

Determination of the Rates of Formation and Hydrolysis of the Schiff Bases Formed by Pyridoxal 5′-Phosphate with L-Tryptophan and Its Methyl and *n*-Butyl Esters

Gerardo. R. Echevarría Gorostidi,* José G. Santos,† Andrea Basagoitia,† and Francisco García Blanco††

Department of Physical Chemistry, University of Alcalá, E-28871 Alcalá de Henares, Spain

†Faculty of Chemistry, Pontificia Universidad Católica de Chile, Casilla 306, Santiago 22, Chile

††Department of Physical Chemistry, Faculty of Pharmacy, Complutensian University, E-28040 Madrid, Spain

(Received January 21, 2002)

The apparent rate constants of the formation (k_1) and hydrolysis (k_2) of the Schiff bases formed by pyridoxal 5′-phosphate with L-tryptophan and their methyl and *n*-butyl esters at a variable pH, 25 °C, and an ionic strength of 0.1 M were determined, along with the equilibrium constant (K_{pH}). The individual rate constants of formation and hydrolysis of the Schiff bases of systems corresponding to different chemical species present in the medium as a function of its acidity were also determined, as were the pK values for the Schiff bases. The influence of the α-carboxyl group on the formation and hydrolysis constants of the Schiff bases, and also on their pK values, is demonstrated.

One form of vitamin B_6 is Pyridoxal 5′-phosphate (PLP). This molecule plays an important part as a coenzyme in many reactions, such as transaminations, transiminations, dealdolations, and eliminations, which contribute to amino acid metabolism.[1–3] PLP participates in the formation of a carbinolamine (α-hydroxy amine) intermediate through a reaction of its carbonyl with the ε-amino moiety of the L-lysine residue in the peptide chain.[1,4] This intermediate decomposes to yield a Schiff base and one molecule of water in an acid-catalyzed process.[4,5]

In almost all PLP-dependent enzymes, the first step of the process mentioned above is a transimination reaction, which involved a transformation of the PLP-lysine imine into a PLP-substrate imine.[6] This means that the covalent bond in the Schiff base should be cleaved during the catalytic cycle. Thus, a new base is formed by the reaction of the coenzyme with the substrate (commonly an amino acid).[1]

The literature abounds with references to Schiff bases formed by PLP and various compounds bearing amino groups, such as amines, amino acids, and polypeptides.[7] However, very few kinetic studies of the reactions of PLP with amino acids as a function of the pH have so far been conducted.[8]

In this work, the stability and the kinetics of the formation and hydrolysis of the Schiff bases of pyridoxal 5′-phosphate with L-tryptophan and their methyl and *n*-butyl esters (viz. the PLP–TRP, PLP–MTRP, and PLP–BTRP systems) were determined, and the results were compared with those previously obtained for other amino acids.[8,9] In order to shed additional light on the interactions of the α-carboxyl group, the results were examined in terms of the individual rate constants for the species involved in the process (see Scheme 1).

Material and Methods

L-Tryptophan (TRP), L-tryptophan methyl ester (MTRP), and L-tryptophan *n*-butyl ester (BTRP) were purchased from Sigma Chemical Co. Pyridoxal 5′-phosphate (PLP) and all other chemicals used were of reagent-grade and purchased from Merck.

Acetate, phosphate, and carbonate buffers were used over appropriate pH ranges. The buffer concentration used was typically 0.02 M and the ionic strength was maintained at 0.1 M by adding appropriate amounts of KCl to the medium.

The hydrolysis reaction of MTRP and BTRP was monitored by measuring the presence of TRP by HPLC in a Star chromatography workstation Varian 4.51 equipped with a Merck-Hitachi LaChrom L-6250 pump and a Merck-Hitachi LaChrom L-7420 UV detector. Conditions: LC-18-DB (25 cm, 5 mm) Supelco column, eluant methanol–water 50:50, pH = 6.5, and isocratic mode 0.5 mL min^{-1}. For BTRP in the whole studied pH range and for MTRP at pH < 8, the hydrolysis was less than 1% after the time necessary for the preheating and more than 10 half-lives of the reactions. Nevertheless, at pH > 8 the hydrolysis of MTRP was more important (e.g. at pH = 10, 3% of TRP was found in 10 minutes).

