Study of the hydrolysis and ionization constants of Schiff base from pyridoxal 5'-phosphate and n-hexylamine in partially aqueous solvents

An application to phosphorylase *b*

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Formation and hydrolysis rate constants as well as equilibrium constants of the Schiff base derived from pyridoxal 5'-phosphate and n-hexylamine were determined between pH 3.5 and 7.5 in ethanol/water mixtures (3:17, v/v, and 49:1, v/v). The results indicate that solvent polarity scarcely alters the values of these constants but that they are dependent on the pH. Spectrophotometric titration of this Schiff base was also carried out. We found that a pK_a value of 6.1, attributed in high-polarity media to protonation of the pyridine nitrogen atom, is independent of solvent polarity, whereas the pK_a of the monoprotonated form of the imine falls from 12.5 in ethanol/water (3:17) to 11.3 in ethanol/water (49:1). Fitting of the experimental results for the hydrolysis to a theoretical model indicates the existence of a group with a pK_a value of 6.1 that is crucial in the variation of kinetic constant of hydrolysis with pH. Studies of the reactivity of the coenzyme (pyridoxal 5'-phosphate) of glycogen phosphorylase *b* with hydroxylamine show that this reaction only occurs when the pH value of solution is below 6.5 and the hydrolysis of imine bond has started. We propose that the decrease in activity of phosphorylase *b* when the pH value is less than 6.2 must be caused by the cleavage of enzyme-coenzyme binding and that this may be related with protonation of the pyridine nitrogen atom of pyridoxal 5'-phosphate.

INTRODUCTION

Pyridoxal 5'-phosphate (PLP) is the coenzyme of a great number of enzymes, acting on different specific substrates and catalysing a wide range of reactions.

Schiff bases of PLP and its analogues with amino acids and primary amines have been extensively studied, because of their important biological role in the metabolism of amino acids [1]. However, most of these studies have been carried out in aqueous solutions: determinations of rate and equilibrium constants for the processes of formation and hydrolysis [2–11] and of ionization constants [12–14], calorimetric studies on their formation [15] and conformational studies through circular dichroism [16].

For several years we have been studying the behaviour of glycogen phosphorylase *b*, its coenzyme (PLP) and its effectors. In glycogen phosphorylases, PLP is bound to an ϵ -amino group of a lysine residue, forming a Schiff base in a highly hydrophobic environment [17].

Fischer *et al.* [18] showed that the double bond between the coenzyme and the enzyme residue could be reduced with NaBH_4 without loss of activity. Therefore the role of the PLP in phosphorylase is different from that established for the typical PLP-dependent enzymes, for which the chemistry is well understood.

The activity of glycogen phosphorylase *b* reaches its

peak between pH 6.2 and 6.8, depending on the type of buffer solution [19–21]. This activity is drastically dependent upon an enzymic group with a pK_a of approx. 6.5 [22]. To date, the group responsible for this remains unknown.

The 5'-phosphate group of the coenzyme is directly involved in the catalytic mechanism, and, since in the active state this phosphate group remains in the dianionic form, the pK_a so highly relevant for enzymic activity has been attributed to the loss of the second proton of this group [23–25].

Recent data from Withers *et al.* [26] prove the existence in glycogen phosphorylase *b* of a direct interaction between the phosphate group of the coenzyme and the phosphate group of the substrate, glucose 1-phosphate, in a hydrophobic environment. Under these conditions, the pK_a value for the second proton of the phosphate from the PLP would be higher than 6.5.

Few studies on the formation of Schiff bases and their formation constants in solutions of low dielectric constant have so far been performed [27–29]. In order to deduce whether the stability of imine double bond between PLP and the lysine residue is responsible for the loss of activity in phosphorylase *b* when the pH is lowered, we have now studied the hydrolysis process of the Schiff base of PLP and n-hexylamine in low-polarity media. We have found that the stability of this Schiff base

Abbreviation used: PLP, pyridoxal 5'-phosphate.

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in ethanol/water (3:17, v/v) is very similar to that in ethanol/water (49:1, v/v).

MATERIALS AND METHODS

Stock solutions of PLP (approx. 10 mM) in distilled water were prepared. The concentration of PLP was determined spectrophotometrically in 0.1 M-NaOH at 388 nm by using a molar absorption coefficient of $6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [30].

