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Kinetic and thermodynamic study of the reaction of pyridoxal 5′-phosphate with L-tryptophan

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ABSTRACT: The apparent rate constants for formation (k_1) and hydrolysis (k_2) of the Schiff bases formed by reaction of pyridoxal 5′-phosphate with L-tryptophan were determined at various pH values, at different temperatures and at constant ionic strength (0.1 M). Also obtained were the elementary rate constants for formation and hydrolysis of the Schiff bases corresponding to the different chemical species present in the media, and the pK values of the Schiff's bases. The activation and thermodynamic parameters for the formation and hydrolysis of the Schiff's bases also were determined. Some of the ΔH^0 and ΔS^0 values for the individual processes were found to be positive. In basic media the enthalpic factor is unfavorable but the entropic contribution leads to a negative ΔG^0. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: Schiff base; L-tryptophan; PLP

INTRODUCTION

An important number of enzymes related to amino acid metabolism require pyridoxal 5′-phosphate (PLP) as coenzyme.[1–3] The first step in the formation of a Schiff base from PLP involves an aminomethanol intermediate by attack of the amino group on the carbon atom of the carbonyl group of PLP. Dehydration of the aminomethanol gives the Schiff base in an acid-catalyzed process.[4,5]

Although the kinetics and mechanism of formation and hydrolysis of the Schiff's bases formed by PLP and various compounds bearing amino groups, such as primary amines, hydrazine derivatives, amino acids and polypeptides, have been the subject of many reports,[6] very few kinetic studies have been carried out on the reactions of PLP or its analogues (e.g. 5′-deoxypyridoxal) with amines or amino acids as a function of pH and temperature.[7–13]

This paper reports the results of a study on the kinetics of formation and hydrolysis of the Schiff bases formed by PLP and L-tryptophan (PLP–TRP system) at variable pH values and temperatures. The activation parameters for both processes and the thermodynamic parameters for the reaction are examined in terms of the elementary rate constants for the species involved in the process (see Scheme 1).

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EXPERIMENTAL

L-Tryptophan was purchased from Sigma Chemical Co. and pyridoxal 5′-phosphate and all other chemicals used were 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 PLP solutions were prepared in appropriate buffers and stored in the dark. Their exact concentrations were determined by dilution in 0.1 M HCl and subsequent measurement of its absorbance at 295 nm ($\varepsilon = 6700\,\mathrm{l\,mol^{-1}\,cm^{-1}}$)[14] and were found to be in the region of 2×10^{-5} M. The L-tryptophan solutions in the concentration range 5×10^{-4}–2×10^{-2} M were also prepared, on a daily basis, by diluting appropriate amounts of stock solutions in the corresponding buffer.

Kinetic measurements were carried out at various pH values by using a Hewlett-Packard 8453 diode array spectrophotometer and an 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 L-tryptophan solutions at the desired temperature and pH.

Table 1. Kinetic parameters[a] for the formation and hydrolysis of the Schiff bases formed by reaction of PLP and L-tryptophan

10 °C			20 °C			30 °C			37 °C		
pH	k_1	$k_2 \times 10^2$	pH	k_1	$k_2 \times 10^2$	pH	k_1	$k_2 \times 10^2$	pH	k_1	$k_2 \times 10^2$
2.80	—	30.0	2.66	—	50.4	2.88	—	162	3.95	20.0	126
3.81	—	30.0	3.91	6.06	46.8	4.00	9.72	96.7	4.89	25.6	138
4.56	5.27	18.0	3.91	5.40	47.7	4.96	18.2	96.7	5.64	54.6	216
6.04	20.8	36.0	4.96	11.7	52.5	5.76	43.5	138	6.62	169	122
6.95	35.8	24.0	5.80	29.8	77.4	6.79	118	90.6	7.18	190	72.0
7.64	43.3	12.0	6.76	73.7	55.5	7.42	150	42.6	7.94	228	15.6
7.91	46.0	5.40	7.36	83.9	30.5	8.06	153	16.8	8.67	241	—
9.06	71.6	2.30	7.99	108	5.70	8.92	165	8.50	9.34	218	19.2
9.48	69.2	2.00	8.76	112	2.50	9.78	140	28.3	9.48	172	36.0
9.75	60.3	2.20	9.16	121	2.80	—	—	—	9.83	181	66.0
10.30	48.5	6.30	9.47	110	4.60	—	—	—	9.89	193	66.0
—	—	—	10.17	80.3	34.8	—	—	—	—	—	—

[a] Units: $mol^{-1}\,min^{-1}$ for k_1 and min^{-1} for k_2. The estimated errors of k_1 and k_2 are not greater than 5%.