PLP solutions were made in appropriate buffers and stored in the dark. Their exact concentrations were determined by dilution with 0.1 M HCl,[10] and were found to be in the region of 2×10^{-5} M. TRP solutions spanning the concentration range from 5×10^{-4} to 2×10^{-2} M were also prepared, on a daily basis, by diluting appropriate amounts of stock solutions in the corresponding buffer. In order to prevent the hydrolysis of MTRP and BTRP, the solutions were prepared inmediatly prior to use in the corresponding buffer, spannning the same concentration range as that of the TRP solutions.

Kinetic measurements were made at a variable pH by using a Hewlett-Packard 8453 diode array spectrophotometer and a Uvikon 941-Plus spectrophotometer furnished with thermostated cells of 1-cm light path. In each case, the reaction was started by adding a known volume of PLP buffered solution to prethermostated TRP, MTRP (pH $<$ 8), and BTRP solutions at (25 $\pm$ 0.05) °C. For the reaction of PLP with MTRP at pH $>$ 8, where the hydrolysis of MTRP is important, freshly prepared PLP and MTRP stock solutions at pH = 7 and 25 °C were added to a preheated solution of the buffer at the desired pH.

The presence of TRP in the reactions was checked at the end of the reactions, and was never greater than 3% (in most cases $<$ 1%). Those reactions showing TRP $>$ 3% at the end of the reaction were disregarded.

The difference between the initial and final pH in the reaction cell never exceeded 0.03 units. pH measurements were made by using a Crison pH-meter equipped with a Metrohm EA120 electrode that was previously calibrated with aqueous buffers at 25.0 °C.

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

$$R_1-CHO + NH_2-R_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R_1-CH{=}N-R_2 + H_2O, \quad (1)$$

where k_1 and k_2 are the overall rate constants of formation and hydrolysis, respectively, of the Schiff base. The rate Eq. 1, taking into account the Beer–Lambert law and that the kinetics of both the forward and the reverse reactions are second-order, is Eq. 2 with the application of Eq. 3, where a and b are the initial aldehyde and amine concentration, respectively:

$$\ln\frac{A_\infty - A_0}{A_\infty - A} = -\ln\frac{ab - xx_e}{x_e^2} + k_{obs}t, \quad (2)$$

$$k_{obs} = \left\{[k_2 + k_1(a+b)]^2 - 4abk_1^2\right\}^{1/2}. \quad (3)$$

Here, A_0, A, and A_∞ are the absorbances at times 0, t, and ∞, respectively; k_1 and k_2 are the overall formation and hydrolysis rate constants for the Schiff base.

The k_{obs} values were obtained from the slopes of the plots of $\ln(A_\infty - A)$ versus time, since $ab >> xx_e$. The k_1 and k_2 values were calculated from the k_{obs} values obtained at a given pH for different $(a + b)$ values.[11] Their ratio coincides with the equilibrium constant ($K_{pH} = k_1/k_2$). Nucleophilic rate constants (k_N) were obtained from the slopes of linear plots of k_{obs} vs the free amine concentration, or by dividing the k_1 values into the corresponding free amine mole fractions.

The deprotonation equilibrium constant (K_N) values for TRP, MTRP, and BTRP were determined potentiometrically by titration with 0.1 M NaOH of 0.01 M solutions of the corresponding amine and 0.01 M HCl, using a Radiometer autotitrator equipped with a PHM-62 pH-meter, an ABU-11 autoburette, a TTT-60 titrator, a REA-160 recorder, a TTA-60 thermostatic support, a G-2040 glass electrode, and a K-4040 calomel electrode. The experimental conditions used were the same as those for the kinetic measurements. The pK_N values for TRP, MTRP, and BTRP thus found were 9.2, 7.6, and 8.0, respectively.

Results

Figures 1–3 show the experimental results in the form of the variation of the logarithmic overall rate constants of formation (k_1) and hydrolysis (k_2), and the equilibrium constant (K_{pH}), for the Schiff bases of PLP–TRP, PLP–MTRP, and PLP–BTRP systems as a function of the pH.