The Schiff base was formed in ethanol. The n-hexylamine concentration was 100 times that of aldehyde, and the concentration of PLP was one-tenth that in the stock solution. The pH value of the solutions was adjusted to about 8–8.5. Under these conditions no hydrolysis of the imine occurred, since there was no variation of absorbance as a function of time at any wavelength.

The Schiff base obtained by following this procedure was diluted 10-fold in ethanol/water mixtures of a determined volume ratio and adjusted to the desired pH value. Variations in the absorbances of these solutions with time were monitored to follow the kinetics of the hydrolytic process of the Schiff base in these media. Identical samples were used to check pK .

Spectrophotometric measurements were performed with a Zeiss DMR 11 spectrophotometer with 1 cm quartz cuvettes. In all cases the temperature was maintained at 25 °C.

The pK_a values were measured with a Crison pH-meter with a combined electrode that had been calibrated with aqueous buffer solutions at $\text{pH } 4.01 \pm 0.01$ and $\text{pH } 7.00 \pm 0.01$ at 25 °C. To transform the pH values read for each sample, pH(R) , into real pH, pH^* ($-\log a_{\text{H}}$), the following equation was used:

$$\text{pH}^* = \text{pH(R)} - \delta \quad (1)$$

according to the notation of Bates *et al.* [31]. The values of δ have also been those given by Bates *et al.* [31] for different ethanol/water mixtures.

Phosphorylase *b* was obtained from rabbit skeletal muscle by the method of Krebs *et al.* [32]. The enzyme concentration was measured spectrophotometrically by using an absorption coefficient, $A_1\%_{\text{cm}}$, of 13.2 at 280 nm [33]. The buffer solution used was 50 mM-KCl/0.2 mM-EDTA/50 mM-glycylglycine adjusted to the desired pH.

The reaction between hydroxylamine and the enzyme was started by mixing identical volumes of both reagents dissolved in the buffer described above at a fixed pH.

Fluorescence studies were carried out in a FICA model 55 spectrofluorimeter, recording the change in fluorescence of the characteristic coenzyme band: excitation was at 425 nm and emission at 535 nm [21]. The slit width was 7.5 nm.

All chemical reagents were purchased from Merck.

RESULTS

Kinetic measurements for the hydrolysis of the Schiff base

When the Schiff base between PLP and n-hexylamine, prepared as described in the Materials and methods section, is dissolved in ethanol/water (3:17) no change occurs in the intensity of the absorption bands from 240 to 500 nm throughout the pH region from 10.5 to 7.7.

A decrease of pH to below 7.7 yields a clear modification in the absorption spectrum of the imine. The band at 415 nm shifts towards the blue region, and at the same time the intensity of the peak at 275 nm diminishes. Absorption spectra for the PLP–n-hexylamine adducts are plotted in Fig. 1. The respective pH values are indicated on each curve. The spectrum at 7.7, with peaks at 275 nm and 415 nm, has the typical profile of a Schiff base from PLP in its ketoamine form [27]. At pH 4.1 the spectrum cannot be assigned to the imine, but rather to free PLP in a solution with a high dielectric constant and acidic pH [34].

Therefore hydrolysis of the Schiff base takes place in this milieu, a process that is pH-dependent.

Fig. 1. Absorption spectra for 0.1 mM-PLP and 10 mM-n-hexylamine in ethanol/water (3:17) at different pH values

Numbers beside each curve indicate the pH of the solution. Spectra were recorded after equilibrium was reached.

The equilibrium of hydrolysis and formation for the Schiff base can be written:

where k_a is the rate constant of the formation and k_b the rate constant of hydrolysis; K_H is the equilibrium constant ($K_H = k_b/k_a$).

Variation of the Schiff-base concentration (x) with time (t) is given by the expression:

$$\frac{dx}{dt} = -k_b x + k_a [\text{R}'\text{-CHO}] [\text{R-NH}_2] \quad (3)$$

Integration of this equation leads to:

$$\ln\left(\frac{xc - ab}{(ac - ab)(x - c)}\right) = \left(\frac{k_a(ab - c^2)}{c}\right)t - \ln(a - c) \quad (4)$$

where a is the initial concentration of Schiff base, b is the initial concentration of amine and c is the Schiff-base concentration when equilibrium is reached.

