Kinetic and Thermodynamic Parameters for Schiff's Base Formation between Pyridoxal 5′-Phosphate and n-Hexylamine

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The kinetic and thermodynamic parameters for the reaction between pyridoxal 5′-phosphate and n-hexylamine in aqueous medium as a function of pH, at constant ionic strength (0.1) and temperatures of 10, 15, 20, and 30 °C have been calculated. The variation observed in the overall kinetic formation and hydrolysis constants of the corresponding Schiff's base can be accounted for on the basis of the protonation and reactivity of the substrates present (pyridoxal 5′-phosphate, n-hexylamine, and Schiff's base) in the medium as a function of pH. The first protonation, both of pyridoxal 5′-phosphate and of the Schiff's base, modifies the energies of activation corresponding to the formation and hydrolysis processes, respectively. The less negative charge the pyridoxal 5′-phosphate molecule bears, the more negative the ΔH and ΔS values become.

Pyridoxal 5′-phosphate (PLP) is a cofactor for various enzymes which catalyse a large number of reactions.[1,2] For all the PLP-dependent enzymes studied, this compound is present in the form of a Schiff's base resulting from the coupling of its carbonyl group to the ε-amino function of a lysine residue from the polypeptide chain. There are other possible mechanisms for stabilizing the bond between PLP and the protein.[3–5] It is therefore essential to know the thermodynamic parameters associated with the formation of the Schiff's base in order to be able to evaluate the energy parameters related to the interactions occurring once the above mentioned bond has stabilized, or the energy linked to possible conformational changes resulting from the binding.[3–6]

However, the description at the molecular level of enzymatic processes is, in general, very complicated, though it is often simplified by the use of model systems adequately simulating the biological behaviour.

One such model which simulates the formation of PLP Schiff's base in proteins is formed by PLP and n-hexylamine, since this amine has the same number of carbon atoms and roughly the same protonation constants as L-lysine. This model has been studied kinetically by Cortijo *et al.*[7] at 25 °C, although these authors did not determine the corresponding thermodynamic parameters. This paper describes the results obtained in the study of the kinetics of formation the Schiff's base formed between PLP and n-hexylamine as a function of pH, at different temperatures (10, 15, 20, and 30 °C) and constant ionic strength (0.1). This study has allowed us to calculate the overall kinetic and thermodynamic parameters, as well as those corresponding to the different ionic species involved in the process.

Experimental

Pyridoxal 5′-phosphate, n-hexylamine, and all other chemicals were reagent grade and purchased from Merck, Darmstadt.

PLP solutions were prepared daily in a suitable buffer and kept in the dark. Their exact concentration was determined by dilution with 0.1M-HCl and subsequent measurement of its absorbance[8] at 295 nm (ε 6 700 l mol^{-1} cm^{-1}). The concentration found was *ca.* 10^{-3}M.

Acetate, phosphate, or carbonate buffers with an ionic strength of 0.1M in every case were used according to the pH required.[9]

n-Hexylamine solutions were also prepared daily by diluting the appropriate amount of concentrated amine in the corresponding buffer and adjusting their pH with 0.1M-HCl when needed. Their concentration ranged between 0.01 and 0.1M.

The kinetics of formation the Schiff's base was monitored by measuring the variation in the absorption at 430 nm on a Zeiss DMR 11 spectrophotometer furnished with thermostatted cells of 1 cm light path. The reaction was started by adding PLP solution (50—100 μl) to the measuring cell, previously thermostatted and at the same pH. The amine concentration in the measuring cell was 50—100 times as high as that of PLP, although this ratio was less in basic pH. The amine was present at an approximate concentration of 10^{-4}M.

The initial and final pH in the reaction cell was monitored throughout; at no time was its variation > ±0.03 units. This parameter was measured by means of a CRISON pH meter, using a METROHM EA 120 combined electrode.

The temperature was kept constant in every case, with a precision of ±0.05 °C.