Reaction measurements could only be made up to pH 10.5 because more alkaline media resulted in secondary imine intramolecular cyclization by an attack of the indole group on the azomethine carbon, thereby hindering the reaction (as previously observed in that between PLP and histidine[12,13]) and precluding an examination beyond this alkalinity level.

In PLP–MTRP and PLP–BTRP systems, k_1 increased with increasing pH in acid and neutral media, and peaked at a pH of about 7.0; however, for the PLP–TRP system, k_1 increased with increasing pH, but no peak was observed. Below pH 9, k_1 were greater for the ester systems than for the PLP–TRP sys-

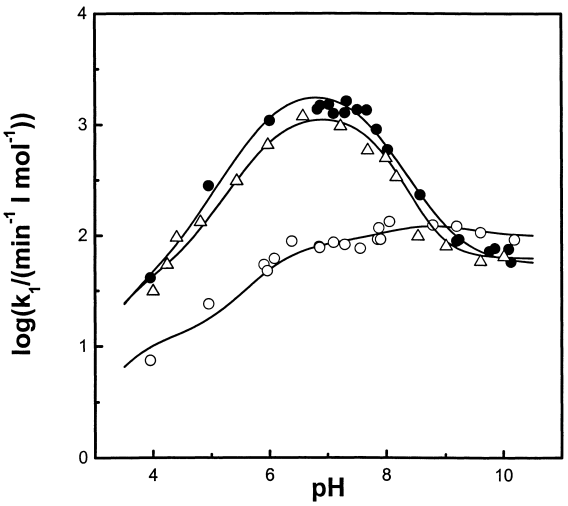


Fig. 1. Plot of log k_1 vs pH for the PLP–TRP (○), PLP–MTRP (●), and PLP–BTRP (△) systems. Curves calculated using Eq. 7 and data from Table 1.

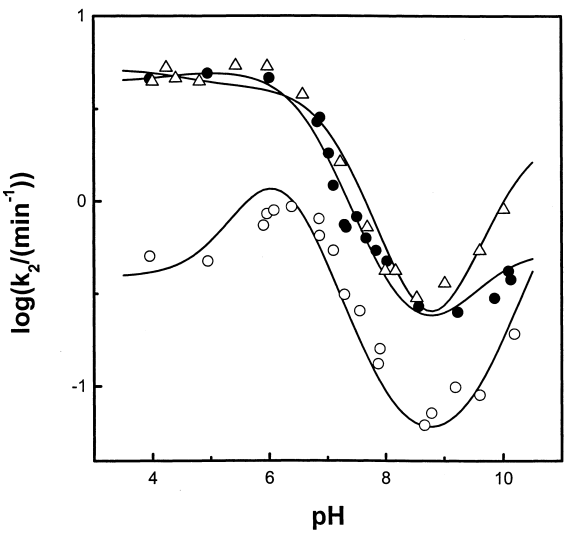


Fig. 2. Plot of log k_2 vs pH for the PLP–TRP (○), PLP–MTRP (●), and PLP–BTRP (△) systems. Curves calculated using Eq. 8 and data from Table 1.

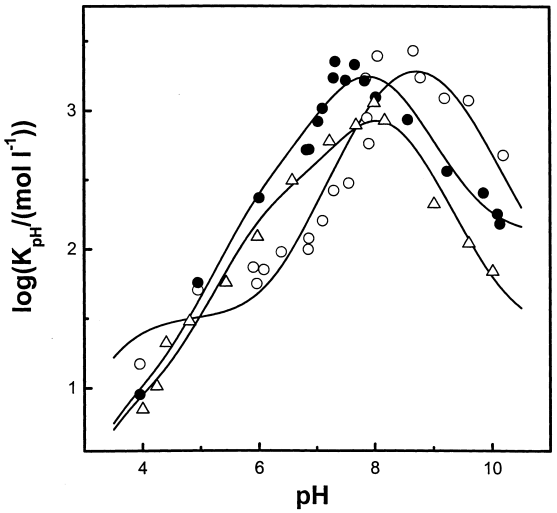


Fig. 3. Plot of log K_{pH} vs pH for the PLP–TRP (○), PLP–MTRP (●), and PLP–BTRP (△) systems. Curves calculated using Eq. 9 and data from Table 1.