In ethanol/water (3:17) hydrolysis of the imine was monitored through variation of its intensity of absorption at 415 nm. At any given time the intensity detected can

be obtained by adding separately the absorption of free PLP and the Schiff base, since at this wavelength the amine does not absorb.

Hence imine concentration can be expressed:

$$x = \frac{A_{415} - ae_{415}^{\text{PLP}}}{e_{415} - e_{415}^{\text{PLP}}} \quad (5)$$

where A_{415} is the absorption intensity at 415 nm, e_{415}^{PLP} is the molar absorption coefficient of PLP at 415 nm and e_{415} is the molar absorption coefficient of the Schiff base at the same wavelength [35].

The initial concentrations of the Schiff base (a) and of amine (b) are previously known and the equilibrium concentration of Schiff base (c) can easily be calculated by using the values of absorbance when the equilibrium is reached (A_{415}):

$$c = \frac{A_{415} - ae_{415}^{\text{PLP}}}{e_{415} - e_{415}^{\text{PLP}}} \quad (6)$$

Experiments on the hydrolysis of the Schiff base were performed at several pH values between 3.5 and 7.5. Fig. 2 shows how some of these kinetics fit with eqn. (4), x being calculated according to eqn. (5). Straight lines were obtained in all cases, proving that chemical eqn. (2) is valid in the pH region considered. From their slopes it was possible to determine k_a at each pH value. This k_a value allows one to calculate k_b , the rate constant of hydrolysis, and K_H , the equilibrium constant.

We have analysed these experimental values according to a scheme put forward by Sánchez-Ruiz *et al.* [9] for this same system in water. The ionic forms existing in solution in the pH range studied (above 3.5 and below 8) are given in Scheme 1, in which P and B indicate PLP and its imine respectively and the subindices indicate the number of the net negative charges on the molecule. F is the monoprotonated form of the amine and G its deprotonated form (see Scheme 1).

For this model, eqns. (7) and (8) can readily be obtained:

$$k_b = \frac{k_b^2 + \frac{h}{K_{2B}}k_b^1 + \frac{h^2}{K_{2B}K_{1B}}k_b^0 + \frac{h^3}{K_{2B}K_{1B}K_{0B}}k_b^{-1}}{1 + \frac{h}{K_{2B}} + \frac{h}{K_{2B}K_{1B}} + \frac{h^3}{K_{2B}K_{1B}K_{0B}}} \quad (7)$$

$$k_a = \frac{k_a^2 + \frac{h}{K_{2P}}k_a^1 + \frac{h^2}{K_{2P}K_{1P}}k_a^0}{\left(1 + \frac{h}{K_N}\right)\left(1 + \frac{h}{K_{2P}} + \frac{h^2}{K_{2P}K_{1P}}\right)} \quad (8)$$

where $h = 10^{-\text{pH}}$

The logarithms of the kinetics and equilibrium constants are plotted in Fig. 3 as a function of pH. The continuous lines are those obtained from the theoretical model by using the values shown in Table 1. These values were calculated through a non-linear-regression method and minimizing the functions:

$$U_1 = \sum (\log k_{b,e} - \log k_{b,t})^2 \quad (9)$$

$$U_2 = \sum (\log k_{a,e} - \log k_{a,t})^2 \quad (10)$$

where the subscripts e and t refer to experimental and theoretical data.

When the Schiff base of PLP and n-hexylamine is dissolved in ethanol/water (49:1) and the pH value is

Fig. 2. Fitting of the kinetics of hydrolysis for the Schiff base derived from PLP and n-hexylamine in ethanol/water (3:17)

$W = (xc - ab)/(ac - ab)(x - c)$ (see the text). Numbers beside each graph indicate the pH for the hydrolysis process of the imine.

Fig. 3. Dependence of $\log k_a$, $\log k_b$ and $\log K_H$ on pH in ethanol/water mixtures

The lines were calculated from eqn. (7) for k_a , from eqn. (8) for k_b and from k_b/k_a for K_H by using the values given in Table 1. — and +, Ethanol/water (13:7); ---- and ●, ethanol/water (49:1). + and ● are experimental values.