The overall reaction between PLP and n-hexylamine to yield a Schiff's base can be represented by (1) the rate equation of

$$\mathrm{R^1CHO + R^2NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R^1CH{=}NR^2 + H_2O} \qquad (1)$$

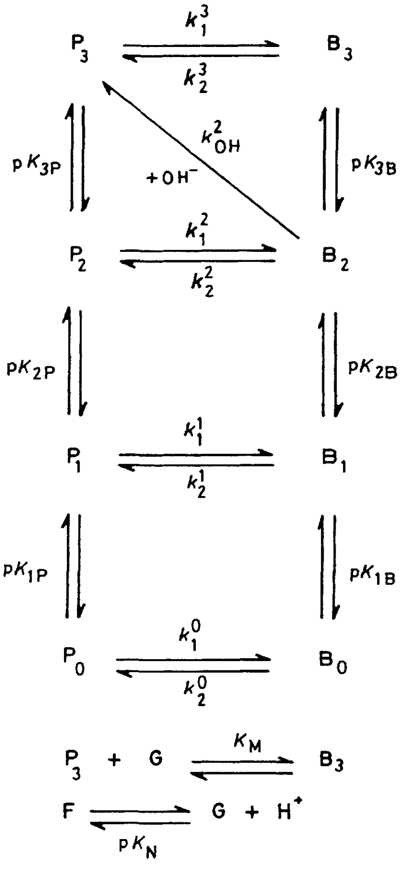
which, taking into account the Beer–Lambert law and that the kinetics of both the forward and the reverse reaction are second-order, is (2) with the application of equation (3) where *a* and *b*

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

$$k_{obs} = \{[k_2 + k_1(a + b)]^2 - 4abk_1^2\}^{\frac{1}{2}} \qquad (3)$$

are the initial aldehyde and amine concentration, respectively; x and x_e are the Schiff's base concentrations at times t and ∞, respectively; A_0, A, and A_∞ are the absorbances at times 0, t, and ∞, respectively; and k_1 and k_2 are the overall formation and hydrolysis rate constants for the Schiff's base.

The k_{obs} values were obtained from the slopes of the plots of $\ln(A_\infty - A)$ *versus* time, since $ab \gg xx_e$. The correlation coefficient obtained was always greater than 0.999. The k_1 and k_2 values were calculated from the k_{obs} values obtained at a given pH for different $(a + b)$ values.



Scheme.

The equilibrium constant corresponding to each pH, K_{pH}, was calculated as the ratio k_1/k_2.

The overall hydrolysis and formation rate constants for the aldimine can be described in terms of the rate constants corresponding to the individual ionic species present in each case. This ionic species existing in solution in the pH range studied are given[7,10] in the Scheme, in which P and B indicate PLP and its aldimine, respectively. The subindices (0—3) indicate the net negative charge on the molecules; F and G are the protonated and non-protonated forms of n-hexylamine, respectively; and K_{N} is the ionization constant of n-hexylamine. Equations (4)—(6) were derived from the Scheme where

$$k_1 = \frac{k_1^3 + k_1^2\dfrac{c}{K_{3\mathrm{P}}} + k_1^1\dfrac{c^2}{K_{3\mathrm{P}}K_{2\mathrm{P}}} + k_1^0\dfrac{c^3}{K_{3\mathrm{P}}K_{2\mathrm{P}}K_{1\mathrm{P}}}}{\left(1 + \dfrac{c}{K_{\mathrm{N}}}\right)\left(1 + \dfrac{c}{K_{3\mathrm{P}}} + \dfrac{c^2}{K_{3\mathrm{P}}K_{2\mathrm{P}}} + \dfrac{c^3}{K_{3\mathrm{P}}K_{2\mathrm{P}}K_{1\mathrm{P}}}\right)} \tag{4}$$