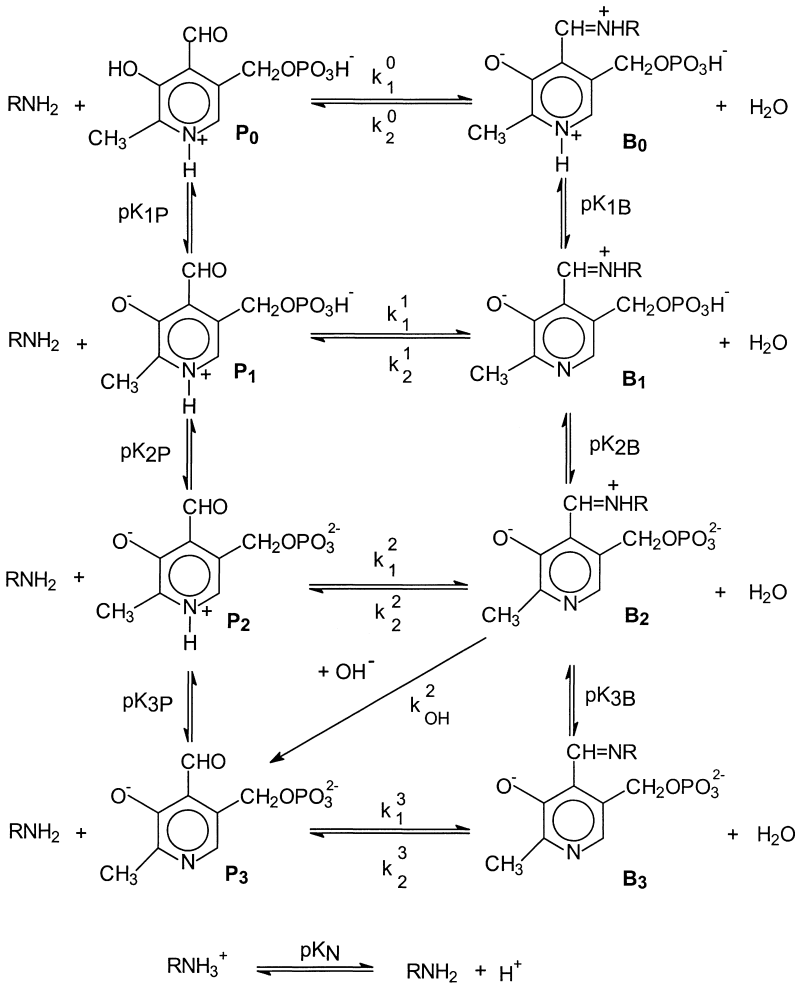
tem (Fig. 1).

On the other hand, k_2 was greater for the ester systems throughout the pH range studied (Fig. 2). Because the sole difference between the two systems was the presence of a carboxyl (or carboxylate) group or an ester function in the carbon α, it must have been the origin of the observed differential behavior.

Figure 3 is consistent with the results for other similar systems. As can be seen, K_{pH} increases with increasing pH up to a value in between that of pK_N for the amine and the value of the pK_{3P} for PLP (see Scheme 1);[14] the experimental maximum lies at about 8.7 for the PLP–TRP system and 7.8, and 8.0 for the PLP–MTRP and PLP–BTRP systems, respectively (the pK_N values for TRP, MTRP, and BTRP are 9.2, 7.6, and 8.0, respectively, and the pK_{3P} for the PLP[15] is 8.2).

The apparently increased reactivity of MTRP and BTRP with PLP relative to TRP with PLP below pH 9 (see Fig. 1) arises from the differential values of pK_N for the amino group in the three compounds. One way of comparing their reactivity is by making the formation rate constants for the Schiff bases independent of pK for the amino group. Figure 4 shows the variation of the nucleophilic rate constant, k_N, with pH; as can be seen, TRP is more reactive than MTRP and BTRP throughout the pH range studied, but in the 6 to 8 pH range is very symilar.