Fig. 4. Variation of initial absorbance for the Schiff base derived from PLP and n-hexylamine as a function of pH

Schiff-base concentration was 0.17 mM. (a) In ethanol/water (3:17) at 415 nm; (b) in ethanol/water (49:1) at 415 nm; (c) in ethanol/water (49:1) at 250 nm. Absorbances were measured at zero time, before hydrolysis started, and the continuous lines are the theoretical curves obtained, fitting the experimental values, by using values of molar absorption coefficients shown in Table 2.

In ethanol/water (49:1) two pK_a values were also found, 6.02 and 11.3 (Fig. 4). From these data we can infer that polarity of the solution scarcely affects the pK_a of the pyridine nitrogen atom. Through potentiometric titrations, Blazquez *et al.* [38] found that with PLP and pyridoxal the pK_a associated with protonation of the pyridine nitrogen atom is relatively unaffected by solvent

polarity. Sánchez-Ruiz *et al.* [39], using dioxan/water mixtures, reached the same conclusion.

Few studies concerning the titration of the Schiff bases with PLP or its analogues in solvent with a low dielectric constant can be found in the literature. Schiff bases between PLP and amino acids were titrated in methanol by Lehtokari *et al.* [29]. Under their conditions they

Table 2. Values of the molar absorption coefficients used and pK values obtained in the fitting of experimental values of the Schiff-base titration

ϵ values are in units of $M^{-1} \cdot cm^{-1}$.				
Ethanol/water (3:17)				
415 nm				
$\epsilon(1)$	4350	$pK(1)$	12.48 ± 0.05	
$\epsilon(2)$	7170	$pK(2)$	6.11 ± 0.02	
$\epsilon(3)$	10320			
Ethanol/water (49:1)				
250 nm			415 nm	
$\epsilon(1)$	7640	$pK(1)$	11.40 ± 0.08	$\epsilon(1)$ 2830 $pK(1)$ 11.3 ± 0.07
$\epsilon(2)$	8970	$pK(2)$	6.05 ± 0.05	$\epsilon(2)$ 1740 $pK(2)$ 5.95 ± 0.05
$\epsilon(3)$	6520			$\epsilon(3)$ 5670

found a pK_a of about 6, which they attributed to the carboxylate group in the amino acid, an assignation that does not apply in our case.

Lastly, the pK_a value in low-polarity solution (11.3) is lower than that found in solution of higher polarity (12.5). This pK_a , corresponding to the monoprotonated form in a very polar medium, could be attributed to protonation of the phenolate group [8].

Fluorescence of glycogen phosphorylase *b*

The stability of the bond between the PLP and glycogen phosphorylase *b* was studied by removing the coenzyme with hydroxylamine at different pH values. At low concentrations of phosphorylase and whenever the absorption at the excitation wavelength is negligible (there is no internal quenching), fluorescence can be considered to be proportional to the amount of PLP bound to enzyme.

When phosphorylase *b* is incubated with hydroxylamine, no decrease in fluorescence of enzyme with incubation time can be observed if the pH value of the solution is maintained above 6.5. Decrease of the pH below 6.5 induces a fall of the relative fluorescence of glycogen phosphorylase *b* (Fig. 5).

The influence of pH on the reaction of hydroxylamine and the coenzyme of phosphorylase can be analysed if we take into account:

$$v = -\frac{d[PLP-enz]}{dt} = k[PLP-enz]^a[NH_2OH]^b[H^+]^n \quad (12)$$

$$\frac{F}{F_0} = k' \left(\frac{[PLP-enz]}{[PLP-enz]_0} \right) \quad (13)$$

where $[PLP-enz]$ and $[PLP-enz]_0$ are the concentrations of native phosphorylase at any time and at zero time respectively, a , b , n , k and k' are constants, and F and F_0 are the relative fluorescence at any time and at zero time respectively.

Fig. 5. Fluorescence ratio of the enzyme (1 mg/ml) plotted versus incubation time in 0.1 M-hydroxylamine at 28.5 °C

Curve A, pH 5.4; curve B, pH 5.8; curve C, pH 6.0; curve D, pH 6.3; curve E, pH 7, 7.5 and 8.3.

Fig. 6. Eqn. (14) (see the text) plotted in order to calculate the order of reaction for the dissociation of the PLP-enzyme bond with respect to protons.

