169. On the Stability of the *Schiff* Bases of Pyridoxal 5′-Phosphate with Polypeptides Containing L-Lysine

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We determined the apparent equilibrium constant of formation, K_{pH}, of the *Schiff* bases of pyridoxal 5′-phosphate (PLP) and poly- and copolymers containing L-lysine, as a function of pH at 25° and a constant ionic strength of 0.1M. The K_{pH} values obtained at acidic and neutral pH were larger that those reported for *Schiff* bases of PLP and hexylamine. We determined calorimetrically ΔH of formation of *Schiff* bases of PLP and poly(L-lysine) (−4.5 kcal/mol), and PLP and hexylamine (−3.4 kcal/mol) at pH 7.00. Semi-empirical theoretical calculations (INDO and AM1 methods) of a model compound of *Schiff* base of PLP and polypeptide containing L-lysine show the capability of specific interactions between groups of PLP and the peptide skeleton.

Introduction. – A number of enzymes related to α-amino-acid metabolism require pyridoxal 5′-phosphate (PLP) as coenzyme [1]. In every PLP-dependent enzyme studied so far, PLP is bound to the protein *via* a *Schiff* base (imine) in a moiety of variable polarity [2–4].

The reaction yielding the *Schiff* base involves the carbonyl group of PLP and the ε-amino function of an L-lysine residue of the polypeptide chain, and gives rise to a carbinolamine that is subsequently dehydrated to the final imine [5]. Both steps are reversible and subject to general acid-base catalysis. In addition, the dehydration of carbinolamines from PLP and its analogues is subject to intramolecular acid catalysis [6–14].

The *Schiff* bases of PLP and its analogues with primary amines [8–21], amino-alkylphosphonic acids [22–25], and amino acids [16] [26–37] have been the subject of extensive research basically concerned with the stability, formation, and hydrolysis of the *Schiff* bases, both in aqueous media [8] [10] [12] [14] [16–36], and in H_2O/alcohol [11] [15] [16] and H_2O/dioxane mixtures [9] [13], and with the quantification of the different forms present in the medium, the degree of ionization of which depends on the groups, which can be

protonated [12] [20] [37]. The formation of the *Schiff* bases has also been studied in relation to the reaction temperature [10] [14] [16] [29] and the stability of chelates formed by the *Schiff* bases of PLP and its analogues with transition metals [22–27] [32]. In any case, the ultimate goal was always to compare the spectroscopic and thermodynamic behavior of PLP in these systems with that it exhibits on binding to different proteins.

In a recent work [38], we reported a new, more accurately descriptive model for PLP–enzyme binding that was applied to the formation and hydrolysis of *Schiff* bases of PLP and poly(L-lysines). The micro-environment surrounding the *Schiff* base was found to influence decisively its rate constants of formation and hydrolysis.

In the present work, we analyzed the factors increasing the stability of this type of compounds by studying the stability of *Schiff* bases of PLP and poly(L-lysine) with different degrees of polymerization (DP), and those of PLP, and L-lysine/L-alanine or L-lysine/glutamic acid copolymers of different compositions. The results obtained allowed us to determine the effect of the presence of neutral and charged groups in the amino-acid side chains on the stability of the PLP–enzyme bond. Such results are discussed on the basis of information obtained from calorimetric determinations and semi-empirical theoretical calculations, and the potential interactions involved are analyzed.

Experimental. – The polypeptides used were supplied by *Sigma Chemical Co.* The molecular weight of the poly(L-lysines) used as determined viscometrically were 3500 (DP = 17), 58000 (DP = 277), 100500 (DP = 481), and 240000 (DP = 1150) Daltons, while those of the random L-lysine copolymers were as follows: poly(Lys·HBr,Ala) 1:1 40000 (DP = 280), poly(Lys·HBr,Ala) 4:1 38000 (DP = 210), and poly(Glu, Lys·HBr) 1:4 200000 (DP = 1000) Daltons. Both PLP and all other reagents used were *Merck* reagent-grade chemicals.

Acetate, phosphate, and carbonate buffers adjusted to ionic strength 0.1M were used throughout.

PLP solns. were prepared daily in the appropriate buffer and stored in the dark. Their exact concentrations were determined from their absorbances at 295 nm ($\varepsilon = 6700$ l mol^{-1}cm^{-1}) after dilution in 0.1M HCl [39]. The working concentrations used ranged between 5.10^{-5} and 10^{-4} M.

The polypeptide solns. were also prepared daily by dissolving the required amount of polymer in the appropriate buffer. The concentrations used ranged between $5 \cdot 10^{-2}$ and 10^{-3} M.