Pseudo first-order kinetic curves were observed (L-tryptophan concentration was more than 10-fold larger than PLP) by monitoring the absorbance at 435 nm due to the Schiff base. The observed rate constants, k_{obs}, were determined by means of the spectrophotometer kinetic software for first-order reactions. 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 calibrated previously with aqueous buffers at 25 °C.

The overall reaction between an aldehyde and an amine can be depicted 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 for formation and hydrolysis, respectively, of the Schiff base. The procedure used to calculate these two constants is described in detail elsewhere.[7] Their ratio coincides with the equilibrium constant ($K_{pH} = k_1/k_2$).

The pK_N values for L-tryptophan at the different temperatures studied were determined potentiometrically by titration of a solution of L-tryptophan (0.01 M) and HCl (0.01 M) with NaOH (0.1 M) using a Radiometer autotitrator equipped with a PHM-62 pH-meter, an ABU-11 autoburette, a TTT-60 titrator, an 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 thus obtained were 9.6, 9.3, 9.0 and 8.8 at 10, 20, 30 and 37 °C, respectively, and were confirmed by semi-neutralization.

RESULTS

Table 1 shows the experimental results obtained at different pH values and temperatures for the overall rate constants for formation (k_1) and hydrolysis (k_2) of the Schiff bases of PLP with L-tryptophan.

Reaction measurements could be carried out only at the pH values shown in Table 1 because more alkaline media resulted in intramolecular cyclization of the secondary imine by attack of the indole group on the azomethine carbon, which hindered the reaction and precluded examination beyond this alkalinity level.[6b]

As shown in Scheme 1, the overall rate constants for formation and hydrolysis of the Schiff bases can be described in terms of the elementary constants for the different chemical species present in the medium at each pH.

Table 2 gives the elementary rate constants for formation (k_1^i) and hydrolysis (k_2^i, k_{OH}) and the pK_{iB} obtained by fitting the experimental k_1, k_2 and K_{pH} values to Eqns (2), (3) and (4) derived from Scheme 1:[7]

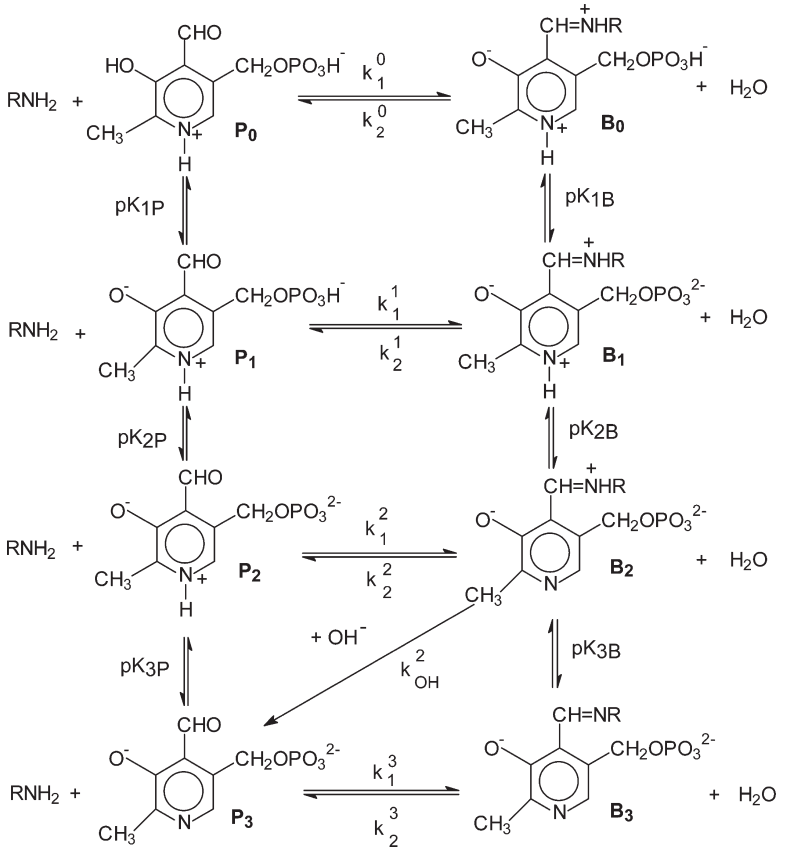
$$k_1 = \frac{k_1^3 + k_1^2 \cdot a/K_{3P} + k_1^1 \cdot a^2/(K_{3P} \cdot K_{2P}) + k_1^0 \cdot a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P})}{(1 + a/K_N)[1 + a/K_{3P} + a^2/(K_{3P} \cdot K_{2P}) + a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P})]} \quad (2)$$