When $t = 0$, we obtain:

$$-\log \left[-\left(\frac{d(F/F_0)}{dt} \right)_0 \right] = \text{constant} + n \cdot \text{pH} \quad (14)$$

On plotting the first term of eqn. (14) versus pH (Fig. 6) it can be observed that the reaction is not dependent on pH at pH above 6.5, whereas the process has an order of 1.5 with respect to protons at pH below 6.5.

DISCUSSION

The essential factor in the hydrolysis process of the Schiff base derived from PLP and n-hexylamine seems to be the pH value more than the dielectric-constant value. A pH value around 6 stands as a crucial point in the variation of the logarithm of k_b (rate constant of hydrolysis) with pH in the mixtures studied.

Since the pK_a value of 6.11 obtained in ethanol/water

(3:17) and the other of 6.0 obtained in ethanol/water (49:1) have been attributed to protonation of the pyridine nitrogen atom, the drastic decrease in the hydrolysis constant for the imine might be caused by deprotonation of this nitrogen atom of PLP.

In glycogen phosphorylase *b*, a group with pK_a about 6.2 [22] is responsible for its catalytic activity. This pK_a has been attributed to loss of the second proton from the phosphate group of the coenzyme PLP, since in the active state of phosphorylase the phosphate group of PLP remains in the dianionic form [22–25]. We believe that the phosphate group of the coenzyme is directly involved in the catalytic mechanism of phosphorylase *b*, but that deprotonation of the monoanionic phosphate cannot possibly be the process with a pK_a value of 6.2 on which the enzymic activity is so dependent, because in phosphorylase *b* the PLP is buried in a highly hydrophobic environment, and under these conditions the pK_a for the second proton of the phosphate group must be higher than 7. So that, if we assign the pK_a of 6.2 that is responsible for the enzymic activity to the phosphate group from the coenzyme, this group must be placed outside the hydrophobic pocket, in contact with the solvent, in an aqueous milieu. But the studies carried out by Withers *et al.* [26] on the activated state of phosphorylase *b* discount this possibility.

In the Results section we showed that there is a decrease in the fluorescence of glycogen phosphorylase *b* when it is incubated with hydroxylamine and the pH of solution is less than 6.5. In carrying out the fluorescence measurements the excitation wavelength used was 415 nm. This is the wavelength of maximum absorption of ketoamine tautomeric form of the imine formed between PLP and phosphorylase [17].

The decrease in the fluorescence of glycogen phosphorylase *b* can be produced either by shifting of tautomeric equilibrium of the Schiff base between PLP and enzyme towards the enolimine form or by breakdown of this linkage [40]. Light-scattering measurements indicate that in glycogen phosphorylase *b* hydroxylamine reacts with PLP better at pH 6.2 than at pH 8 [41], so that the hydrophobic pocket of the coenzyme must be more closed at pH 8 than at pH 6.23 and the decrease in fluorescence shown in Fig. 6 must be due to cleavage of the PLP–enzyme bond.

The data concerning stability and titration of the Schiff base in ethanol/water mixtures presented in this paper suggest that the protonation of the pyridine nitrogen atom of PLP may be the process on which the activity of phosphorylase *b* is so dependent.

Chang & Graves [42] have suggested that in the active form of phosphorylase *b* the pyridine nitrogen atom of PLP must be deprotonated. This suggestion is in agreement with our results showing that protonation of the pyridine nitrogen atom induces cleavage of the PLP–enzyme bond and, obviously, loss of the enzyme activity. The authors cited do not attribute the pK value of 6.2 on which the activity of phosphorylase *b* depends to the pyridine nitrogen atom of PLP, because their studies with phosphorylase *b* reconstituted with 6-fluoro-PLP instead of with PLP show the same variation of activity with the pH of the medium.

However, no investigations have been performed with the Schiff bases of 6-fluoro-PLP, and nothing is known regarding the variation of the pK_a in the aromatic ring by the formation of the double bond $C = N$.

At all events, the fluorescence results described in the present paper reveal a general trend of cleavage of the enzyme–coenzyme bond when the pH of the medium is under 6.5, and the reaction between hydroxylamine and free PLP takes place at the same time as the enzymic inactivation process and, according to the results obtained for the rate constant of hydrolysis of the Schiff base between PLP and *n*-hydroxylamine, while the pyridine nitrogen atom of the coenzyme is protonated.

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