As shown in Scheme 1, the overall rate constants of formation and the hydrolysis of the Schiff bases can be described in



Scheme 1.

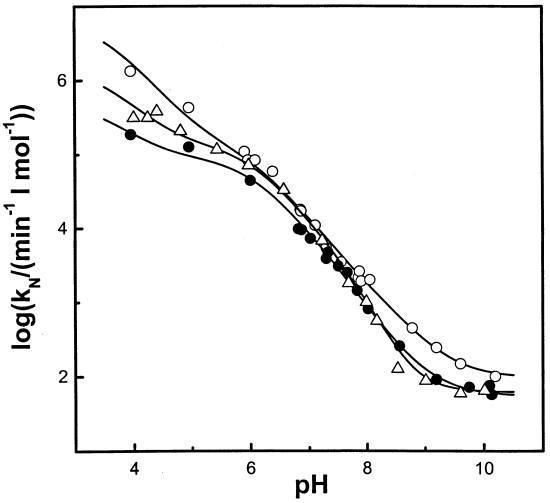


Fig. 4. Plot of log k_N vs pH for the PLP–TRP (○), PLP–MTRP (●), and PLP–BTRP (△) systems. Curves calculated using theoretical values of k_1.

terms of the individual constants for the different chemical species present in the medium at each pH.

Thus, k_1^i and k_2^i (with $i = 0$, 1, 2, or 3) are the individual rate constants of formation of the Schiff bases and of their hydrolysis by H_2O, and k^2_{OH} is the rate constant of hydrolysis of species B_2 (a Schiff base with a net charge of -2) by OH^- ions. P_i (with $i = 0$, 1, 2, or 3) denotes the different chemical species of PLP, and pK_{3P}, pK_{2P}, and pK_{1P} the different pK values that relate them. B_i (with $i = 0$, 1, 2, or 3) are the different chemical species of the Schiff bases, and pK_{3B}, pK_{2B}, and pK_{1B} are the pK values that relate them. Finally, K_N is the deprotonation equilibrium constant of the $-NH_3^+$ group in TRP, MTRP, or BTRP.

In Scheme 1 the hydrolysis reactions of the forms B_i ($i = 0$, 1) for OH^- have been omitted because of the very small concentration of OH^- at the pH at which the concentration of B_i ($i = 0$, 1) is important.

The rate of formation of the Schiff base is given by

$$v_1 = [RNH_2]\sum_{i=0}^{3} k_1^i[P_i] = k_1[RNH_2]_T[PLP]_T, \quad (4)$$

where T denotes the concentration of all the species.

The hydrolysis of the Schiff base conforms to Eq. 5:

$$v_2 = k_{OH}{}^2[B_2][OH^-] + \sum_{i=0}^{3} k_2^i[B_i] = k_2[\text{Schiff Base}]_T. \quad (5)$$

The equilibrium constant (K_{pH}) is given by

$$K_{pH} = [\text{Schiff Base}]_T/([RNH_2]_T[PLP]_T). \quad (6)$$

Taking into account the equilibria in Scheme 1 and the fact that the equilibrium formation constant for the Schiff base at very high pH values is given by $K_M = k_1^3/k_2^3$, Eqs. 4–6 can be readily transformed into

$$k_1 = \frac{k_1^3 + k_1^2 \cdot a/K_{3P} + k_1^1 \cdot a^2/(K_{3P} \cdot K_{2P}) + k_1^0 \cdot a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P})}{(1 + a/K_N)(1 + a/K_{3P} + a^2/(K_{3P} \cdot K_{2P}) + a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P}))}, \quad (7)$$

$$k_2 = \frac{k_{OH} + k_2^2 \cdot a/K_{3B} + k_2^1 \cdot a^2/(K_{3B} \cdot K_{2B}) + k_2^0 \cdot a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B})}{1 + a/K_{3B} + a^2/(K_{3B} \cdot K_{2B}) + a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B})}, \quad (8)$$

$$K_{pH} = \frac{(1 + a/K_{3B} + a^2/(K_{3B} \cdot K_{2B}) + a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B}))K_M}{(1 + a/K_N)(1 + a/K_{3P} + a^2/(K_{3P} \cdot K_{2P}) + a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P}))}, \quad (9)$$

where $k_{OH} = k_2^3 + k_{OH}{}^2 (K_W/K_{3B})$, $a = 10^{-pH}$ and K_W is the ionic product of H_2O.

