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Spectroscopic study of the Schiff bases of dodecylamine with pyridoxal 5′-phosphate and 5′-deoxypyridoxal

A model for the Schiff bases of pyridoxal 5′-phosphate in biological systems

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We recorded the absorption spectra of the Schiff bases of pyridoxal 5′-phosphate (PLP) and 5′-deoxypyridoxal (DPL) with dodecylamine (DOD) at different pH values. By applying deconvolution techniques to the spectra and analysing their different components we found that the above-mentioned Schiff bases in aqueous solutions of pH 7 adopted a conformation in which the pyridine ring is embedded in a very hydrophobic medium from which water is virtually completely excluded. This conformation is the same as that adopted by PLP when it acts as coenzyme for some enzymes such as glycogen phosphorylase. The experimental results obtained also show such a conformation to be highly favoured but sensitive to the protonation of the pyridine nitrogen, which makes the aromatic ring more readily accessible to the solvent.

INTRODUCTION

Pyridoxal 5′-phosphate (PLP) acts as coenzyme for a host of enzymes which catalyse a wide variety of reactions involved in the metabolism of amino acids (racemization, transamination, β-elimination, etc.) and other biological compounds such as glycogen (Snell, 1986).

In every PLP-dependent enzyme, the coenzyme binds to the protein to form an imine with the ϵ-amino group of a lysine residue. Information on the state of the coenzyme in the holoenzyme has largely been obtained by using u.v.–visible absorption spectroscopy or spectrofluorimetry. Thus the spectroscopic features of vitamin B_6 derivatives (Matsushima & Martell, 1967; Metzler *et al.*, 1973; Harris *et al.*, 1974; Scott *et al.*, 1985) and related Schiff bases (Metzler *et al.*, 1980; Donoso *et al.*, 1986; Mitra & Metzler, 1988; Morozov *et al.*, 1988) have been widely studied.

In solution, Schiff bases of PLP present different ionization states depending on the pH of the media. Moreover, some of these ionic species undergo tautomeric equilibria which are affected by medium polarity in such a way that zwitterionic forms are favoured by high-polar media whereas non-polar forms are far more stable in hydrophobic solvents (see Schemes 1 and 2). The absorption bands of these compounds can be accurately described on the basis of log normal distribution curves (Metzler *et al.*, 1980). Insofar as the band shapes are highly predictable, mixtures of tautomeric forms can be readily resolved from the mixed spectra.

This fact helped us to detect the production of pyridoxamine 5′-phosphate when Schiff bases of PLP are formed in water with α-amino acids, since their spectra present longer absorption wavelengths than those of Schiff bases formed by PLP and non-α-amino acids or primary amines. Pyridoxamine 5′-phosphate is produced via a transamination reaction which is favoured by the more acid character of the α-proton (Vázquez *et al.*, 1990).

In native enzymes and because of the high sensitivity of absorption and fluorescence spectra of PLP to medium polarity, the ratio between the absorption of the bands at 320–330 nm (non-polar tautomeric forms) and 430–450 nm (zwitterionic forms) yielded by PLP-dependent enzymes has been reported as a measure of the polarity of the microscopic environment of the coenzyme in the system (Llor & Cortijo, 1977). Furthermore the individual contribution of coenzymes to the absorption spectra of holoenzymes as determined from log normal distribution curves is proving to be of great use in obtaining information on the different tautomers of a given enzyme involved in the corresponding catalytic mechanism (Metzler & Metzler, 1987; Metzler *et al.*, 1988; Miura *et al.*, 1989).

Over the past few years, we have carried out experiments on Schiff bases of PLP, using them as model compounds with the main aim of obtaining information that could help in interpreting the behaviour of PLP-dependent enzymes. On the basis of kinetic experiments on the formation and hydrolysis of Schiff bases of PLP, we have shown the kinetic constants of hydrolysis involved to be sensitive to the presence of hydrophobic groups in the vicinity of the imino bond (Vázquez *et al.*, 1990); this, however, does not alter the proportion of the tautomers present in solution at a given pH and ionic strength.

The work reported here was undertaken to check whether the binding of PLP via a Schiff base to the amino group of a molecule at a position liable to result in strong hydrophobic interactions would by itself alter any of the major tautomeric species occurring in solution. In order to check this assumption we chose dodecylamine (DOD), which is very likely to give rise to such interactions on account of its long hydrocarbon chain.

The behaviour of deoxypyridoxal (DPL) Schiff bases was also investigated, since this compound is a very good analogue of PLP, and the lack of a phosphate group makes it very useful for checking the importance of such a group (Vázquez *et al.*, 1989).

EXPERIMENTAL

Materials

DPL was synthesized from pyridoxine hydrochloride as described by Ikawa (1968). Dioxane was purified by refluxing over metallic sodium. The peroxide test (2% KI), as performed with

Abbreviations used: PLP, pyridoxal 5′-phosphate; DPL, 5′-deoxypyridoxal; DOD, dodecylamine; HEX, n-hexylamine; 6-FPLP, 6-fluoropyridoxal 5′-phosphate.

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freshly distilled dioxane, was always negative. PLP and all other reagents were purchased from Merck and were used with no further treatment.

Acetate, Hepes, phosphate and carbonate buffers were used throughout except at high-pH values, where we used NaOH. PLP and DPL solutions were prepared in the corresponding buffers and their exact concentrations were determined by dilution with 0.1 M-NaOH or -HCl (Peterson & Sober, 1954).