The reaction between an aldehyde and an amine to form a *Schiff* base can be represented by:

$$R^1\text{–CHO}+NH_2\text{–}R^2 \underset{k_2}{\overset{k_1}{\rightleftarrows}} R^1\text{–CH=N–}R^2+H_2O \qquad (1)$$

where k_1 and k_2 are the overall rate constants of formation and of hydrolysis of the *Schiff* base, respectively.

The *Schiff* bases were obtained by adding known volumes of PLP soln. to the corresponding polypeptide soln., which was previously thermostated at 25 ± 0.05°. Base formation was monitored by measuring absorption changes at 280, 380, or 440 nm as a function of pH on a *Perkin-Elmer* spectrophotometer furnished with thermostated cells of 1 cm light path.

The equilibrium constant at each pH, K_{pH}, was calculated as the k_1/k_2 ratio. These formation constants in turn were determined by a method reported in [11].

Calorimetric measurements on the reactions of PLP with poly(L-lysine) and hexylamine were made on a set-up consisting of an *LKB* batch microcalorimeter and a titration assembly [40]. The syringe system used was calibrated by weighing the amount of H_2O displaced. The reaction vessel was loaded with 4 ml of $1.01 \cdot 10^{-5}$ M poly(L-lysine) ($4.86 \cdot 10^{-3}$ M in NH_2 groups) or with 4 ml of $4.86 \cdot 10^{-3}$ M hexylamine, both of 0.1M ionic strength, while the reference vessel was loaded with 4 ml of the appropriate buffer. The two 0.5-ml *Hamilton* syringes were filled with $9.029 \cdot 10^{-3}$ M PLP in every case. Blank runs were performed in order to determine differential compression-mixing effects. Both vessels were loaded with 4 ml of buffer. The resulting heating effects were used to correct the heats measured in the experiments.

The large size of the systems involved compelled us to use semi-empirical methods, which, unfortunately, provide inaccurate results for systems containing second-row atoms. The absence of phosphate-group interactions, which can be ruled out from the results reported below, led us to use {*N*-[6-(formylamino)hexyl]-3-hydroxy-2,5-dimethylpyridin-4-yl}methylideneamine as a model compound for the systems studied and analyze the different conformations of the chain:

$$-CH=N-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH-CHO$$

The INDO method [41] provides good-enough geometric and energy results for systems similar to those studied here (see [42] and ref. cit. therein). However, it also systematically overestimates H-bonding energies, which compelled us to determine theoretical structures and energies by the AM1 method [43] (this is more accurate than the INDO method in this respect; see [44] for an exception to this rule) as well.

All calculations were performed at the Computational Centre of the Autonomous University of Madrid by using the programme GEOMOS QCPE 584, developed by *D. Rinaldi*, *E. Cartier*, and *P.A. Hoggan*, of the University of Nancy (France). The programme was provided by one of its developers and was run on an *IBM 4381* computer under the VN/SP operating system. Geometries were fully optimized within the INDO and AM1 frameworks.

Results and Discussion. – The stability of the *Schiff* bases of PLP and its analogues with various amines, over wide pH ranges, has been studied by several authors on the basis of kinetic formation and hydrolysis and absorption-spectroscopy data [8–14] [16] [33] [45]. As a rule, the stability of the *Schiff* bases increases with the presence of electron-releasing groups in the amine and electrophilic groups in the carbonyl compound. The maximum stability constants of *Schiff* bases in aqueous solutions at 25° range between $10^{-0.3}$ M for the PLP/aminoacetonitrile system to $10^{3.2}$ M for the PLP/hexylamine system. In nonaqueous media (*e.g.* H_2O/EtOH or H_2O/dioxane mixtures), such stability constants are normally larger (occasionally as large as $K_{pH} = 10^4$).

In this work, we performed a kinetic study on the stability of the *Schiff* bases of PLP and polypeptides containing L-lysine.

