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# Fluorescence of the Schiff Bases of Pyridoxal and Pyridoxal 5'-Phosphate with L-Isoleucine in Aqueous Solutions

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The present study reports on the absorption and emission properties of the Schiff bases formed by pyridoxal and pyridoxal 5'-phosphate with L-isoleucine in aqueous solutions. Species protonated at the imine and ring nitrogen are the most fluorescent in both Schiff bases with a quantum yield of 0.02, i.e., 20-fold the value found for species in alkaline solutions. In agreement with other studies, species protonated at the imine nitrogen shows an emission around 500 nm upon excitation at 415 nm. In contrast to previous observations on other PLP Schiff bases, emissions at 560 nm (PL-Ile) and 540 nm (PLP-Ile) are observed upon excitation at 365 and 415 nm, respectively. The emission at 470 nm found in PLP-Ile Schiff base upon excitation at 355 nm is ascribed to a multipolar monoprotonated species. An estimation for the $pK_a$ of the imine in the excited state ( $\approx$ 8.5) for both Schiff bases is also reached. Our results suggest that fast protonation reactions on the excited state are responsible for the observed fluorescence. These effects, in which the hydrogen bond and the phosphate group seem to play a role, could be extended to understanding coenzyme environments in proteins.

**KEY WORDS:** Pyridoxal; pyridoxal-5'-phosphate; Schiff base; L-isoleucine; fluorescence; fast protonation in the excited state.

## INTRODUCTION

Spectroscopic properties of pyridoxal-5'-phosphate (PLP) and its Schiff bases have often been considered in discussions of PLP-dependent enzymes.[1,2] Hydration, acid–base, and tautomeric equilibria of the coenzyme as well as those of its Schiff bases have been well established. Absorption spectroscopy and deconvolution by lognormal fitting of the UV–visible bands have been very useful in describing PLP properties in the ground state[3] and they have been also applied in the study of PLP-dependent enzymes.[4,5]

Fluorescence spectroscopy, as a powerful and sensitive tool in studies of macromolecules, has also been applied.[6–10] Earlier studies reported the fluorescence of some PLP Schiff bases as a simple model of PLP bound to proteins.[7,8] For instance, some studies dealt with the finding that PLP–hexylamine Schiff base reproduces, in organic solvent, the large Stokes shift found in glycogen phosphorylase.[8]

Recently we have studied the reactivity of these molecules. The approach involves quantitative characterization of the formation of the Schiff base[11] and some proton recombination reactions of the ring and imine nitrogens, through electrochemical techniques.[12–15] Some results induced us to carry out a deeper study on the fluorescence properties of PLP Schiff bases, in particular, those derived from α-amino acids. The present study reports on the absorption and emission properties of pyr-

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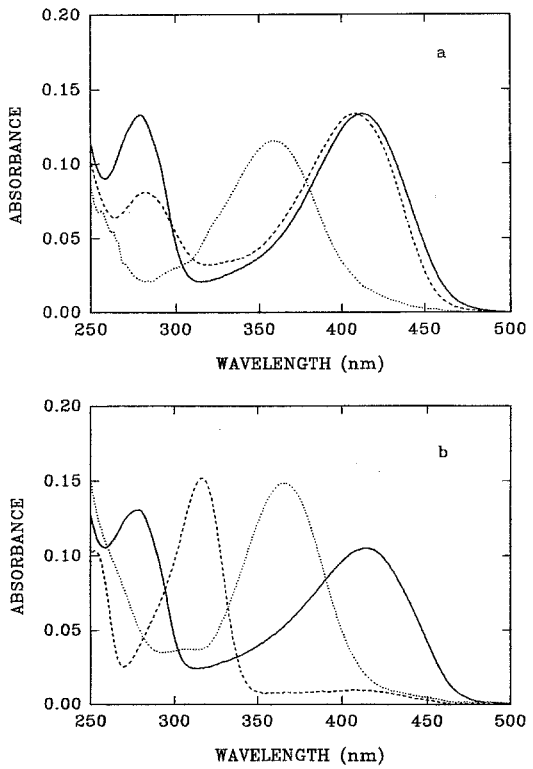



**Fig. 1.** Absorption spectra of PLP and PL with Ile mixtures. (a) PLP–Ile: (···) pH 13; (—) pH 9; (---), pH 5. (b) PL–Ile: (···) pH 12; (—) pH 9; (---) pH 5.

idoxal, pyridoxal-5'-phosphate, and L-isoleucine Schiff bases in aqueous solutions. Some observed effects on the nitrogen basicity, which may be useful in understanding coenzyme environments, are discussed.

## EXPERIMENTAL

Pyridoxal-5'-phosphate (PLP), pyridoxal (PL), and L-isoleucine (Ile) were purchased from Sigma. All other chemicals used were from Merck, p.a. grade. Buffered solutions consisting of 0.02 *M* phosphate buffer were used. The pH was adjusted with KOH and the ionic strength was adjusted to 0.1 moldm$^{-3}$ with KCl.