The water-insolubility of DOD compelled us to synthesize it in its hydrochloride form by using an HCl stream. The hydrochloride thus obtained was recrystallized in a mixture of ethanol/acetone/ether (1:1:1, by vol.). DOD solutions were prepared by dissolving a given amount of the hydrochloride in the minimum volume of ethanol (100 μl) under ultrasonic irradiation. The solutions thus obtained were diluted with the corresponding buffer. The ethanol content of the medium was never higher than 0.1 %. The concentration of DOD in the reaction medium was always lower than the critical micelle concentration, and very close to it at very low pH values (Galera & Ballesteros, 1989).

n-Hexylamine solutions were also prepared from the corresponding hydrochloride, which was dissolved in water containing no ethanol.

The procedure followed to prepare the solutions for spectroscopic measurement has been described elsewhere (Vázquez *et al.*, 1991). The ionic strength of the solutions was maintained at 0.1 mol/l as far as possible. After mixing, the solutions were left for 25 min in order to ensure that equilibrium was reached. The pH of each solution was measured with a Crison pH-meter by using Metrohm EA 120 combined electrodes which had previously been calibrated with aqueous buffers at 25 °C. Full spectra of the solutions were recorded on a Uvikon 940 spectrophotometer, while fluorescence spectra were obtained on a Perkin–Elmer MPF 66 spectrofluorimeter.

Methods

The overall reaction between the aldehyde and the amine can be depicted as follows:

$$R\text{-}CHO + R'\text{-}NH_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R\text{-}CH{=}N\text{-}R' + H_2O$$

the equilibrium constant of which can be written as:

$$K_{pH} = [SB]_e/[P]_e[L]_e \quad (1)$$

where $[SB]_e$, $[P]_e$ and $[L]_e$ are 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 results exclusively from 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:

$$A(\lambda) = [P]_e\epsilon_p(\lambda) + [SB]_e\epsilon_{SB}(\lambda) \quad (2)$$

where $\epsilon_p(\lambda)$ and $\epsilon_{SB}(\lambda)$ are the molar absorptions of the aldehyde and Schiff base respectively.

The spectra of the Schiff bases were obtained by a computer-assisted method fitting the experimental results to eqn. (2). The concentrations $[P]_e$ and $[SB]_e$ were determined from eqn. (1). The ϵ_p and K_{pH} values used for this purpose were determined in our laboratory or taken from Harris *et al.* (1974).

Spectra were deconvoluted into log normal curves by using a method reported by Metzler *et al.* (1973). The wave number of maximum absorption, the maximum molar absorption, the bandwidth and its skewness were the four input data required by the computer in each case. The program minimizes the sum of the squares of the deviations and obtains the output data from the best fitting, which allows the area (integrated intensity) of the absorption band of each tautomer to be determined. Once these areas (a_i) and the molar areas of the tautomers have been calculated, the fraction of Schiff base occurring as each tautomer can be determined from the relationship:

$$x_i = a_i/a_i^0$$

We had to calculate the molar area of each tautomer (a_i^0). For this purpose, and taking account of the fact that this parameter depends on neither the temperature nor the solvent used, we recorded the spectra of the Schiff bases in anhydrous dioxane and in various dioxane/water mixtures at different temperatures (15, 20, 25 and 30 °C). Taking account of the fact that $\Sigma x_i = 1$, we may write:

$$\Sigma_i(a_{ji}/a^0_{ji}) = 1 \quad (3)$$

where j is the number of each chemical species and i denotes the corresponding tautomer of species j. The molar areas were obtained by fitting the results obtained with the different solvents and temperatures.

RESULTS AND DISCUSSION

Analysis of the band shapes

The analysis carried out on the spectra of the Schiff bases of n-hexylamine (HEX) and PLP (HEX–PLP) in aqueous solutions of pH between 4.5 and 12.5 allows us to conclude that there are three macroscopic pK values which correspond to four ionic species, namely B_0, B_1, B_2 and B_3, where the subscripts denote the number of net negative charges on the molecule. Such macroscopic pK_a values (5.54, 6.62 and 11.90) are consistent with those obtained for the same system by kinetic methods (Vázquez *et al.*, 1991*b*), as well as with those obtained for related systems (Metzler *et al.*, 1980; García del Vado *et al.*, 1987; Mitra & Metzler, 1988; Vázquez *et al.*, 1990).

By way of example, Fig. 1 shows the spectrum recorded for one of the species and the log normal curves obtained by fitting as described in the Experimental section. Table 1 lists the values of the typical parameters of the log normal curves into which the spectra were divided. The results obtained for the HEX–PLP system are consistent with those reported for the Schiff bases of PLP and amino acids (Mitra & Metzler, 1988; Morozov *et al.*, 1988; Vázquez *et al.*, 1991*a*).

The different curves resulting from the deconvolution of the overall spectra can be assigned to the tautomeric forms in which

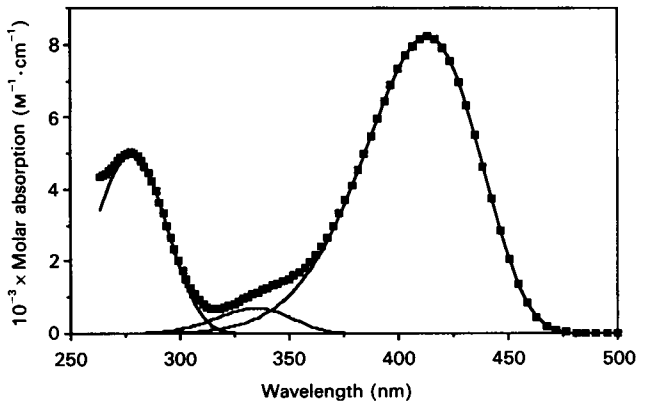


Fig. 1. Absorption spectrum of ionic species B_1 of Schiff base HEX–PLP in aqueous solution at 25 °C fitted with log normal distribution curves

