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Kinetics of the formation and hydrolysis of the Schiff bases of pyridoxal 5′-phosphate and copolypeptides containing L-lysine and aromatic L-amino acids [1]

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Abstract

We determined the apparent rate constants of formation (k_1) and hydrolysis (k_2) of the Schiff bases formed between pyridoxal 5′-phosphate and copolymers of L-lysine–L-tyrosine and L-lysine–L-phenylalanine, at different pH values, at temperature of 25°C and an ionic strength of 0.1 M. The values of the rate constant of formation obtained for the systems of the present work are greater than these for the PLP–poly-L-lysine system in all the range of pH studied, due to the presence of aromatic groups in the lateral chains of the polypeptide. The existence of these groups favors the presence of a few polar environment for the Schiff bases.

Keywords: Schiff base; PLP; L-lysine; L-tyrosine; L-phenylalanine

1. Introduction

Pyridoxal 5′-phosphate (PLP) acts as a coenzyme for a variety of enzymes that catalyze chemical reactions involved in aminoacid metabolism [1–3], as well as in glycogen phosphorylase [4]. In every PLP dependent enzyme studied to date, PLP is bound to the ε-amino group of an L-lysine residue of the polypeptide chain via its carbonyl group, forming a Schiff base (aldimine), which exits in a more or less hydrophobic environment [5–7].

The Schiff bases of PLP and its analogues with various $-NH_2$ bearers have been commonly used as a model for the binding of PLP to the enzymes [8–32]. Recently, we have carried out studies of the Schiff bases of PLP or analogues with poly-L-lysine in various degrees of polymerization [33,34] and those of PLP with L-lysine copolymers [poly(Lys, Ala) and

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poly(Lys, Glu)] [35,36]. These systems are much more similar models to the enzymic behaviour. We have found that the Schiff bases of PLP and polypeptides containing L-lysine are much more stable as a result of hydrogen-bonds interactions between groups of PLP and the peptide skeleton [35].

The presence of residues with aromatic lateral chains in the proximities of the active site of the PLP-dependent enzymes, such as the Tyr70 and Tyr225 of the ArAT (aromatic amino acid aminotransferase) [37] or the Tyr573 of the glycogen phosphorylase [38], can favor the existence of the other type of interactions that modify the stability of the carbon–nitrogen double bond of the Schiff bases.

In this work we studied the stability and kinetics of formation and hydrolysis of the Schiff bases of pyridoxal 5′-phosphate and copolymers of L-lysine with aminoacids containing aromatic lateral chains [poly(Lys, Tyr) 4:1 and poly(Lys, Phe) 1:1]. The results are compared with the ones obtained for the systems PLP–poly-L-lysine, PLP–poly(Lys, Ala) and PLP–poly(Lys, Glu). A band analysis of electronic spectra of Schiff bases of pyridoxal 5′-phosphate and copolymers [poly(Lys, Tyr) 4:1 and poly(Lys, Phe) 1:1] has also been accomplished in order to investigate the polarity of the microenvironment of the Schiff base in these medium.

2. Experimental

The polypeptides were purchased from Sigma Chemical. The molecular weights (based on viscosity determination) of poly(Lys, Tyr) 4:1 and poly(Lys, Phe) 1:1 used was: 24000 (DP = 120) and 50000 (DP = 281) Da, respectively. Pyridoxal 5′-phosphate and all the other chemicals were reagent grade and purchased from Merck.

Acetate, phosphate and carbonate buffers were used in appropriate pH ranges. The buffer concentrations used were typically 0.02 mol/l and the ionic strength was kept constant and equal to 0.1 mol/l by adding appropriate amounts of KCl to the medium.

PLP solutions were prepared in appropriate buffers and were stored in the dark. Their exact concentrations were determined by dilution [39] with 0.1 mol/l HCl and were found to be in the region of 2×10^{-5} mol/l. Polypeptide solutions were also daily prepared by diluting the appropriate amount of polymer in the corresponding buffer. Their concentration ranged between 2×10^{-2} and 5×10^{-4} mol/l.