The experimental values of k_1, k_2, and K_{pH} were fitted simultaneously to Eqs. 7–9 by a nonlinear regression method involving minimization of the functions U_1, U_2, and U_{pH}:

$$U_1 = \sum(\log k_{1,e} - \log k_{1,t})^2, \quad (10)$$

$$U_2 = \sum(\log k_{2,e} - \log k_{2,t})^2, \quad (11)$$

$$U_{pH} = \sum(\log K_{pH,e} - \log K_{pH,t})^2, \quad (12)$$

where subscripts e and t denote the experimental and theoretical data, respectively.

The values of the pK of PLP are found in ref. 15 and those for TRP, MTRP, and BTRP were determined in this work (see Material and Methods).

Table 1 gives the individual rate constants of the formation (k_1^i) and hydrolysis (k_2^i, k_{OH}), as well as the pK values obtained in the fitting of k_1, k_2, and K_{pH} to Eqs. 7, 8, and 9. For comparisons, the values for the Schiff bases of PLP with other amino acids are also listed.[8,9]

At this point it is interesting to notice that the reaction mechanism is through a carbinolamine intermediate, as in Eq. 13 (R_1–CHO is P_i (with $i = 0$, 1, 2, or 3) and R_1–CH=N–R_2 is B_i (with $i = 0$, 1, 2, or 3)). The k_1^i rate constants in Scheme 1 involve the formation of carbinolamine rate constants (k_{ci}) and those from the intermediate carbinolamine to form the Schiff base by water elimination (k_{di}) and to return to reactants by amine elimination (k_{-ci}):

$$R_1-CHO + NH_2-R_2 \underset{k_{-ci}}{\overset{k_{ci}}{\rightleftharpoons}} R_1-CH(OH)-NH-R_2 \underset{k_{-di}}{\overset{k_{di}}{\rightleftharpoons}} R_1-CH{=}N-R_2 + H_2O, \quad (13)$$

If $K_{ci} = k_{ci}/k_{-ci}$ is defined as the equilibrium formation constant for carbinolamine, provided that this is formed and split fairly rapidly, and is completely shifted to the reactants, then $k_1^i = K_{ci}k_{di}$ and $k_2^i = k_{-di}$. This is in accord with the linear plots k_{obs} vs [amine] obtained for the three amine-group bearer at different pH values.

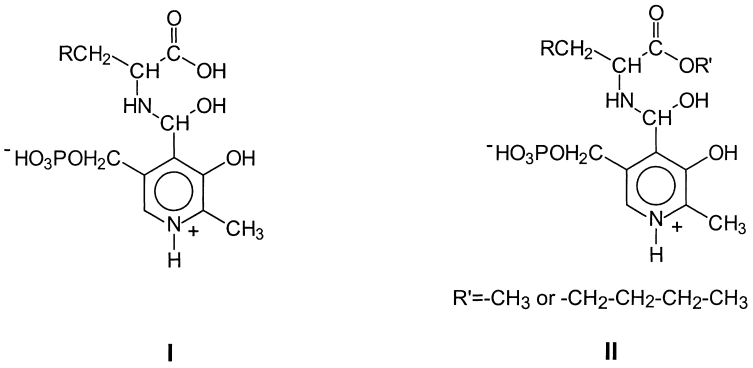
Discussion

As can be seen from Table 1, the k_1^i values for the reactions of PLP with different amino acids are quite similar; consequently, the side chains in the amino acids play no significant role in the formation of Schiff bases with PLP, as has been de-