Table 1. Positions and shapes of absorption bands of four Schiff bases (B) resolved with log normal distribution curves

Schiff base	Band maximum (nm)	Height ($M^{-1} \cdot cm^{-1}$)	$10^{-3} \times$ Width (cm^{-1})	Skewness	$10^{-6} \times$ Area ($mmol^{-1}$)
HEX–PLP					
In water					
B_0	413	8.89	3.72	1.48	362.7
	334	0.73	3.68	1.28	28.8
	280	3.52	5.05	1.26	191.2
B_1	413	8.22	3.72	1.45	334.6
	335	0.70	3.67	1.27	27.7
	278	5.00	5.05	1.26	271.6
B_2	413	6.30	4.11	1.38	281.2
	353	0.36	4.35	1.17	16.7
	335	0.68	4.55	1.30	33.4
	281	5.56	4.76	1.26	284.7
B_3	342	6.60	4.34	1.44	312.9
In dioxane	336	4.67	4.59	1.20	224.6
HEX–DPL					
In water					
B_{-1}	414	8.58	3.71	1.42	347.0
	335	0.56	3.64	1.30	25.1
	282	5.61	5.01	1.22	301.5
B_0	415	6.62	4.08	1.40	296.0
	353	0.43	4.35	1.18	20.0
	340	1.01	4.55	1.22	49.8
	281	7.77	5.08	1.31	426.2
B_1	336	6.46	6.54	1.25	454.1
In dioxane	340	4.46	4.58	1.25	219.6
DOD–PLP					
In water					
B_0	416	4.01	3.72	1.45	163.4
	334	3.61	3.68	1.28	143.6
	282	6.65	5.06	1.21	360.7
B_1	417	0.38	4.11	1.39	16.9
	351	0.07	4.35	1.20	3.3
	336	4.17	4.71	1.31	212.1
	282	1.33	4.76	1.26	68.1
In dioxane	336	4.36	4.72	1.32	222.4
DOD–DPL					
In water					
B_{-1}	415	3.85	3.74	1.43	157.9
	339	3.66	3.65	1.33	144.5
	284	5.98	5.03	1.21	322.5
B_0	418	0.33	4.09	1.40	14.8
	353	0.05	4.35	1.18	2.3
	340	4.10	4.68	1.27	206.5
	282	1.08	4.61	1.23	53.4
In dioxane	340	4.27	4.72	1.29	217.3

the system can occur (Metzler *et al.*, 1980). Scheme 1 shows the structural formulae of such tautomers, as well as their wavelengths of maximum absorption.

The above study was also performed on the imine of DPL and HEX (HEX–DPL), the former of which is a representative analogue of PLP, in order to determine the influence of the phosphate group. The aldimine was found to occur as three rather than four ionic species over the same pH range, namely B_{-1}, B_0 and B_1, which give rise to two macroscopic pK values (6.23 and 11.81) consistent with those of the PLP imine. The tautomers involved are thus equivalent to those depicted in Scheme 1. The results obtained in this respect are also listed in Table 1.

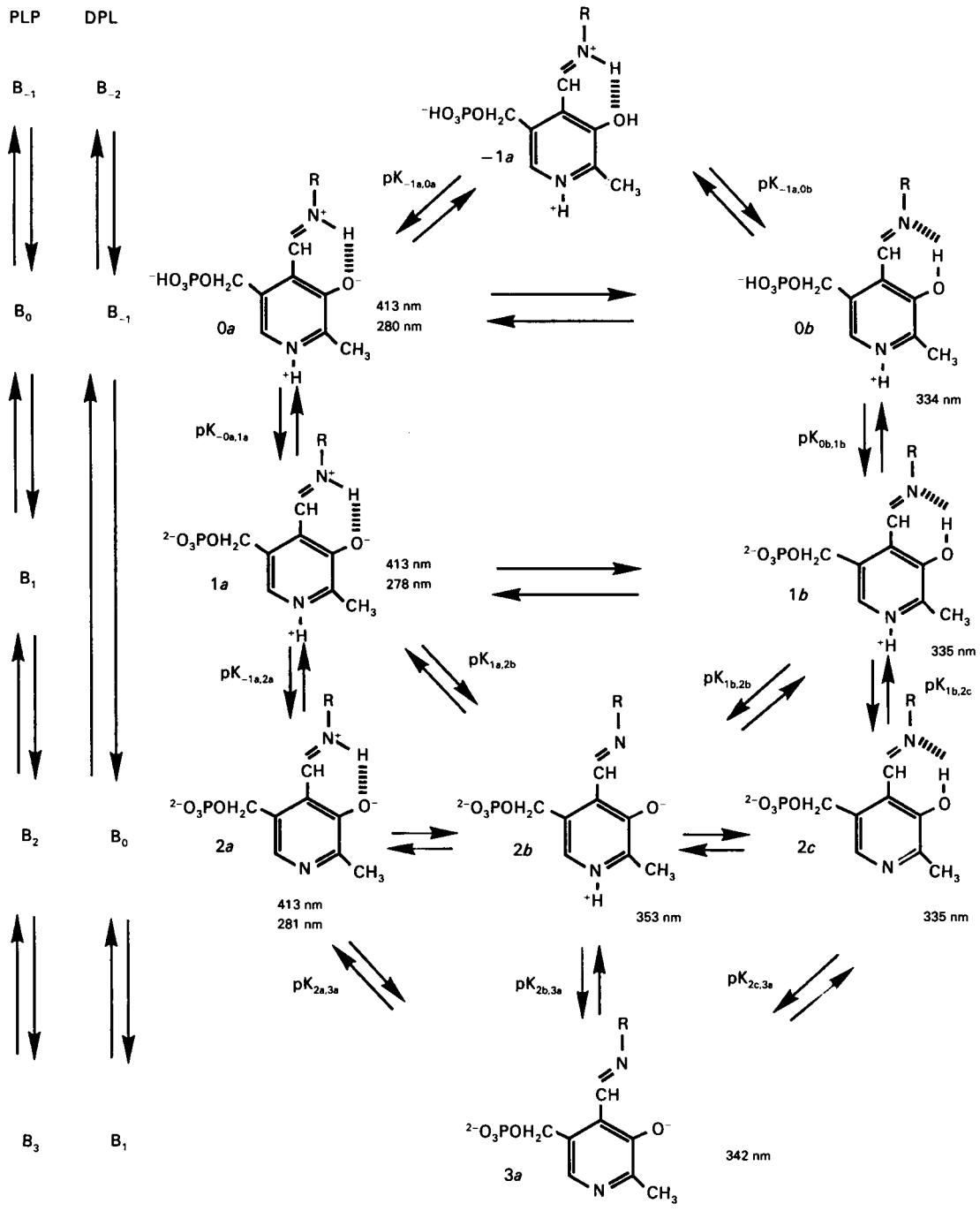
In both imines, the tautomer B_{ja} has two absorption maxima at 413 and 280 nm, whatever the degree of protonation of the pyridine nitrogen. However, the height and area of the band at 413 nm decreases while the corresponding parameters of the band at 280 nm increase in passing from the species with a protonated pyridine nitrogen (B_{-1a} in DPL and B_{1a} in PLP) to that with an unprotonated nitrogen (B_{0a} in DPL and B_{2a} in PLP).