$$k_2 = \frac{k_{\mathrm{OH}} + k_2^2\dfrac{c}{K_{3\mathrm{B}}} + k_2^1\dfrac{c^2}{K_{3\mathrm{B}}K_{2\mathrm{B}}} + k_2^0\dfrac{c^3}{K_{3\mathrm{B}}K_{2\mathrm{B}}k_{1\mathrm{B}}}}{1 + \dfrac{c}{K_{3\mathrm{B}}} + \dfrac{c^2}{K_{3\mathrm{B}}K_{2\mathrm{B}}} + \dfrac{c^3}{K_{3\mathrm{B}}K_{2\mathrm{B}}K_{1\mathrm{B}}}} \tag{5}$$

$$K_{\mathrm{pH}} = \frac{\left(1 + \dfrac{c}{K_{3\mathrm{B}}} + \dfrac{c^2}{K_{3\mathrm{B}}K_{2\mathrm{B}}} + \dfrac{c^3}{K_{3\mathrm{B}}K_{2\mathrm{B}}K_{1\mathrm{B}}}\right)K_{\mathrm{M}}}{\left(1 + \dfrac{c}{K_{\mathrm{N}}}\right)\left(1 + \dfrac{c}{K_{3\mathrm{P}}} + \dfrac{c^2}{K_{3\mathrm{P}}K_{2\mathrm{P}}} + \dfrac{c^3}{K_{3\mathrm{P}}K_{2\mathrm{P}}K_{1\mathrm{P}}}\right)} \tag{6}$$

c is equal to $10^{-\mathrm{pH}}$, $k_{\mathrm{OH}} = k_2^3 + k_{\mathrm{OH}}^2(P_{\mathrm{w}}/K_{3\mathrm{B}})$, and P_{w} is the ionic product of water.

Table 1. pK Values of PLP and n-hexylamine calculated by different techniques

T/°C	p$K_{1\mathrm{P}}$	p$K_{2\mathrm{P}}$	p$K_{3\mathrm{P}}$	pK_{N}
10	3.65[a]		8.5[a]	11.12[c]
15	3.70[a]		8.3[a]	10.96[c]
20	3.77[b]	6.12[b]	8.37[b]	10.70[c]
30	3.46[b]	6.02[b]	8.16[b]	10.47[c]

[a] pK Values calculated by spectrophotometric techniques. [b] pK Values calculated by calorimetric techniques.[14] [c] pK Values taken from refs. 11 and 13.

The experimental values of k_1, k_2, and K_{PH} were fitted simultaneously to equations (4)—(6) by means of a least-squares procedure with the aid of a computer. The initial values of the protonation constants for PLP and n-hexylamine given in Table 1 were used for that purpose; were either obtained by different techniques or taken from the literature.[11–14] The protonation constants for the Schiff's base were estimated from the data reported in ref. 7.

Results and Discussion

The values found for the rate constants corresponding to the formation, k_1, and hydrolysis, k_2, of Schiff's base formed between PLP and n-hexylamine as a function of the pH at different temperatures (10, 15, 20, and 30 °C) are listed in Table 2. Such values are consistent with those reported by Cortijo *et al.*[7] for the same system at 25 °C, as well as with those published by Wiesinger and Hinz,[3] who studied PLP–ε-aminocaproic acid and PLP–serine systems in the pH range 7—8. As observed, k_1 increases with increasing pH, whilst k_2 shows a minimum between pH 8 and 9.

The overall equilibrium constant for the process, K_{pH} (Table 2), is, at any pH, larger than that obtained for analogous systems.[15,16]

The Figure illustrates the variation of this equilibrium constant as a function of pH. The maximum of this plot shifts to more basic pHs as the temperature decreases (it appears at 9.6 at 30 °C, and at 10.2 at 10 °C). Such a maximum, according to Metzler *et al.*,[16] always lies between the more basic pK of the aldehyde and the pK of the amine, also shifting with the temperature in the same way as the pK values (see Table 1).

Table 3 lists the values found for the energy of activation corresponding to the formation and hydrolysis processes, as well as those obtained for the enthalpy of formation of the Schiff's base. All these values were calculated from the data given in Table 2 and in ref. 7.