PLP, PL, and Ile solutions were prepared daily in a suitable buffer and kept in the dark. The imines were formed by the addition of known amounts of PLP to buffered solutions of Ile. The reaction mixture was studied after the equilibrium was reached.

Spectrophotometric measurements were performed on a Perkin Elmer Lambda 3B with 1-cm quartz cuvettes thermostated at 25 ± 0.1°C.

Fluorescence spectra were recorded on a MPF 66 Perkin Elmer spectrophotometer furnished with a 150 W-xenon lamp and thermostated cuvettes at 25 ± 0.1°C. All measurements were carried out with solutions having an absorbance lower than 0.2. Fluorescence quantum yields were determined using quinine sulfate ($<3 \cdot 10^{-5}$ mol · dm$^{-3}$) as standard ($\Phi = 0.546$).[16]

To avoid hydrolysis of the imines, mainly at acid and neutral pH, an initial molar ratio of Ile to PLP as high as 5500 was employed, over the whole pH range. However, since the quantum yield of the imine species in alkaline solutions appears to be one order lower than in a weak acid medium, higher molar ratios were also employed. Moreover, PLP and PL as free aldehyde show a weak emission upon excitation at 388 nm that deserves special attention in dealing with the emission spectrum of the reaction mixture. Thus an initial molar ratio of amino acid to aldehyde of 11000:1 was employed to minimize the contribution of the free aldehydes upon excitation at 360–370 or 415–425 nm.

## RESULTS

The reaction of PL or PLP with Ile can be followed in solution by UV–visible spectroscopy. After the equilibrium is reached, formation of the Schiff base in alkaline solutions is indicated by bands at 414 and 275 nm for the monoprotonated imine. Dissociation of the imine proton produces a new band centred at 350–365 nm, which corresponds to the unprotonated form (Fig. 1a) as reported for similar PLP and PL imines.[1,2]

In acid media, hydrolysis of the PLP–Ile Schiff base[2,4] produces some contribution of the 390- and 295-nm bands of the PLP to the observed absorption, causing small shifts (Fig. 1a).

For PL–Ile in acid media, even under conditions of 5500-fold of Ile to PL, the hydrolysis of the imine is quite apparent and the absorption band (centred at 316 nm) of the PL hemiacetal predominates in solution (Fig. 1b). The spectrum observed at pH 5 practically coincides with that found for PL in the absence of the amino acid.[3]

Table I shows maximum wavelengths of the absorption bands and acid–base p$K_a$'s of the mixtures.

The PLP–Ile mixture displays a fluorescent emission band centered at around 490 nm, upon excitation at 415 nm, at pH 5. Fluorescence intensity and emission wavelength are strongly pH dependent, under conditions of a fixed initial ratio of Ile to PLP (Fig. 2). The bell-shaped variation of the fluorescence intensity shows a maximum at around pH 5.5.

**Table I.** UV–Visible Absorption and Fluorescence Properties of the Reaction Mixtures of PLP and PL with Isoleucine in Aqueous Solutions

| Sample | pH | $\lambda_a$ | | | $pK_a$ | $\lambda_f$ | $\Phi$ | $pK_a^*$ |
|---|---|---|---|---|---|---|---|---|
| PLP–Ile | 3.0 | 294 | 408 | | 3.2[a] | | | |
| | 5.0 | 281 | 408 | | 6.5[b] | 490 | 0.021 | 5.9[b] |
| | 9.0 | 279 | 414 | | 12.6[c] | 530 | < 0.001 | 8.5[c] |
| | 13.6 | | | 352 | | 540 | < 0.001 | |
| PL-Ile | 3.0 | 289 | | | 4.0[a] | | | |
| | 5.0 | 316 | | | 7.0[b] | 490 | 0.021 | 6.2[b] |
| | 9.0 | 275 | 414 | | 10.6[c] | 530 | < 0.001 | 8.5[c] |
| | 12.0 | | | 365 | | 560 | < 0.001 | |

[a] *o*-Hydroxyl group of the PLP or PL.
[b] Ring nitrogen of the Schiff base ($pK_1$).
[c] Imine nitrogen of the Schiff base ($pK_2$).