Fig. 1 shows the variation of K_{pH} of the *Schiff* bases, formed between PLP and poly(L-lysine) in different degrees of polymerization, with pH. For comparison, it also includes the results obtained for the *Schiff* bases of PLP and hexylamine [12] at the same temperature and ionic strength. As can be seen, K_{pH} varies with the degree of polymerization, particularly over the pH range 7.5–9, and is always larger than that of the *Schiff* bases of PLP and hexylamine in media of high [8] [10] [12] and low dielectric constant [11], whether acidic or moderately basic. Above pH 9, the K_{pH} of the PLP/poly(L-lysine) system

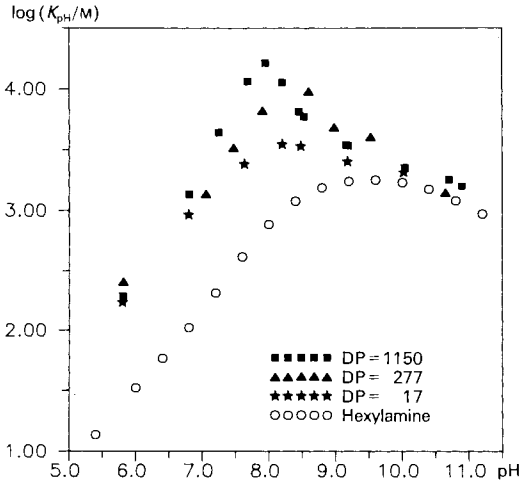

Fig. 1. *Variation of log* K_{pH} vs. *pH for different PLP/poly(L-lysine) adducts of different DP.* Data of PLP/hexylamine taken from [6] [12].

tends to approach that of the PLP/hexylamine system. As can be seen, the maximum of the K_{pH} *vs.* pH plot lies at a lower pH than that for the *Schiff* bases of PLP and hexylamine. In addition, the maximum is much better defined in the former case, and varies with the degree of polymerization. In fact, it shifts to lower pH values and becomes better defined as the degree of polymerization increases. However, K_{pH} does not vary significantly with the degree of polymerization in acidic media.

Fig. 2 shows the experimental K_{pH} values for the formation of the *Schiff* bases of PLP and L-lysine and L-alanine copolymers, together with those of the bases of PLP and poly(L-lysine) of DP = 277 (*i.e.* of the same order as the previous ones). Likewise, *Fig. 3* also shows the K_{pH} values of the *Schiff* bases of PLP and L-lysine and L-glutamic-acid copolymers.

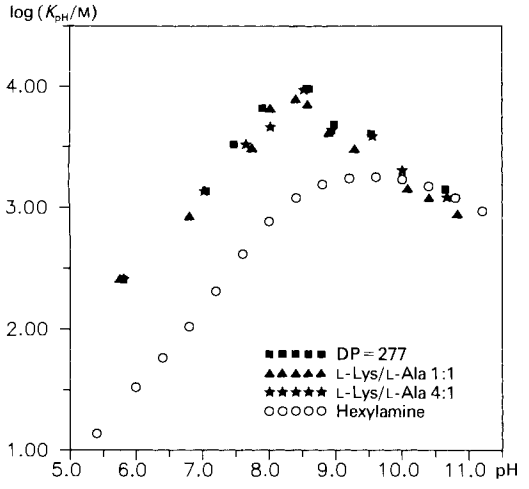

Fig. 2. *Variation of log* K_{pH} vs. *pH for different adducts of PLP and copolymers of* L-*Lys*/L-*Ala of different composition, and PLP/poly(*L-*lysine)* (DP = 277). Data of PLP/hexylamine taken from [6] [12].

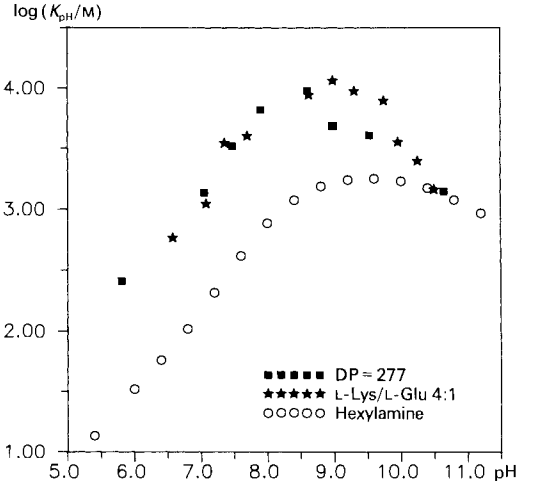

Fig. 3. *Variation of log* K_{pH} vs. *pH for different adducts of PLP and copolymers of* L-*Lys*/L-*Glu of different composition, and PLP/poly(*L-*lysine)* (DP = 277). Data of PLP/hexylamine taken from [6] [12].