Table 1. Best Kinetic Constant Values Obtained in the Fitting of Experimental k_1 (L mol^{-1} min^{-1}), k_2 (min^{-1}), and K_{pH} (L mol^{-1}) Values to Scheme 1, and Those Corresponding to the PLP–GLY,[9] PLP–LEU,[9] PLP–ILE,[9] PLP–PHE,[8] and PLP–ALA[8] Systems[a)]

	PLP–TRP	PLP–MTRP	PLP–BTRP	PLP–GLY	PLP–LEU	PLP–ILE	PLP–PHE	PLP–ALA
$\log k_1^0$	6.83 ± 0.07	5.77 ± 0.09	6.19 ± 0.07	7.30	7.16	7.31	—	—
$\log k_1^1$	5.15 ± 0.03	4.94 ± 0.03	5.11 ± 0.03	5.52	5.05	5.20	4.75	5.01
$\log k_1^2$	3.14 ± 0.07	2.64 ± 0.10	2.26 ± 0.10	3.79	3.36	3.54	2.96	3.23
$\log k_1^3$	1.96 ± 0.08	1.80 ± 0.05	1.76 ± 0.06	2.50	2.25	2.34	2.23	2.39
pK_{1P}	3.46[b)]	3.46[b)]	3.46[b)]	3.58	3.61	3.61	—	—
pK_{2P}	6.02[b)]	6.02[b)]	6.02[b)]	5.90	5.90	5.86	5.95	5.95
pK_{3P}	8.22[b)]	8.22[b)]	8.22[b)]	8.33	8.34	8.33	8.14	8.14
$\log k_2^0$	−0.42 ± 0.10	0.66 ± 0.12	0.67 ± 0.09	0.584	0.170	−0.010	—	—
$\log k_2^1$	0.33 ± 0.05	0.69 ± 0.04	0.65 ± 0.04	0.676	0.080	−0.170	—	—
$\log k_2^2$	−1.16 ± 0.07	−0.70 ± 0.09	−1.08 ± 0.25	−0.550	−0.900	−1.000	0.477	—
$\log k_{OH}$	0.01 ± 0.17	−0.34 ± 0.07	0.32 ± 0.08	1.160	1.080	1.020	0.64	—
pK_{1B}	6.04 ± 0.35	4.34 ± 0.20	4.54 ± 0.14	5.46	5.68	5.67	—	—
pK_{2B}	6.33 ± 0.30	6.72 ± 0.13	7.09 ± 0.11	6.36	6.65	6.66	6.55	—
pK_{3B}	10.58 ± 0.30	9.66 ± 0.22	10.1 ± 0.60	11.35	11.61	11.57	12.3	11.78
$\log K_M$	2.10 ± 0.28	2.10 ± 0.18	1.48 ± 0.60	1.33	1.19	1.31	—	—

a) GLY, LEU, ILE, PHE and ALA denote glycine, L-leucine, L-isoleucine, L-phenylalanine, and L-alanine, respectively. b) From Ref. 15.



I II

Scheme 2.

scribed.[8] A comparison of the k_1^i values obtained for the PLP–MTRP and PLP–BTRP systems reveals the absence of significant differences (see Table 1); therefore, the methyl or *n*-butyl group size is not important in Schiff-base formation.

A comparison of the k_1^i values obtained for PLP–TRP and ester systems (Table 1) reveals the presence of a strong differences in k_1^0 and k_1^2. This effect is also apparent by comparing the k_N curves of Fig. 4, which exhibit appreciable differences in acid and alkaline media, but virtually none in neutral media.

The rate-determining step in the formation of a Schiff base is known to be the dehydration of an intermediate carbinolamine formed by an attack of the amine on the carbonyl group.[4,5] It is also known that, with PLP, the dehydration is subject to intramolecular acid catalysis, and that the phenol group at C-3 on the pyridine ring plays an especially prominent role in acid media.[16] The dehydration of carbinolamines of these systems in an acid medium (structures I and II in Scheme 2) is catalyzed by the phenol group; in addition, structure I, which corresponds to the PLP–TRP system, can release a water molecule by the interaction of the proton in the carboxyl group with the OH group in the carbinolamine. This is impossible in the ester and in those chemical species of TRP that bear a deprotonated carboxyl group (the reported pK for the carboxyl group in tryptophan is[17] 2.4). If this is an explanation to the observed behavior, the Brönsted must be curved up in the acidic media; nevertheless, the plot is linear with $\alpha = 0.77$ (Fig. 5), as in the other α-amino acids[9] $\alpha = 0.74$–0.78, precluding a catalytic effect due to the carboxylic group. On the other hand, the PLP–MTRP and PLP–BTRP show non-linear Brönsted plots (Fig. 5), showing that the ester groups inhibed the Schiff-base formation, probably due to a steric inhibition.