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

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

where k_1 and k_2 are the overall rate constants of formation and hydrolysis of the Schiff base, respectively. The procedure used to calculate these two constants is described in detail elsewhere [14]. The ratio between them represents the equilibrium constant ($K_{pH} = k_1/k_2$),

The kinetics of formation of the Schiff base was monitored by measuring the variation in the absorption at 390 nm (Lys–Phe) and 430 nm (Lys–Tyr) as a function of pH using a Uvikon 941-Plus spectrophotometer furnished with thermostated cells of 1 cm lightpath. In each case, the reaction was started by adding a known volume of PLP buffered solution to prethermostated polypeptide solutions at 25 ± 0.05°C. The difference between the initial and final pH in the reaction cell never exceeded 0.03 pH units. pH measurements were made by using a Crison pH-meter furnished with a Metrohm EA120 electrode that was previously calibrated with aqueous buffers at 25.0°C. After the Schiff base was obtained, in order to carry out the band-shape analysis of the electronic spectra, the solutions were allowed to stay for 25 min so that we to ensure that the equilibrium was reached. Data over the wavelength range 500–200 nm ($\nu = 20.000$–50.000 cm^{-1}) were acquired, absorbances being measured to the nearest 0.001 unit. Full spectra of the solutions were

recorded on a Uvikon 941 spectrophotometer equipped with cells of 1.0 cm light path at a constant temperature of 25.0 ± 0.05°C.

The equilibrium constant, K_{pH}, can be expressed as follows:

$$K_{pH} = \frac{[B]_e}{[P]_e[L]_e} \quad (2)$$

where $[B]_e$, $[P]_e$ and $[L]_e$ denote the equilibrium concentrations of the Schiff base, aldehyde and amine, respectively. In the wavelength range of interest, the absorption of the mixture formed by the aldehyde and its corresponding aldimines is exclusively due to the absorption of these two compounds rather than the free amine present. Therefore, at equilibrium and a given wavelength, the overall absorption of the sample will be given by,

$$A(\lambda) = [P]_e E_P(\lambda) + [B]_e E_B(\lambda), \quad (3)$$

where $E_P(\lambda)$ and $E_B(\lambda)$ are the molar absorptions of the aldehyde and Schiff base, respectively. The spectra of the Schiff base were acquired by using a computer assisted method that fitted the experimental results to Eq. (3). The concentrations $[P]_e$ and $[B]_e$ were determined from Eq. (2); the $E_P(\lambda)$ values used for this purpose were either obtained in previous work [21,26] or taken from Harris et al. [40]. and the K_{pH} were obtained in this work.

Spectra were deconvoluted into lognormal curves by using the method of Metzler et al. [41]. The wavenumber of maximum absorption, the maximum molar absorption, and the bandwidth and its skewness were the four input data required by the computer in all instances. The programme minimized the sum of the squares of the deviations and obtained the output data from the best fit, which allowed the area (integrated intensity) of the absorption band of each ionic species to be determined.

3. Results and discussion.

Figs. 1–3 show the experimental results obtained for the logarithm of the rate of formation

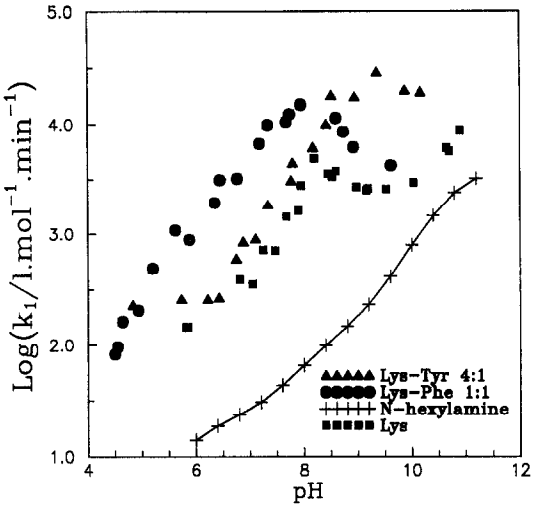


Fig. 1. Plot of log k_1 versus pH for different adducts of PLP with n-hexylamine, homo and copolymers of L-lysine.