Above pH 7, K_{pH} depends on the L-lysine content (*Fig. 2*): K_{pH} increases with increasing pH. On the other hand, the K_{pH} of the *Schiff* bases of PLP and the L-lysine and L-glutamic-acid copolymers varies rather differently (*Fig. 3*), and the pH of the maximum is similar to that of the PLP/hexylamine system. These differences are related to the ease of formation of the α-helix, which depends on the nature of the side chains and is more stable in basic media [46]. Below pH 7, K_{pH} is virtually independent of the polymer composition (*Figs. 2* and *3*).

The difference in K_{pH} between the PLP/polypeptide and PLP/hexylamine systems should, therefore, be ascribed to the occurrence of different interactions in the two systems, which were evidenced in studying the rate constants of formation of the *Schiff* bases [38]. The use of a polypeptide as an NH_2-group carrier results in further effects such as electrostatic interactions between charged PLP groups (phosphate, pyridine-N and phenolic OH) and charged functions on the side chains of the polymer or copolymer

containing groups of pK between 10 and 11 (ε-amino) or between 4 and 5 (γ-acid), or even interactions between groups of PLP and others in the peptide skeleton such as those reported to occur between L-tyrosine residues and the peptide skeleton of some proteins [47].

Below pH 7–7.5, electrostatic interactions between charged groups of the polypeptides used and other charged groups of PLP seem to play no significant role as variations in the composition of the polypeptide side chains, *i.e.* in the number (DP), arrangement (composition), and sign of electric charges (L-lysine/glutamic-acid copolymer), result in no significant differences in K_{pH} (*Figs. 2* and *3*). Therefore, this type of interaction can be assumed not to play a major role in the stability of the *Schiff* bases, at least under the working conditions used here.

The only plausible explanation for the greater stability of the *Schiff* bases of PLP and poly(L-lysine) in neutral media is the occurrence of specific interactions not present in the PLP/hexylamine system. This shows as a difference between the enthalpy of formation of these two *Schiff* bases.

The ΔH value determined calorimetrically for the reaction between PLP and poly(L-lysine) or hexylamine is not merely the heat of formation of the *Schiff* base. In fact, it includes: *a*) the deprotonation of the pyridine N-atom, the pK of which is shifted to lower pH values; *b*) the dehydration of PLP, the unhydrated form of which is actually the reacting species; *c*) the protonation of the phenolic OH group to form a H-bond with the pyridine N-atom. By subtracting the reported ΔH values for processes *a*, *b*, and *c*, we determined the enthalpies of formation at pH 7 to be −4.5 kcal/mol for the PLP/polypeptide system and −3.4 kcal/mol for the PLP/hexylamine system.

The calorimetrically obtained $\Delta H = -4.5$ kcal/mol is of the same order as that one would expect from a H-bonding interaction (between −4 and −6 kcal/mol). The difference between the enthalpies of formation of the two *Schiff* bases, $\delta(\Delta H) = -1.1$ kcal/mol, can be ascribed to the greater strength of the H-bond involving the phenolic OH group and the imine N-atom [48] or to the appearance of one or more new types of interaction that could, in principle, involve groups on the side chains or skeleton of the peptide and phenol or phosphate O^- groups or pyridine NH^+ groups of PLP in the *Schiff* base. However, any potential interactions *via* the phosphate group can be ruled out, since stability assays performed on the *Schiff* bases of pyridoxal and poly(L-lysine) confirmed that the interactions were identical in both cases. Interactions involving the pyridine NH^+ group can also be discarded, because the pK value of this group in the *Schiff* bases is 6.3, and no changes in K_{pH} values observed in this region.

A plausible explanation for the increased stability of the *Schiff* bases with the polypeptides would involve assuming the occurrence of a further interaction between the peptide skeleton and the above-mentioned H-bond. We checked theoretically for the feasibility of structures with a stabilized intramolecular H-bond involving the CO or NH group of the polypeptide skeleton. Likewise, we considered changes in the dipole moment of these structures: the higher this is, the more stabilized by solvation, and hence, the more likely to occur in aqueous phase the structures will be.

Considering the results obtained for the different conformations, only the energetically most stable and that with an extended terminal chain (structure **C**) and thus unable to form an intramolecular H-bond with the terminal NH–CHO group of the model molecule are commented on.

The INDO method allowed us to obtain two very favorable structures (**A** and **B**). Conformations **A**, **B**, and **C** are depicted in *Fig. 4*, and their corresponding energies and dipole moments are listed in the *Table*.