The phosphate group exerts a minimal effect on the position of the absorption bands. Nevertheless the absorption intensity of the band of tautomer *a* at 280 nm is decreased by about 30 % in relation to the form with an unprotonated nitrogen. On the other hand, tautomer *b* is completely insensitive to the protonation.

Table 1 gives the characteristic parameters of the single band in the absorption spectrum of the imines obtained in anhydrous dioxane, which should correspond to the neutral enolic form (Metzler *et al.*, 1980).

Subjecting the Schiff bases of DOD and PLP or DPL to the above-described spectroscopic study was rendered difficult by the decreased solubility of the bases in basic media; thus the imine concentrations present at a pH of about 8 were insufficient to obtain detectable signals. For this reason, basic pK values were determined by spectrofluorimetry.

A first look at the results obtained from the spectra of the



Scheme 1. Structures of tautomers of HEX–PLP Schiff base

Numbers are the characteristic band wavelengths.

Schiff base of DOD and PLP (DOD–PLP) reveals the occurrence of a single macroscopic pK (3.62) over the pH range investigated. Kinetic measurements produced another pK at 7.91 (Vázquez *et al.*, 1991*b*) which was taken into account in deconvoluting the absorption curves. The Schiff base DOD–DPL also features a single pK (3.81) in aqueous solutions.

In order to determine which group should be assigned to this macroscopic pK, we made fluorescent measurements on excitation at 415 and 336 nm at various pH values and recorded the corresponding emission spectra. Fig. 2(*a* and *b*) shows the emission spectra in question; as can be seen, virtually the whole fluorescence change took place below pH 6.5. The fluorescence changes can be ascribed to variations in the protonation state of the pyridine nitrogen (Bridges *et al.*, 1966) rather than to changes in the phosphate group. This indicates that the macroscopic pK value of the phosphate group in the imine of HEX (5.54) increases by 2.5 pH units or more, i.e. one should take into account the fact that the phosphate group is deprotonated much later than the pyridine nitrogen when assigning the ionization states and structural forms to the tautomers of the imines of DOD.

Fig. 3 shows the absorption spectrum of the ionic species B_0 of DOD–PLP and its deconvolution into log normal curves. In Scheme 2, which corresponds to the Schiff base of DOD and

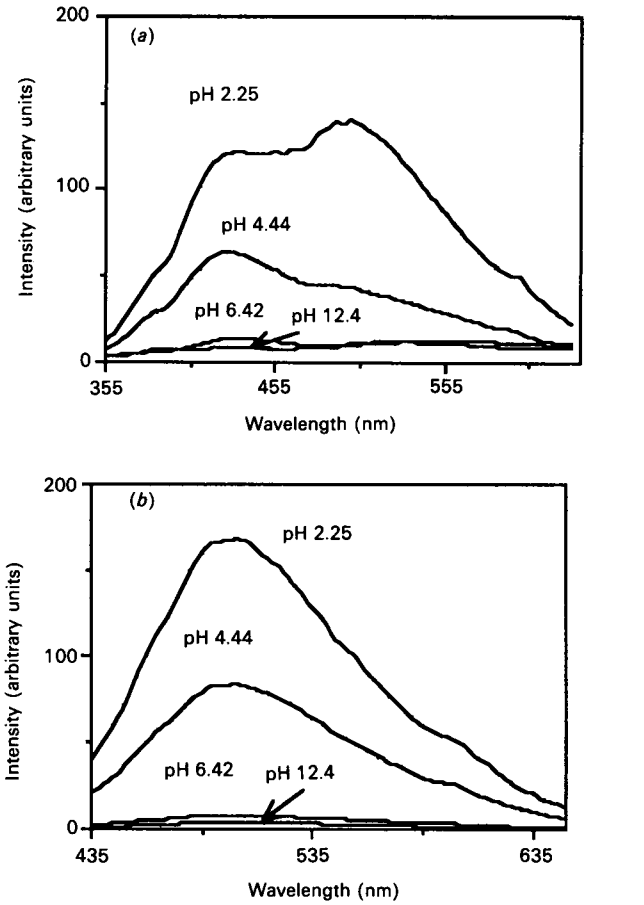


Fig. 2. Fluorescence emission spectra of Schiff base DOD–PLP in water at 25 °C at several pH values

(*a*) $\lambda_{exc.}$ = 415 nm. (*b*) $\lambda_{exc.}$ = 336 nm.