(k_1), for the logarithm of the rate of hydrolysis (k_2) and for the equilibrium constant (K_{pH}) as a function of pH for the Schiff bases of PLP with poly(Lys, Tyr) and poly(Lys, Phe). The figures also include the results obtained for the Schiff bases of PLP with poly-L-lysine homopolymers (degree of polymerization (DP) = 277 and 1150) [33] and for the Schiff bases of PLP with n-hexylamine, at the same temperature and ionic strength [11,14].

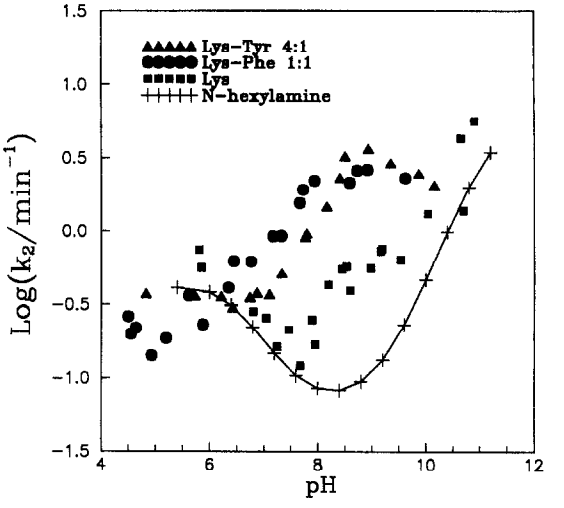


Fig. 2. Plot of log k_2 versus pH for different adducts of PLP with n-hexylamine, homo and copolymers of L-lysine.

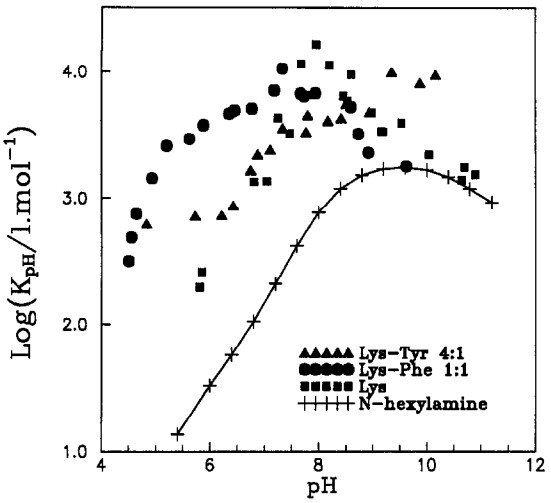


Fig. 3. Plot of log K_{pH} versus pH for different adducts of PLP with n-hexylamine, homo and copolymers of L-lysine.

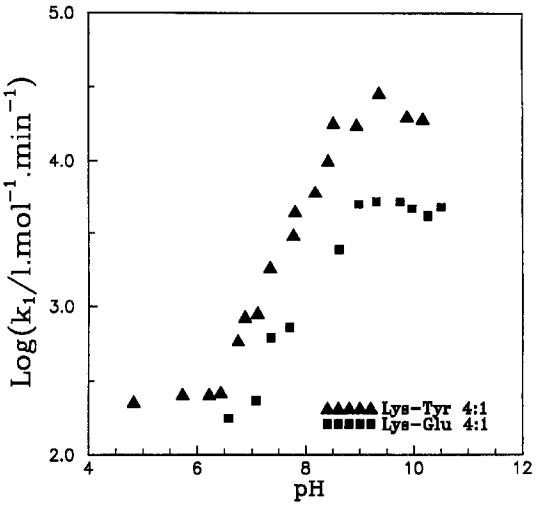


Fig. 4. Plot of log k_1 versus pH for different adducts of PLP with poly(Lys, Tyr) 4:1 and poly(Lys, Glu) 4:1.