The values obtained for the energy of activation of the formation process, $E_{\mathrm{a}(k_1)}$, are of the same order as those reported by Wiesinger and Hinz[3] (between 28 and 54 kJ mol^{-1}), which are the only data available in the literature for the Schiff's bases of PLP.

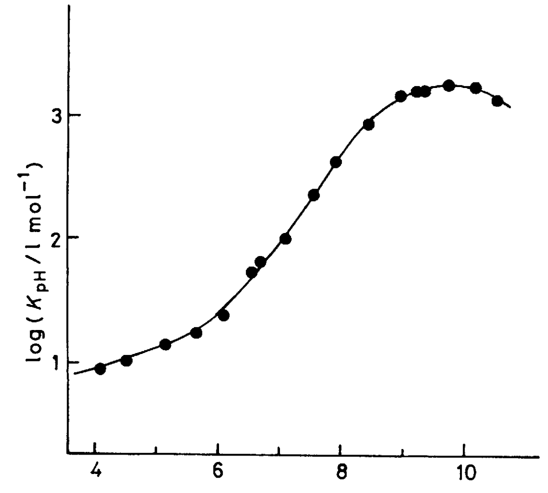
The formation of the Schiff's base is endothermic at neutral pH, which may account for the binding of PLP to tryptophan synthetase and other analogues recently studied by calorimetric techniques.[4] The process becomes exothermic at acid or basic pH values.

The fitting of the kinetic data from Table 2 to equations (4)—(6) allowed us to calculate the individual rate constants, both of formation, k_1^i, and of hydrolysis, k_2^i and k_{OH} (Table 4). We have also determined the different equilibrium constants of

Table 2. Kinetic parameters for Schiff's base formations[a]

10 °C				15°C				20 °C				30 °C			
k_1	k_2	K_{pH}	pH	k_1	k_2	K_{pH}	pH	k_1	k_2	K_{pH}	pH	k_1	k_2	K_{pH}	pH
0.74	0.065	11.40	3.9	1.12	0.126	8.90	4.1	1.26	0.140	8.98	3.8	2.87	0.334	8.59	3.9
0.75	0.057	13.20	4.0	1.51	0.144	10.49	4.5	1.85	0.160	11.56	4.2	4.17	0.490	8.51	4.5
1.11	0.077	14.42	4.5	2.24	0.161	13.91	5.1	2.52	0.178	14.16	4.5	5.01	0.562	8.91	4.8
1.32	0.088	14.97	4.8	3.49	0.185	18.86	5.6	3.80	0.190	19.98	5.0	5.62	0.588	9.56	5.0
1.48	0.093	15.91	5.0	4.59	0.191	24.03	6.0	4.35	0.191	22.77	5.2	7.08	0.602	11.75	5.2
2.31	0.100	23.10	5.3	8.07	0.145	55.66	6.5	5.62	0.188	29.89	5.5	7.58	0.575	13.18	5.4
2.82	0.100	25.63	5.7	9.59	0.151	63.51	6.7	6.31	0.186	33.92	5.6	12.84	0.464	26.67	6.0
3.71	0.112	33.10	6.0	13.96	0.126	110.8	7.1	7.94	0.174	45.63	6.0	16.52	0.372	44.41	6.5
5.41	0.091	59.45	6.5	16.04	0.073	219.7	7.5	11.29	0.151	74.77	6.2	24.27	0.251	96.69	6.8
7.08	0.052	136.1	7.0	20.27	0.051	397.5	7.9	12.71	0.144	86.26	6.5	26.30	0.251	104.8	7.0
8.67	0.039	222.3	7.5	37.15	0.044	844.3	8.4	17.78	0.117	151.9	6.9	31.11	0.188	165.5	7.2
13.23	0.022	601.1	7.8	73.61	0.049	1 502	8.9	20.07	0.080	250.9	7.4	41.72	0.170	245.4	7.4
19.95	0.021	947.6	8.4	93.47	0.061	1 532	9.2	32.11	0.079	406.4	7.7	58.56	0.151	287.8	7.7
31.73	0.021	1 509	9.1	117.3	0.072	1 629	9.3	46.77	0.055	850.3	8.4	112.2	0.129	869.8	8.4
63.47	0.028	2 268	9.3	208.3	0.117	1 780	9.6	85.11	0.068	1 251	8.9	201.5	0.162	1 244	8.9
132.5	0.060	2 200	9.7	467.3	0.251	1 861	10.1	135.1	0.085	1 589	9.2	256.7	0.209	1 228	9.2
271.4	0.093	2 914	10.2	836.5	0.608	1 375	10.5	342.1	0.187	1 829	9.7	398.1	0.316	1 260	9.6
633.1	0.289	2 190	10.7					1 178	0.828	1 424	10.6	588.8	0.631	933.1	10.0