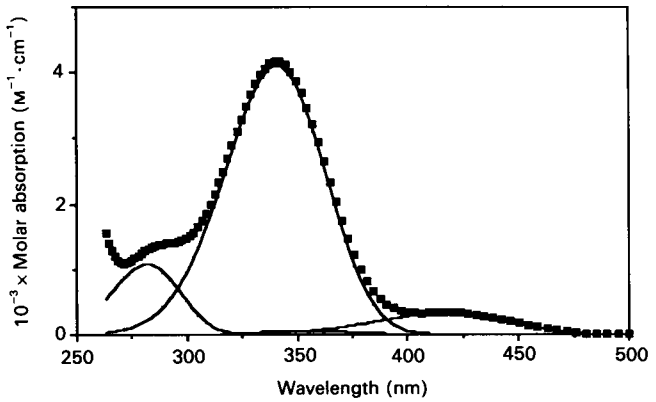


Fig. 3. Absorption spectrum of ionic species B_0 of Schiff base DOD–PLP in water at 25 °C fitted with log normal distribution curves

PLP, the ionic species B_2 and B_3 are included by analogy with those of the HEX–PLP system, even though their spectra could not be obtained.

Table 1 lists the characteristic parameters of the log normal bands of the DOD–PLP and DOD–DPL Schiff bases. Because of the above-described constraints, only the spectra of B_0 and B_1 for DOD–PLP, and of B_{-1} and B_0 for DOD–DPL could be obtained. We should emphasize that the positions of the absorption maxima of the bands are independent of the amine residue in both systems. However, the height and area of such bands are significantly greater than those of the Schiff bases of HEX. Obviously, the prevailing tautomers of these systems do not coincide at all with those of the Schiff bases of DOD.

Table 1 also includes the characteristic parameters of the spectrum of these Schiff bases in dioxane; as expected, the spectrum shows a single band, the absorption maximum of which coincides with that of the forms B_{1c} in DOD–PLP and B_{0c} in DOD–DPL, and so does its height and area, thereby indicating that, in aqueous solutions, the Schiff bases of DOD occur as tautomers stable in non-polar hydrophobic media.

Tautomerization ratios and microscopic ionization constants

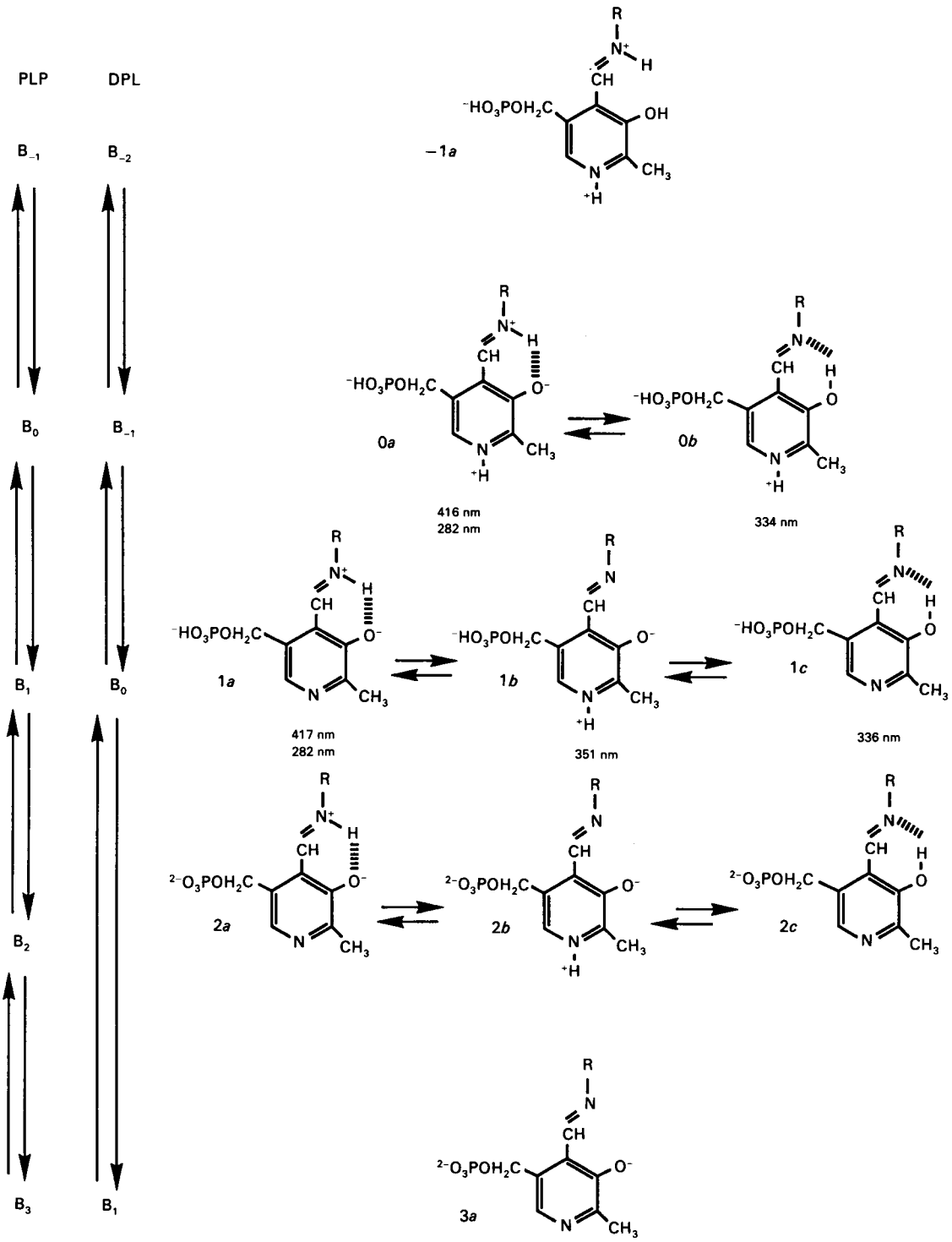
From the molar area of each band we calculated the proportion in which the different tautomers occur in the system. Table 2 lists the tautomerization ratios (x_i) for the Schiff bases of HEX, which are consistent with those reported for other amino acids (Metzler *et al.*, 1980; Mitra & Metzler, 1988; Vázquez *et al.*, 1990). The zwitterionic forms (B_{-2a}, B_{-1a} and B_{0a}) are prevalent in aqueous solutions. Such is not the case with the Schiff bases of DOD. Thus tautomer B_0 (*c* in Scheme 2) accounts for 95% of DOD–DPL, while forms *b* and *a* account for only 1 and 4% respectively. This indicates that the microenvironment of DPL in this imine is quite different from that of the imine of HEX and favours the occurrence of the tautomer with no charge separation, i.e. that obtained in anhydrous dioxane (Table 1). The situation is exactly the same for the DOD–PLP system, in which the tautomer B_{1c} accounts for 94% of the Schiff base and is thus virtually the sole form present.