The k_1 values obtained in the formation of Schiff bases of PLP with polymers containing L-lysine (Fig. 1), are between 30 and 100 times greater than those which are obtained for the Schiff bases from PLP with n-hexylamine. This increase is due to the presence of $-NH_3^+$ groups in the lateral chains of the polymer that favors the acid catalysis of dehydratation of the carbinolamine intermediate, through a acid catalysis, and therefore the formation of Schiff base. Furthermore the polypeptide skeleton of the polymers can establish hydrogen bonds with molecules of the solvent, providing proton carrier molecules to the environment where Schiff base will be formed, which favors the catalysis. To basic pH, when the groups amino terminal of the lateral chains are unprotonated, the values of k_1 for the systems of the Fig. 1 are similar.

The k_1 values obtained in this work, to acids pH, as a rule they are greater than those of the PLP–poly-L-lysine system (Fig. 1) [33], PLP–poly(Lys, Ala) system (Fig. 4) [36] and PLP–poly(Lys, Glu) system (Fig. 5) [36]. As to acids pH, k_1, is independent of the grade of polymerization [33] and of the number of charges in the lateral chains of the copolymers, as well as of its sign and distribution [36], the differences which were found should be related to the composition of those lateral chains, that is to say to the existence of aromatic groups in them.

The presence of aromatic groups in the lateral chains of the polypeptide favors the formation of Schiff bases, by the interaction with the aromatic ring of the molecule of PLP. It can be thought about some type of stacking between the aromatic ring of the L-phenylalanine and the L-tyrosine and the pyridinic ring of the PLP. Thus, for the copolymer of L-tyrosine, the values of k_1 are similar to those of the systems

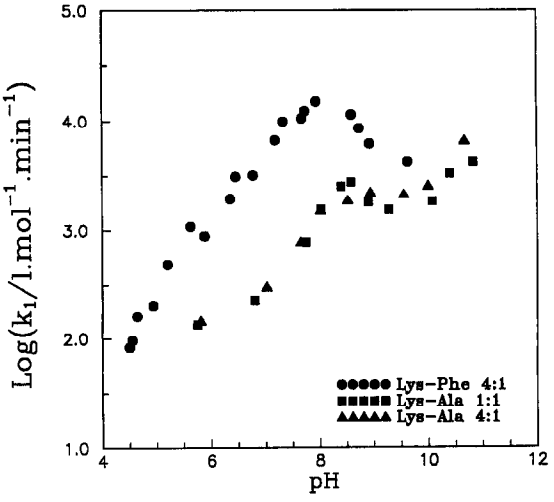


Fig. 5. Plot of log k_1 versus pH for different adducts of PLP with poly(Lys, Phe) 4:1, poly(Lys, Ala) 4:1 and poly(Lys, Ala) 1:1.

PLP–poly-L-lysine (Fig. 1) and PLP–poly(Lys, Glu) (Fig. 5) due to the fact that the number of aromatic groups in its composition is small (20%), while for the copolymer of L-phenylalanine, the values of k_1 are greater than for the systems PLP–poly-L-lysine (Fig. 1) and PLP–poly(Lys, Ala) (Fig. 4) due to the fact that the number of aromatic groups in its composition is increased (50%).

