyclization of PLP-histidine Schiff base proceed through the $BPLP_4$ species and it is surprising because the cyclization reaction has been classified as Mannich type and a priori $BPLP_3$ species should have the highest constant.

Bruice and Lombardo (1969) have found a similar behavior for ring closure of PL-histamine Schiff base. They postulate the existence of an anionic species of Schiff base in which the imidazole group is completely deprotonated. This group could attack the carbon of azomethine and condensation would involve the intramolecular addition of an enamine anion. That explanation is perfectly suitable for the system here studied.

Acknowledgements

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