These results indicate that, in aqueous solutions, water is virtually completely excluded from the vicinity of the imino bond and from the aldehyde part in the imines of DOD. PLP and DPL are embedded in highly hydrophobic environments, which accounts for the fact that the pK of the phosphate group of PLP in this imine is higher than that of the imines of other amino acids; in fact, in a hydrophobic medium, of low polarity, the pK of an acid group such as phosphate should be higher, about 2 pH units for dioxane (Bates *et al.*, 1963).

In this situation, a Schiff base embedded in a highly hydrophobic medium must be rather stable since the next species in the acid–base sequence can only be arrived at on a marked increase in the proton concentration (pK_{0b} = 3.62). Even under these conditions, species *b* prevails over *a*, contrary to what happens in HEX–PLP. The results obtained for the DOD–DPL system support the above reasoning.

From the macroscopic pK values and the tautomerization rates, the microscopic pK_a values of the tautomers were calculated, which are listed in Table 3. We should note that the microscopic pK values were virtually the same as their macroscopic counterparts for the major forms. Thus, the pK_{1b} of HEX–PLP (6.62) is virtually identical with its $pK_{1a,2a}$ (6.66) since the prevalent form is B_{ja}. This is also the case with the imine of DOD, the pK_{0b} of which (3.62) is virtually identical with the $pK_{0b,1c}$ (3.47); also, the pK_{-1b} and $pK_{-1b,0c}$ of the Schiff base of DOD and PLP are very similar, as the prevalent tautomers are the forms *c* and *b* (see Table 3).

However, it is significant that the macroscopic pK values of the DOD–PLP system diverge from those of the HEX–PLP system, particularly as the latter coincide with those of the Schiff bases of PLP and other amino acids. This can be accounted for on two bases. On the one hand, species B_1 in HEX–PLP is not equivalent to that in DOD–PLP as the phosphate group occurs as PO_4^{2-} in the former and as HPO_4^- in the latter. In any case, we do not believe that this is sufficient to decrease the macroscopic pK from 5.54 to 3.62. In addition, the Schiff base of DPL does not contain



Scheme 2. Structures of tautomers of DOD–PLP Schiff base

Numbers are the characteristic band wavelengths.

the phosphate group and yet behaves identically (its pK_{1b} is decreased from 6.23 in HEX–DPL to 3.81 in DOD–DPL).

We believe that the true reason lies in the high stability achieved by DOD–PLP and DOD–DPL in adopting a conformation in which the pyridine ring is embedded in a hydrophobic environment, where it can only be destroyed if the proton concentration of the medium is so high that the pyridine nitrogen is protonated, and interactions with the solvent and hence adoption of a given conformation are favoured. For this reason, the fraction of typical tautomers of a hydrophilic environment (e.g *a* forms) increases at lower pH values: from 5% (1*a*) to 33% (0*a*) in DOD–PLP, and from 4% (0*a*) to 41% (−1*a*) in DOD–DPL.

The microscopic p*K* values obtained from fluorescence measurements are listed in Table 3. These measurements were made on excitation at wavelengths typical of a single tautomer and thus provide information on the ionization constant of each form.

Fig. 4 shows the variation of the fluorescence intensity at 525 nm with the pH obtained by exciting aqueous solutions of DOD–PLP at 415 nm. Fitting of these experimental results provided two pK values, namely 2.68 and 4.26. On the basis of Scheme 1, and taking into account the fact that excitation at 415 nm only provides information on the tautomer forms B_{ja}, the latter pK (4.26) must correspond to the ionization of B_{0a} to B_{1a} as it coincides with the data previously obtained by deconvolution of the absorption spectra.