The system PLP–poly(Lys, Phe) shows the presence of a maximum value for k_1 when pH is about 8 (Fig. 1). This behavior is similar to those of other Schiff bases of PLP and polymers that contain L-lysine (Figs. 1 and 5) and it is related to the change of conformation of the polypeptide. Above pH 8, the formation of the α-helix, the most stable conformation of poly-L-lysine in these media [42], makes k_1 dependent on the copolymer composition. The Schiff bases of PLP and poly(Lys, Phe) to pH superior to pH = 8 are found with the protonable groups of the different lateral chains without charge, what permits the stabilization of these conformation. The same effect is observed in the formation of the Schiff bases of PLP and copolymers of L-lysine and L-alanine (Fig. 5) [36]. It is interesting to point out that the stabilization of the α-helix conformation begins to the same pH for the three systems shown in the Fig. 4. The Schiff bases of the L-tyrosine copolymers have the largest k_1 values to pH superior to 8 (Fig. 4). Inasmuch as the hydroxyl group of the L-tyrosine rest unprotonated and hence charged at these pH values, the copolymer will hardly adopt the α-helix conformation, i.e. the statistical chain is more reactive than the α-helix. Further support for this assumption is provided by the k_1 values obtained for the copolymers containing L-glutamic acid [36].

The values of the rate constants of hydrolyisis of the Schiff bases, k_2, obtained for the copolymers scope in this work to basic pH are greater than the corresponding to the Schiff bases of PLP and n-hexylamine (Fig. 2) being also superior to the of those system PLP–poly-L-lysine. Since the α-helix is stable to basic pH, the Schiff bases scope of this study are more hydrolyzable in this conformation. To neutral

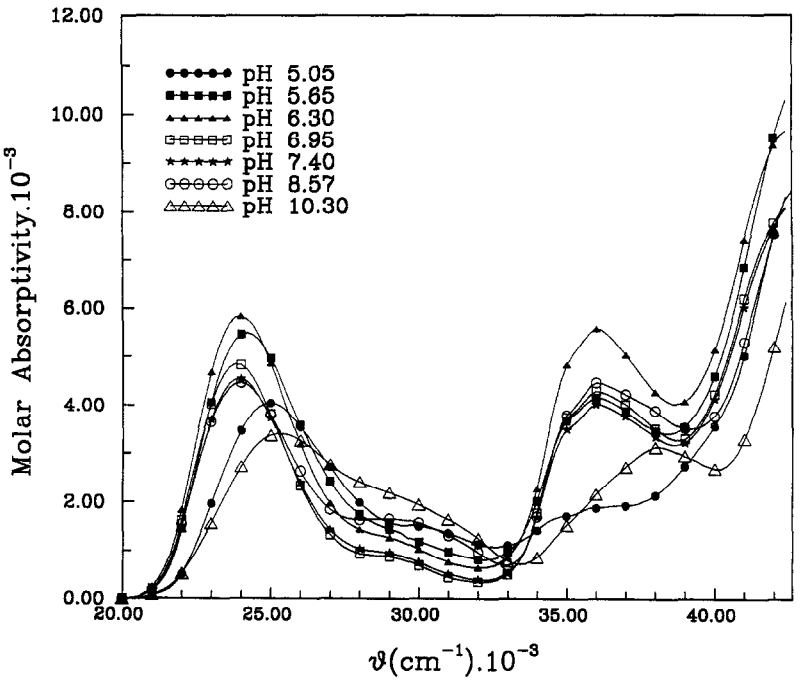
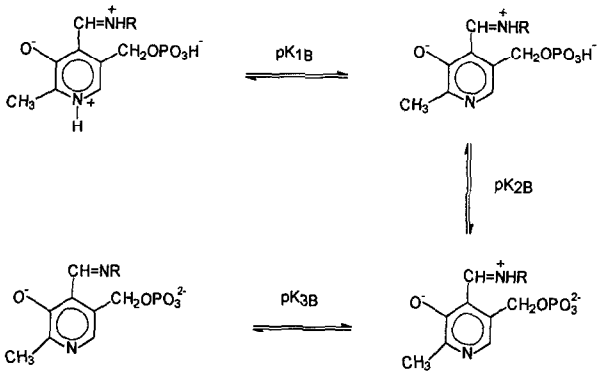


Fig. 6. UV-visible absorption spectra of the Schiff base of PLP with poly(Lys, Tyr) at various pH.



Scheme 1.

pH, that is to say physiological, the rate constants of hydrolysis are similar for all the systems, being therefore equally hydrolyzable all the Schiff bases of PLP with homo and copolymers of L-lysine.