[a] k_1/l mol^{-1} min^{-1}, k_2/min^{-1}, K_{pH}/l mol^{-1}.



Variation of the equilibrium constant for the formation of the Schiff's base between PLP and n-hexylamine as a function of the pH, at 15 °C. ● Experimental values calculated from k_1/k_2. The line is calculated from equation 6 and the parameters are given in Table 5

protonation for the aldehyde, amine, and Schiff's base, as well as the equilibrium formation constant for the Schiff's base at very basic pH, $K_M = k_1^3/k_2^3$ (see Scheme). These values are given in Table 5. The set of kinetic results and equilibrium constants obtained is self-consistent and satisfies equations (7)—(9) which can be readily derived from the Scheme.

$$-\mathrm{p}K_{1P} + \log k_1^1 - \log k_2^1 = -\mathrm{p}K_{1B} + \log k_1^0 - \log k_2^0 \quad (7)$$

$$-\mathrm{p}K_{2P} + \log k_1^2 - \log k_2^2 = -\mathrm{p}K_{2B} + \log k_1^1 - \log k_2^1 \quad (8)$$

$$-\mathrm{p}K_{3P} + \log k_1^3 - \log k_2^3 = -\mathrm{p}K_{3B} + \log k_1^2 - \log k_2^2 \quad (9)$$

It is also possible to check that equations (4)—(6), derived from the parameters given in Tables 4 and 5, conform thoroughly to the experimental results (see Figure).

Table 3. Energy of activation for Schiff's base formation and hydrolysis and enthalpy of formation process

pH	$E_{a(k_1)}$/ kJ mol^{-1}	$E_{a(k_2)}$/ kJ mol^{-1}	ΔH/ kJ mol^{-1}
10	48.1	86.5	−38.4
9	60.6	86.5	−25.9
8	65.6	54.3	11.3
7	64.0	34.7	29.3
6	60.6	53.5	7.1
5	49.0	65.2	−16.2
4	46.4	65.2	−18.8

The increase in k_1^i with decreasing pH observed at all the temperatures used is due to the involvement of all the protonable groups in PLP in intramolecular acid catalysis of the dehydration of the corresponding carbinolamine. Such catalysis could be promoted by the 3-hydroxy function of PLP, as suggested by various authors.[7,10,17] On the other hand, plots of log (rate constant) as a function of pK at different temperatures have very similar slopes (α 0.64—0.68).

The rate constant k_2^3 can be calculated from the ratio k_1^3/K_M; the values thus obtained coincides with that of k_{OH}. Therefore, under these conditions, the term k_{OH}^2 (P_w/K_{3B}) must be much less than k_2^3. From the K_{3B} values given in Table 5 and those of P_w reported in the literature,[18] and taking into account the precision with which k_{OH}, k_1^3, and K_M are obtained, it can be estimated that k_{OH}^2 will be 40 times as large as k_2^3 at the most.