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

$$K_{pH} = \frac{[1 + a/K_{3B} + a^2/(K_{3B} \cdot K_{2B}) + a^3/(K_{3B} \cdot K_{2B} \cdot K_{1B})]K_M}{(1 + a/K_N)[1 + a/K_{3P} + a^2/(K_{3P} \cdot K_{2P}) + a^3/(K_{3P} \cdot K_{2P} \cdot K_{1P})]} \quad (4)$$

where $k_{OH} = k_2^3 + k_{OH}^2(K_W/K_{3B})$, $a = 10^{-pH}$, K_W is the ionic product of water and K_M is the equilibrium constant of formation of the Schiff's base at very high pH $(= k_1^3/k_2^3)$.

Note that, owing to intramolecular cyclization of the Schiff base,[6b] only few experimental points were determined in the alkaline pH range; therefore, values of pK_{3B} and k_{OH} are affected by a significant error.



Scheme 1

DISCUSSION

Table 1 shows that at each temperature, as the pH increases, the k_1 value increases up to a maximum and then decreases. The maxima in the k_1 values are the result of the pK_N for the amino group (the fraction of free amine increases with increasing pH) and of the sequence $k_1^0 > k_1^1 > k_1^2 > k_1^3$ (the reactivity decreases with increasing pH). The net balance between these opposing effects leads to the maxima in the k_1 values.

The rate-determining step of the formation of a Schiff base is known to be the dehydration of an aminomethanol

Table 2. Best kinetic constants and pK values (Scheme 1) obtained by fitting of experimental values of k_1, k_2 and K_{pH} to Eqns (2), (3) and (4)

	10 °C	20 °C	30 °C	37 °C
log k_1^0	6.73 ± 0.03	6.49 ± 0.03	6.57 ± 0.05	6.73 ± 0.04
log k_1^1	4.91 ± 0.02	5.08 ± 0.02	5.06 ± 0.03	4.96 ± 0.03
log k_1^2	2.91 ± 0.03	3.16 ± 0.03	3.22 ± 0.06	3.24 ± 0.04
log k_1^3	1.60 ± 0.05	1.85 ± 0.05	2.07 ± 0.07	2.22 ± 0.04
pK_{1P}[a]	3.61	3.77	3.46	3.39
pK_{2P}[a]	6.32	6.12	6.02	6.05
pK_{3P}[a]	8.68	8.37	8.16	8.13
log k_2^0	−0.74 ± 0.08	−0.23 ± 0.1	0.02 ± 0.1	0.03 ± 0.09
log k_2^1	−0.23 ± 0.04	0.13 ± 0.1	0.54 ± 0.13	0.80 ± 0.07
log k_2^2	−1.85 ± 0.08	−1.37 ± 0.13	−1.05 ± 0.2	−0.94 ± 0.2
log k_{OH}	0.041 ± 0.10	0.46 ± 0.17	0.21 ± 0.28	0.44 ± 0.07
pK_{1B}	5.85 ± 0.18	5.85 ± 1.1	5.73 ± 0.28	5.89 ± 0.32
pK_{2B}	6.88 ± 0.17	6.55 ± 1.1	6.23 ± 0.28	6.05 ± 0.30
pK_{3B}	11.7 ± 2.5	11.3 ± 4.2	10.7 ± 1.0	10.46 ± 0.42
log k_M	1.68 ± 0.03	1.72 ± 0.08	1.76 ± 0.03	1.86 ± 0.04
pK_N	9.60	9.30	9.00	8.80

[a] From refs 7 and 15.