The minima that are shown in the representations of k_2 versus pH (Fig. 2) appear to more acids pH (pH = 5 system PLP–poly(Lys, Phe) and pH = 6.5 system PLP–poly(Lys, Tyr)) than for the system PLP–poly-L-lysine (pH = 7–7.5) and for the Schiff bases of PLP and n-hexylamine (pH = 8.5).

Because of the large k_1 values obtained, the corresponding K_{pH} values are also quite large (Fig. 3). Above pH 9, the K_{pH} values of the PLP–poly-L-lysine system and the PLP–poly(Lys, Phe) tends to approach those of the Schiff bases of PLP and n-hexylamine. Only the

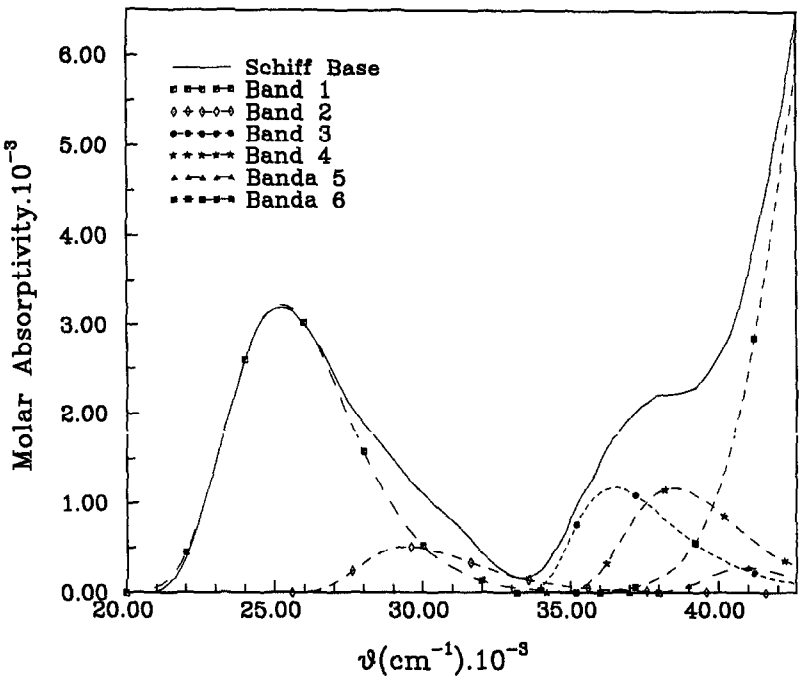


Fig. 7. UV-visible absorption spectrum (deconvoluted into lognormal curves) of the chemical specie B of the Schiff base of PLP with poly(Lys, Tyr).

system PLP–poly(Lys, Tyr) does not continue this behavior due to the no stabilization of the conformation of α-helix.

In the Fig. 6 are shown some spectra of the Schiff bases of PLP and poly(Lys, Tyr) in different media of pH understood between 5.05 and 10.94, in the range of 500 to 235 nm (of 20 to 42.6×10^3 cm^{-1}). It is deduced from its analysis that in that range of pH three macroscopic pK exist, that correspond to the unprotonation equilibriums of the ionic species of the Schiff bases. These ionic species we will designate as B, BH, BH_2 and BH_3 depending on the number of protons that may have linked (Scheme 1).

The macroscopic pK obtained correspond to the values of 10.29, 8.90 and 5.64. These values are similar to the ones obtained in analogous systems of Schiff bases from PLP and copolymers from L-lysine (10.92, 7.74 and 6.62) [36]. However the most basic pK, that corresponds to the protonation of the iminic nitrogen, according to the assignment usually accepted [43], and the intermediate pK that corresponds to the protonation of the phosphate group, are displaced to values more acids and more basics respectively. This type of shift in the pK, have already been observed in the study of the Schiff bases of PLP or 5'-deoxypyridoxal with n-hexylamine, when the polarity of the medium was varied [18,24]. If the polarity of the environment, where the Schiff base is found, is less than the polarity of water, the pK of the iminic nitrogen and of the phosphate group suffer shifts as the described. The Schiff bases of PLP and n-hexylamine in a medium water–ethanol 1:4 (w/w) present pK of 10.20 and 8.69 [18], very similar to the obtained for the Schiff bases of the system PLP–poly(Lys, Tyr).