On the other hand, the former pK (2.68) can be ascribed to the ionization of the phenol group, i.e. to $pK_{-1a,0a}$, since the ionization of the phosphate group should not alter the fluorescence of the molecule. As applied to aqueous solutions of DOD–DPL, the fluorescence experiments provided two pK values as well, namely 4.66 and 2.87, which obviously correspond to the ionization of the same groups.

By plotting the fluorescence intensity at 525 nm against the pH on exciting DOD–PLP at 336 nm we also obtained two microscopic pK values (Fig. 4), namely 3.45 and 3.01, which must correspond to the ionization of tautomers b and c, i.e. to $pK_{0b,1c}$ and $pK_{-1a,0b}$ respectively.

Fig. 5 shows the variation of the maximum emission wavelength with the pH obtained on excitation at 336 nm. The result is a typical deprotonation plot, fitting of which provided a pK (11.34) that corresponds to the ionization of B_{2c} to B_{3a}. The same experiment was performed on DOD–DPL, the pK values obtained in this case being listed in Table 3.

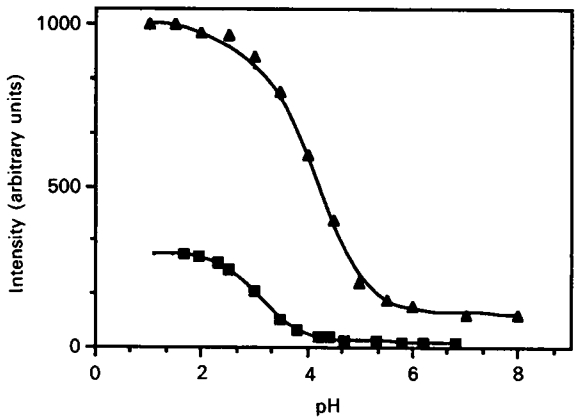


Fig. 4. Fluorescence intensity of Schiff base DOD–PLP in water versus pH at two different excitation wavelengths (415 nm, ▲, and 336 nm, ■)

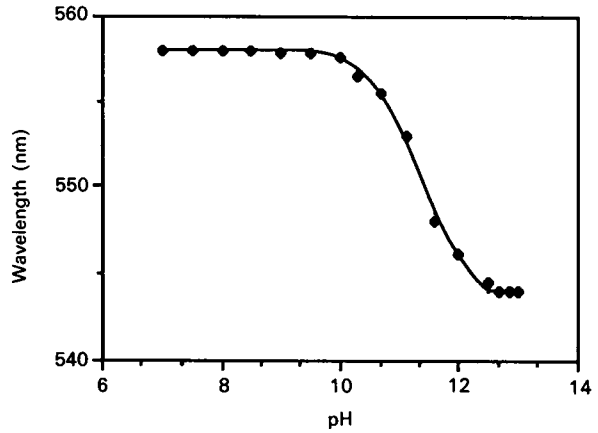


Fig. 5. Wavelength of maximum intensity of fluorescence emission of Schiff base DOD–PLP in water versus pH on excitation at 336 nm

Table 2. Molar areas ($mmol^{-1} \times 10^{-6}$) and tautomeric ratios for the PLP and DPL Schiff bases

	DOD–DPL	HEX–DPL		DOD–PLP	HEX–PLP
a^0_{-1a}	385	385	a^0_{0a}	380	407
a^0_{-1b}	245	255	a^0_{0b}	252	262
a^0_{0a}	370	373	a^0_{1a}	375	376
a^0_{0b}	335	335	a^0_{1b}	330	253
a^0_{0c}	217	220	a^0_{1c}	222	–
x_{-1a}	0.41	0.90	a^0_{2a}	–	351
x_{-1b}	0.59	0.10	a^0_{2b}	–	330
x_{0a}	0.04	0.79	a^0_{2c}	–	225
x_{0b}	0.01	0.06	x_{0a}	0.33	0.89
x_{0c}	0.95	0.15	x_{0b}	0.67	0.11
			x_{1a}	0.05	0.89
			x_{1b}	0.01	0.11
			x_{1c}	0.94	–
			x_{2a}	–	0.80
			x_{2b}	–	0.05
			x_{2c}	–	0.15

Table 3. Macroscopic and microscopic ionization constants of PLP and DPL Schiff bases

	HEX–DPL	DOD–DPL		HEX–PLP	DOD–PLP
pK_{-1b}	6.23	3.81	pK_{0b}	5.54	3.62
pK_{0b}	11.81	–	pK_{1b}	6.62	(7.91†)
$pK_{-1a,0a}$	6.28	4.82 (4.66*)	pK_{2b}	11.90	–
$pK_{-1a,0b}$	7.40	5.42	$pK_{0a,1a}$	5.54	4.44 (4.26*)
$pK_{-1b,0c}$	6.13	3.60 (3.68*)	$pK_{0b,1b}$	5.54	5.44
$pK_{-1b,0b}$	6.53	5.58	$pK_{1a,2a}$	6.66	$pK_{0a,1b}$ 5.14
$pK_{0a,1a}$	11.71	–	$pK_{1a,2b}$	7.86	$pK_{0b,1c}$ 3.47 (3.45*)
$pK_{0b,1a}$	10.59	–	$pK_{1b,2b}$	6.96	–
$pK_{0c,1a}$	10.99	(11.24*)	$pK_{1b,2c}$	6.52	–
			$pK_{2a,3a}$	11.80	–
			$pK_{2b,3a}$	10.60	–
			$pK_{2c,3a}$	11.08	(11.34*)

* Data from fluorescence results.
† Data from kinetic results.