The k_{OH} or k_2^3 values obtained are larger than those of k_2^i; this is indicative of the ease with which the Schiff base can be hydrolysed, which shows in the k_M value found. On the other hand, the incorporation of the first proton into the Schiff's base gives rise to the ionic species B_2 (see Scheme). The rate constants of hydrolysis of this species, k_2^2, show the lowest values among all those obtained for k_2^i. This indicates that species B_2 is the most stable one against the hydrolysis by H_2O. In aqueous media, this species corresponds to the Schiff's base with the protonated imine nitrogen, the proton in question forming a bridge with the 3-phenoxide group of the aromatic ring which has been described in n.m.r. studies.[19]

The Arrhenius plot of the corresponding rate constants

Table 4. Best kinetic constants obtained in the fitting of experimental k_1 and k_2 values to equations (4) and (5)

T/°C	log k_1^0	log k_1^1	log k_1^2	log k_1^3	log k_2^0	log k_2^1	log k_2^2	log k_{OH}
10	7.39	5.60	4.02	3.20	−1.20	−0.90	−1.80	0.70
15	7.54	5.75	4.21	3.38	−0.90	−0.70	−1.47	0.83
20	7.60	5.84	4.41	3.47	−0.80	−0.51	−1.27	0.85
30	7.86	5.95	4.70	3.55	−0.35	−0.19	−0.95	0.88

Table 5. Best pK and K_M values obtained in the fitting of experimental k_1, k_2, and K_{pH} values to equations (4)—(6)

T/°C	pK_{1P}	pK_{2P}	pK_{3P}	pK_{1B}	pK_{2B}	pK_{3B}	pK_N	log K_M
10	3.61	6.32	8.68	5.69	7.00	11.97	11.03	2.50
15	3.50	6.20	8.47	5.50	6.97	11.60	10.83	2.55
20	3.40	6.08	8.34	5.40	6.70	11.40	10.75	2.62
30	3.23	5.98	8.02	5.30	6.47	11.10	10.60	2.67

Table 6. Thermodynamic parameters corresponding to the elementary processes

i	$E_{a(k_1^i)}$/ kJ mol^{-1}	$E_{a(k_2^i)}$/ kJ mol^{-1}	ΔH/ kJ mol^{-1}	ΔS/ J mol^{-1} K^{-1}
0	35.1	67.3	−32.2	−92 ± 4
1	31.8	56.7	−24.9	−69 ± 3
2	56.4	68.6	−12.2	−27 ± 1
3	29.3	8.0	21.3	80 ± 3

i = The net negative charge on the molecules.

obtained allows a calculation of the energy of activation of the individual processes of formation and hydrolysis of the Schiff's base (Table 6), which is markedly altered by the incorporation of the first proton to completely ionized PLP or Schiff's base.

The k_1^0 and k_1^1 values reported in ref. 7 are slightly different from those obtained in this study, probably due to an error made in calculating the former which the authors of ref. 7 consider too small.

The ΔH values for the elementary processes calculated from the energies of activation given in Table 6 clearly show that the formation of the covalent bond in the Schiff's base becomes more exothermic as the number of protons bound to PLP increases.

We have also calculated the ΔS values corresponding to the elementary processes from their rate constants and ΔH (Table 6). As can be seen, such values become more negative as the number of protons bound to the Schiff's base increases. Wiesinger and Hinz[3] obtained ΔS -40 kJ mol^{-1} K^{-1} at pH 7.5 for the Schiff's base formed between PLP and ε-aminocaproic acid.

The differences between the pK values listed in Tables 1 and 5 arise from the use of a different method of calculation in each case. Thus, the values shown in Table 1 are macroscopic, *i.e.* they are mean values of those corresponding to the hydrated and unhydrated forms of PLP, whereas those in Table 5 correspond only to the unhydrated forms of PLP. As could be expected, the most marked differences correspond to the region of acid pH, where the occurrence of hydrated forms is particularly favoured.[19]

Finally, we note that the use of overall thermodynamic parameters for a given process may lead to errors in interpretation unless they are analysed by using a model as similar as possible to the process, as in the case of ΔH. The calculated thermodynamic parameters corresponding to the individual processes can be applied to the study of the formation of the covalent bond between PLP and various enzymes in aqueous media, since the energy parameters corresponding to bond formation do not depend significantly on the particular compound bearing the amino group.

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