In the Fig. 7 the spectrum of the ionic specie B of the Schiff base of PLP–poly(Lys, Tyr) is shown as well as the bands lognormal obtained in the adjustment that is described in the experimental section. In Table 1 the values of the parameters of the bands lognormal of the adjustments of the species B, BH, BH_2 and BH_3 are shown. The results are similar to the ones described for other Schiff bases, as much of primary amines as of amino acids and PLP [21,43], as the obtained for the Schiff bases from PLP and poly-L-lysine [33,44]. The more meaningful of the data of Table 1 is the presence so much for the species BH and BH_2 of bands to approximately 29×10^3 cm^{-1} that correspond to exist-

Table 1
Position and shapes of absorption bands of two Schiff bases resolved with lognormal distribution curves

Schiff base	Band maximum $\nu \cdot 10^{-3}$ (cm^{-1})	Height (M^{-1} cm^{-1})	Width $\cdot 10^{-3}$ (cm^{-1})	ρ
PLP–poly(Lys, Tyr)				
B	25.24	3.2412	4.8030	1.296
	29.49	0.5188	4.6350	1.542
	36.48	1.1876	4.1140	1.684
	38.51	1.1811	4.4700	1.456
	40.96	0.2840	3.3590	1.215
	43.77	7.2196	4.0310	0.839
BH	34.37	3.5850	5.9600	1.455
	29.07	5.8485	3.1680	1.026
	31.68	7.0493	4.1100	1.419
	35.65	6.7067	3.2540	1.141
	38.45	10.9110	3.1720	1.068
	42.94	6.1357	3.0430	1.223
BH_2	23.88	5.1815	3.5150	1.307
	28.40	0.2618	3.2640	1.205
	35.95	4.2763	4.4830	1.756
	40.40	0.8096	3.2320	2.253
	43.10	8.7180	5.3370	1.255
BH_3	25.09	4.6926	4.6170	1.338
	30.40	1.4331	4.6520	1.462
	33.43	0.6579	4.2250	1.449
	35.43	1.5614	3.7180	1.309
	39.12	2.5438	4.5300	1.320
	43.64	9.5001	5.2470	1.227
PLP–poly(Lys, Phe)				
BH	23.35	1.1913	3.0630	1.149
	28.64	0.1007	3.0430	1.023
	29.80	1.8285	3.9830	1.018
	32.86	0.4293	4.1100	1.419
	35.94	1.4067	4.0610	1.184
	39.71	2.6885	3.9980	1.162
BH_2	23.60	1.1030	3.5410	1.300
	29.40	2.5008	3.8640	1.195
	31.89	0.7501	2.7730	1.219
	35.35	1.8618	4.3630	1.229
	38.99	4.6103	3.2730	1.034
	42.90	6.5462	5.8770	1.450

ing forms in media of low polarity [45,46] confirming the existence of an environment more hydrophobic for the Schiff bases of the system PLP–poly(Lys, Tyr).

In a similar way it was accomplished the study of the electronic spectra of the Schiff bases of the system PLP–poly(Lys, Phe), being found three macroscopic pK with values of 10.42, 7.60 and 5.08. Now the environment continues being apolar but quite more polar than that of the Schiff bases PLP–poly(Lys, Tyr). This fact remains confirmed by the band-shape analysis of electronic spectra of the species BH and BH_2 of these Schiff bases (Table 1).

Finally the aromatic rings presence in the lateral chains of the polypeptide does not only favor the formation of the Schiff bases, but also favors the existence of a hydrophobic environment.

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