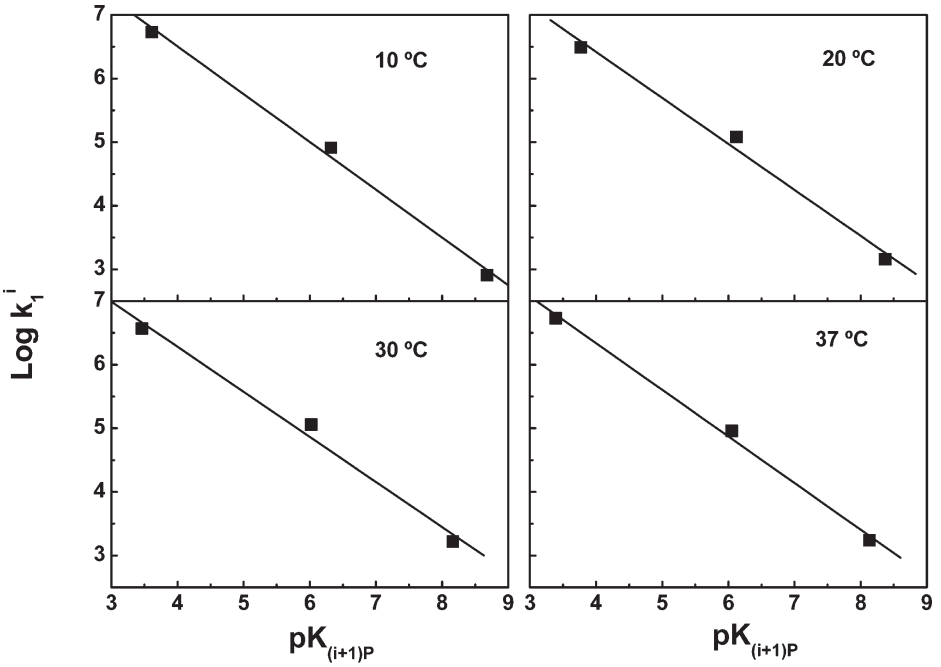


Figure 1. Brønsted plots for the PLP–TRP system at different temperatures

intermediate formed by attack of the amine on the carbonyl group.[4,5] The increase in k_1^i with decreasing pH observed at all the temperatures used (Table 2) is due to the involvement of all the protonable groups in PLP in intramolecular acid catalysis on the formation of the Schiff base. The linear Brønsted plot (Fig. 1) confirms the intramolecular acid catalysis; the values of $\alpha = 0.71$–0.75 are similar to those reported for the formation of Schiff bases of PLP with amines and amino acids.[6,7,13,16] On the other hand, experiments carried out by Llor *et al.*[17,18] exclude the possible influence of general acid catalysis on this reaction by the buffer systems. A similar observation was made by Auld and Bruice[19,20] in the Schiff base formation from 3-hydroxypyridine-4-carbaldehyde.

The rate constant k_2[2] is the smallest among the hydrolysis constants obtained at each temperature (Table 2). Accordingly, species B_2 is the most stable against hydrolysis by water: because its imine nitrogen is protonated, it forms a hydrogen bond with the 3-phenoxy group on the pyridine ring.[21] In other words, the incorporation of a proton in B_3 stabilizes the Schiff base; on the other hand, the incorporation of the second proton to form B_2 facilitates its hydrolysis by water. Figure 2 shows the calculated curves of log k_2 vs. pH at three different temperatures. It can be observed that the minimum shifts from pH 9.2 at 10 °C to pH 8.5 at 37 °C; this shift can be due to a greater shift for pK_{3B} than for pK_{2B} with temperature (see Table 2), in the same way as the shift described by solvent polarity.[22]

Table 2 also shows the pK values for the various forms of the Schiff's bases. As can be seen, pK_{1B} is very similar throughout the temperature range studied. This is quite consistent with expectation because the pK values for phosphoric acid are known to be independent of temperature.[23] However, pK_{2B} decreases with an increase in temperature. This behavior corresponds to the deprotonation of pyridinic nitrogen, as reported for the Schiff's bases of PLP with *n*-hexylamine,[7] PLP with GABA[13] and 5′-deoxypyridoxal with *n*-hexylamine.[8]