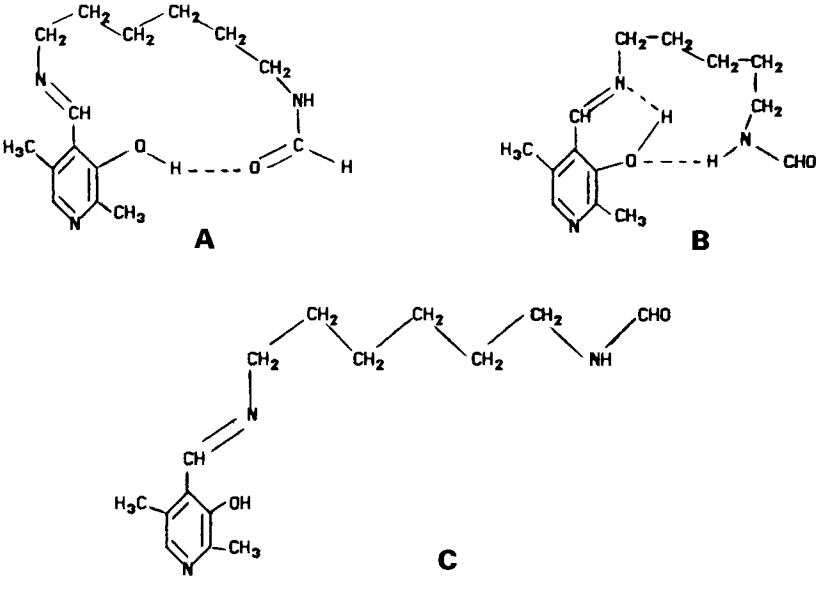

Fig. 4. *Conformations of the optimized geometries by INDO and AM1 methods*

Table. *Energies and Dipole Moments of Some of the Optimized Geometries Provided by the INDO and AM1 Methods*

Structure	Energy [kcal/mol]	Increment	Dipole moment [Debye]	Gradient
Optimized INDO geometries				
A	−118822.9	−13.9	7.75	0.000007
B	−118829.2	−10.2	9.68	0.000006
C	−118819.0	−0.0	4.62	0.000004
Optimized AM1 geometries				
D	−69.4	−2.1	6.08	0.000006
E	−69.1	−1.8	7.67	0.000007
F	−67.3	0.0	1.55	0.000007

None of the three conformations is fully planar. Conformation **A** features a H-bond between the proton in the group and the terminal C=O group, while conformation **B** has two H-bonds: one between the OH proton and the imine N-atom at one end of the chain (six-membered pseudo-cycle), and the other between the OH O-atom and the proton bonded to the terminal N-atom in the chain. Conformation **C** is a fully stretched chain. Structures **A** and **B** have larger dipole moments, so their occurrence in an aqueous solution will be more favorable.

The systematic overestimation of H-bonding energies by the INDO method makes structures **A** and **B** much more favorable than **C**. Even so, in view of the experimental

results, we believe that the occurrence of an intramolecular H-bond stabilized by a different type of interaction is also favored.

Calculations were subsequently refined and structures re-optimized by the AM1 method. The results obtained for the most significant structures are summarized in the *Table*.

First of all, it should be stressed that the AM1 method does not overestimate H-bonding energies to the extent of the INDO method. Rather, it occasionally provides underestimated values. Structures **D** (similar to **A** obtained by the INDO method) and **E** are not planar and possess a H-bond between the OH proton and the O-atom in the CHO group, even though the orientation of the OH group is different in the two structures. Structure **E** possesses another H-bond between the OH proton and the imine N-atom at one end of the chain. The data obtained for structure **F** (identical with structure **C** yielded by the INDO method) show that structures **D** and **E** are by 2 kcal/mol more stable and have much larger dipole moments, which favors their occurrence in an aqueous medium.

It should be stressed that the most favorable structures provided by the INDO method are roughly coincident with those yielded by the more modern AM1 method. In fact, both predict the occurrence of a H-bond stabilized by another intramolecular H-bond. Thus, both the experimental evidence and the theoretical calculations performed by the INDO and AM1 methods support the occurrence of conformations with an intermolecular H-bond additionally stabilized by a rest of the peptide skeleton.

In summary, the *Schiff* bases formed between PLP and polypeptides in an aqueous medium are stabilized by specific interactions between the phenol group of PLP and a C=O or NH group of the peptide skeleton. The K_{pH} value of these systems is typically larger by *ca.* one order of magnitude than those of other model systems in nonaqueous media, even if binding to low-molecular-weight polymers is considered. The assumption that the stability of the *Schiff* bases is only dependent on the dielectric constant of the medium must, therefore, be revised. The influence of C=O and NH group on the peptide skeleton must be considered in accounting for the stability of the linkage of PLP to a protein.

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