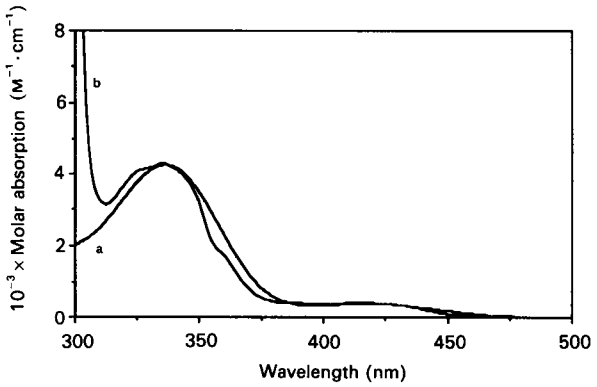


Fig. 6. Comparison between the absorption spectrum of (a) Schiff base DOD–PLP in water at pH 6.5 and (b) the difference absorption spectrum holophosphorylase *b* − apophosphorylase *b*

Data from spectrum b were taken from Donoso (1984).

Comparison of the spectra of DOD–DPL and glycogen phosphorylase

One of the most relevant conclusions that can be drawn from this work is that, in aqueous solutions, the Schiff base formed between PLP and the amino residue of a sufficiently large hydrocarbon chain preferentially occurs as the conformation that takes up the shape of a hydrophobic pocket from which water is virtually completely excluded. This has been reported to occur in glycogen phosphorylase, in which the Schiff base formed between the coenzyme (PLP) and an α-amino residue of Lys-679 is enclosed in a hydrophobic pocket (Shaltiel & Cortijo, 1970; Fletterick & Madsen, 1980).

The absorption spectrum of the coenzyme in glycogen phosphorylase *b* is shown in Fig. 6, which also shows that obtained for DOD–PLP in an aqueous solution at pH 6.5; the two nearly completely overlap. The differences between these two spectra below 315 nm arise from the contribution of the aromatic amino acid residues in the protein.

Glycogen phosphorylase *b* requires binding to its allosteric activator, AMP, and other effectors, such as Mg^{2+}, to become active (Graves & Wang, 1972). On binding to AMP, phosphorylase *b* induces a change in the state of its coenzyme which in turn results in a differential absorption spectrum with a minimum at 330 nm and a maximum at 360 nm (Donoso *et al.*, 1985). Such a spectrum is increased by the presence of Mg^{2+}, glucose 1-phosphate and inorganic phosphate (Buc-Caron *et al.*, 1974; Bresler & Firsov, 1968; Bresler *et al.*, 1966). This must be ascribed to a displacement of the tautomeric equilibrium from B_{1c} (an enolimine), which is stable in the absence of effectors, to B_{1b}, an ion dipolar form (see Scheme 2). The form B_{1b} prevails in neither model system, HEX–PLP (hydrophilic environment) or DOD–PLP (hydrophobic environment). In this context, we should note that the dipolar ion form prevails in other PLP-dependent enzymes such as transaminases, and that the close binding of the protonated nitrogen in the aromatic ring to an aspartate ion at the active site of aspartate aminotransferase has been postulated to stabilize such a form, and the oxygen in the phenolate ion to interact with other groups by forming hydrogen bonds, thus exerting an additional stabilizing effect (Braunstein *et al.*, 1985).

Results shown here seem to be in disagreement with those coming from ^{19}F-n.m.r. studies on phosphorylase *b* reconstituted with 6-fluoropyridoxal 5′-phosphate (6-FPLP) which have shown that the enzyme is active with the pyridine nitrogen in the neutral form and that the state of ionization of that nitrogen is unlikely to be affected by the binding of substrates (Chang & Graves, 1985; Chang *et al.*, 1986).

The 6-FPLP-reconstituted phosphorylase *b* is very similar to the native phosphorylase but it was found to be far less active and similar to pyridoxal-reconstituted phosphorylase *b*, their $V_{max.}$ values being 17.8, 66.7 and 15.4 μmol/min per mg respectively (Chang *et al.*, 1987). Such a loss in activity was explained on the basis of a lower basicity for the pyridine nitrogen of the former enzyme and of a change in the interaction between the pyridine nitrogen with some residue on the enzyme (Chang & Graves, 1985).

The drastic change in the basicity of pyridine nitrogen in 6-FPLP (Korytnyk & Kravastava, 1973) will considerably alter the constant values in Scheme 2 in such a way that the binding of AMP and other substrates to 6-FPLP-reconstituted phosphorylase could not render the same coenzyme tautomer as in the native phosphorylase. The fact that the catalytic activity of the 6-FPLP-reconstituted enzyme is not totally abolished has been taken as proof that the interaction between the pyridine nitrogen of PLP and the protein is not essential for catalytic activity and that the role of the ring nitrogen of PLP is structural rather than catalytic (Chang & Graves, 1985; Chang *et al.*, 1986, 1987). Nevertheless the difference in absorption spectrum observed on the binding of AMP and substrates to native phosphorylase cannot be explained if ionization changes on the coenzyme do not happen.

The stabilization of the B_{1b} form of the coenzyme in the active state of phosphorylase *b* probably arises from the presence of charged amino acid residues in the hydrophobic pocket which stabilize the charge of the phenolate ion and interact with the protonated pyridine nitrogen. The carboxy group of Glu-645 has been suggested as a candidate for this last function (Chang & Graves, 1985). The DOD system, which only possesses a long hydrocarbon chain, is inadequate to simulate this environment.

The results obtained for DOD–PLP allow the conformational behaviour of the coenzyme in PLP-dependent enzymes to be rationalized.

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