The Arrhenius and Eyring plots of the rate constants k_1^i and k_1^i obtained (Fig. 3) allow calculation of the energy of activation (E_a) and activation parameters ($\Delta H^{\pm}$ and $\Delta S^{\pm}$) of the elementary processes of formation and

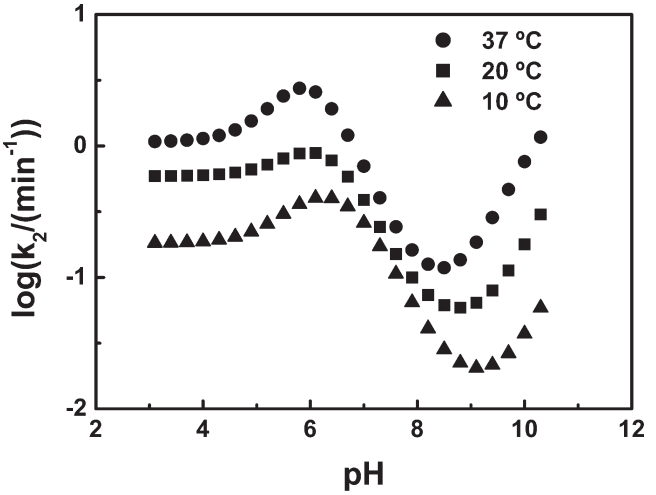


Figure 2. Plot of log k_2 vs. pH for the Schiff bases of pyridoxal 5′-phosphate and L-tryptophan at different temperatures. Points calculated using Eqn (3) and data from Table 2

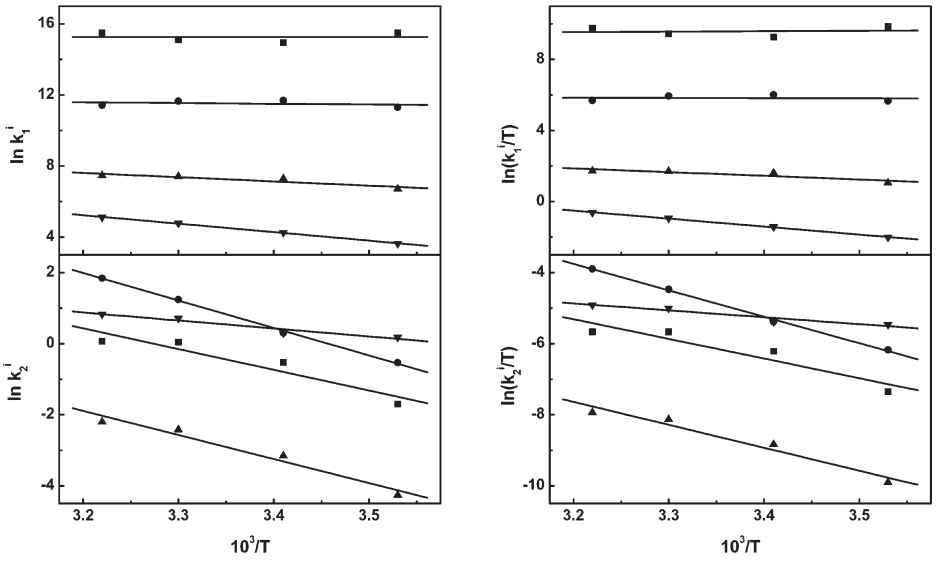


Figure 3. Arrhenius and Eyring plots for the formation (k_1^i) and hydrolysis (k_2^i) of the Schiff bases of pyridoxal 5′-phosphate and L-tryptophan: (■) $i=0$; (●) $i=1$; (▲) $i=2$; (▼) $i=3$

hydrolysis of the Schiff bases (see Table 3). The k_2^3 values were obtained from the relation $K_M = k_1^3/k_2^3$ (see Scheme 1). As can be observed in Table 2, the $k_1{}^0$ values are nearly constant in temperature, suggesting very low $E_a(k_1^0)$ and $\Delta H^{\pm}(k_1^0)$ values in acid media.