The maxima in the k_1 curves (Fig. 1) are a result of the pK_N of the amino group (the mole fraction of free amine increases with increasing pH) and of the sequence $k_1^0 > k_1^1 > k_1^2$ (the reactivity decreases with increasing pH). The net balance between these two opposing effects leads to the maximum in Fig. 1.

Figure 2 reveals an increased stability of the Schiff base of TRP relative to that of esters, consistent with the values of the individual k_2^i constants in Table 1. The presence of non-polar groups in the vicinity of the hydrolysis site reportedly protects the imine bond from hydrolysis,[9] as found, for example, in comparing the Schiff bases of PLP with L-leucine (LEU) and L-isoleucine (ILE), and those of the same substrate with glycine (GLY) and LEU (Table 1). This effect has also been ob-

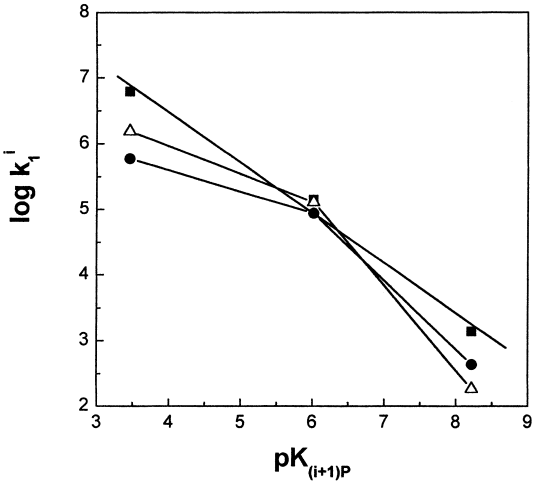


Fig. 5. Brönsted plot for the PLP–TRP (■), PLP–MTRP (●), and PLP–BTRP (△) systems.

served in the Schiff bases of PLP with poly L-lysines,[18] where the polypeptide chain provides a less polar environment and leads to bases of increased stability. Accordingly, the methyl or *n*-butyl group in the ester should result in less readily hydrolyzed bases. However, the behavior of the Schiff bases studied here leads to the opposite conclusion. Thus, the presence of the carbonyl group or the carboxylate anion stabilizes the Schiff base in relation to the alkyl group in the ester.

In the same way, compared to other described models,[7–9] the curves log k_2 vs pH of Fig. 2 show a minimum in accordance with the fact that k_2^2 is the minor of the individual hydrolysis rate constants (see Table 1).

Table 1 also gives the pK values for the various forms of the Schiff bases derived from the different amino acids; as can be seen, there are no marked differences in this respect. However, a comparison of the pK values for the Schiff bases of the PLP–TRP and PLP–MTRP or PLP–BTRP systems reveals significant differences in pK_{1B} and pK_{3B}, which are smaller in the ester. It should be noted that the differences are quite close to that in the pK_N for the amino group in TRP relative to that in MTRP or BTRP. The difference in pK_{3B} is quite normal, because it involves an imine nitrogen; on the other hand, the fact that the effect is passed onto pK_{1B} suggests that this corresponds to the pyridine nitrogen. The effect can be transmitted to this atom, but not to the phosphate group, to which pK_{2B} can be assigned. This result allows one to unequivocally assign the protonation sequence of Scheme 1.

This work was funded by Spain's DGI (Project BQ2000-0646), FONDECYT (Chile, Projects 1990551 and 2990006) and *Programa para la Cooperación con Iberoamérica*.

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