As can be seen, for the formation process, $E_a(k_1^i)$ and $\Delta H^{\pm}(k_1^i)$ increase with the increasing number of negative charges in the PLP molecule. In neutral media the values are lower than those reported for the PLP–*n*-hexylamine system[7] and PLP–GABA system,[13] but in basic media they are greater. This behavior can be due to the presence of the carboxylate anion in the α-position, although a catalytic effect due to the carboxylic group has been disregarded.[6b] Nevertheless, it should be noted that, although dehydration of the aminomethanol intermediate is the rate-determining step, there are several previous equilibrium steps that can be affected by the carboxylate group. Our values are also lower than those reported by Wiesinger and Hinz for the reactions of PLP with ε-aminocaproic acid and L-serine.[9]

With regard to hydrolysis of the Schiff base, the $E_a(k_2^i)$ and $\Delta H^{\pm}(k_2^i)$ values for species B_0 in the PLP–TRP system are smaller than those for the Schiff base of *n*-hexylamine[7] or GABA.[13] On the other hand, these parameters for species B_1 and B_2 are very similar in all cases; for B_3 in hydrolysis of the Schiff base of L-tryptophan the values are larger than in the PLP–GABA system.[13]

Table 3 also shows that, as in the reactions of PLP with *n*-hexylamine[7] and GABA,[13] the $\Delta S^{\neq}$ values are negative in all cases, in accordance with the decreased number of degrees of freedom in the transition state: the removal of a water molecule from the aminomethanol intermediate requires the presence of a catalyst, and hydrolysis of the Schiff base is a bimolecular process.

Table 4 shows the thermodynamic parameters for the elementary processes as obtained from the activation parameters corresponding to each elementary process.

Table 3. Energy of activation and activation parameters[a] for the elementary processes ($i=0$–3)

	0	1	2	3
$E_a(k_1^i)$	—	3 ± 8	20 ± 6	40 ± 1
$\Delta H^{\neq}$ (k_1^i)	—	1 ± 1	18 ± 6	38 ± 1
$\Delta S^{\neq}$ (k_1^i)	-140 ± 40	-160 ± 30	-140 ± 20	-100 ± 4
$E_a(k_2^i)$	49 ± 11	65 ± 2	57 ± 7	19 ± 3
$\Delta H^{\neq}$ (k_2^i)	46 ± 10	62 ± 2	54 ± 8	16 ± 3
$\Delta S^{\neq}$ (k_2^i)	-110 ± 40	-46 ± 7	-100 ± 30	-200 ± 10

[a] Units: E_a and $\Delta H^{\neq}$ in kJ mol^{-1}; $\Delta S^{\neq}$ in J mol^{-1} K^{-1}.

Table 4. Thermodynamic parameters for the elementary processes ($i=0$–3) and equilibrium constants obtained from kinetic (Table 2) and thermodynamic parameters (Table 3)[a]

Process i	ΔH^0	ΔS^0	$\Delta G^0{}_{298\,K}$	$\log K_{eq,298\,K}$	$\log\left(K_{pH}^i = k_1^i/k_2^i\right)$
0	-46 ± 10[b]	-30 ± 80	-37	6.5	7.1
1	-61 ± 10	-110 ± 40	-27	4.7	4.8
2	-42 ± 14	-40 ± 50	-30	5.2	4.5
3	22 ± 46	100 ± 15	-8.6	1.5	1.7

[a] Units: ΔH^0 and ΔG^0 in kJ mol^{-1}; ΔS^0 in J mol^{-1} K^{-1}.
[b] Assuming $\Delta H^{\neq}$ $(k_1^0)=0$ (see text).

It also shows the corresponding ΔG^0 values calculated for the reaction at 25 °C, and the log K values obtained from the ΔG^0 and k_1^i and k_2^i values.[6b] As can be seen, the reaction is exothermic in acid and neutral media but endothermic in basic media, similar to the reaction of PLP with *n*-hexylamine[7] and also in accordance with $\Delta H^0 < 0$ measured calorimetrically for the reaction of PLP with *n*-hexylamine and poly-L-lysine[24] at pH 7.0.

The ΔS^0 value is negative in neutral and acid media but positive in alkaline media. The significance of the entropic factor in alkaline media is noteworthy because it offsets the unfavorable enthalpic factor in ΔG^0, which is thus rendered negative. Because of the entropic contribution to the reaction, the formation of the Schiff base is more favorable in an acid than in a neutral or alkaline medium. No significant differences are observed between the equilibrium constant obtained from thermodynamic parameters and that derived from k_1^i/k_2^i.

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