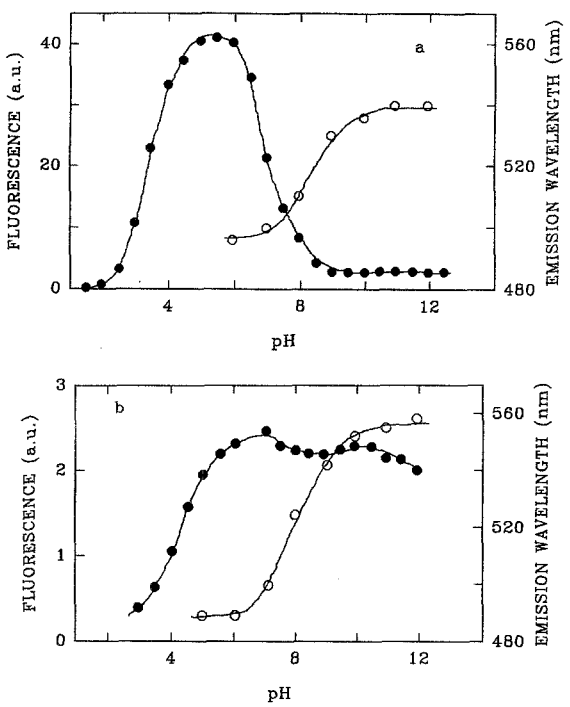



**Fig. 2.** Variation of the fluorescence intensity monitored at the maximum emission wavelength (500–550 nm) (●) and emission wavelength (○) of the Schiff bases with the pH upon excitation at 414 nm. (a) PLP–Ile; (b) PL–Ile.

A decrease in fluorescence intensity at pH below 5.5 is assigned to imine hydrolysis. However, at pH > 5.5, imine formation is favored and then the observed decrease may be produced by a change of the fluorescent species. The acid–base properties deduced from UV visible studies strongly suggest that dissociation of the ring proton is involved.[1,2,4] Basically, the fluorescent properties are similar to those found in earlier studies of PLP

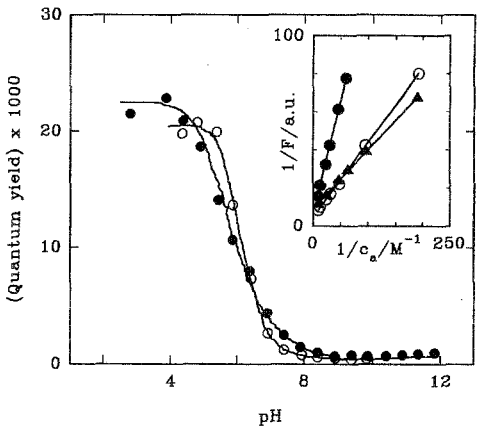



**Fig. 3.** Quantum yield as a function of pH ($\lambda_{exe}$ = 415 nm). (●) PLP–Ile; (○) PL–Ile. Inset: Plot of the fluorescence intensity as a function of the Ile concentration according to Eq. (1). PLP-Ile; (●) pH 3; (○) pH 4; (▲) pH 5.

Schiff bases of L-valine, *n*-butylamine, and *n*-hexylamine.[9,10,11]

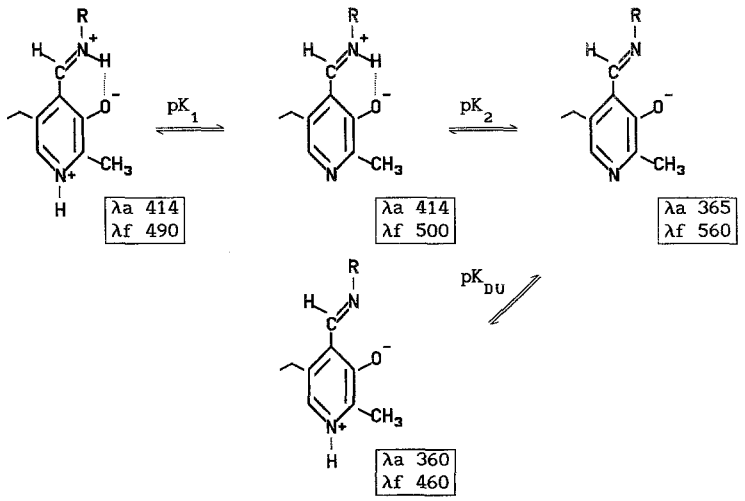
The PL–Ile mixture also displays a fluorescence emission band centered at 490–560 nm upon excitation at 415 nm, with an intensity almost 15-fold lower than that of the PLP mixture under the same experimental conditions (Fig. 2). In this case, the pH variation is not as marked as in PLP-Ile, indicating a low imine concentration at a pH where protonated ring nitrogen species appears.

In spite of this difference on stability, a comparison of the fluorescence of the imine was followed by using a simple aldehyde–Schiff base equilibrium approach [Eq. (1)].

$$1/F = 1/F_0 + (1/F_0K_{pH})\,(1/c_A) \qquad (1)$$

In this equation, $F$ is the observed fluorescence intensity, $F_0$ the fluorescence assuming total conversion of the initial aldehyde to Schiff base, $K_{pH}$ the apparent formation constant at a fixed pH, and $c_A$ the initial amino acid concentration, when an excess of this over aldehyde is provided.

Figure 3 shows the variation of the quantum yield for PLP and PL Schiff bases together with some plots according to Eq. (1). The inflections at pH 5.9 and 6.2 are reasonable estimates of the $pK_1^*$ value for PLP–Ile and PL–Ile, respectively. Accordingly no significant differences are observed for acid–base properties of the ring nitrogen in the ground and first excited singlet state (Table I). Moreover, the quantum yield, 0.021, of the most fluorescent species, is quite similar in PLP and PL Schiff bases and this value is close to that reported for PLP–valine.[9]




Scheme 1

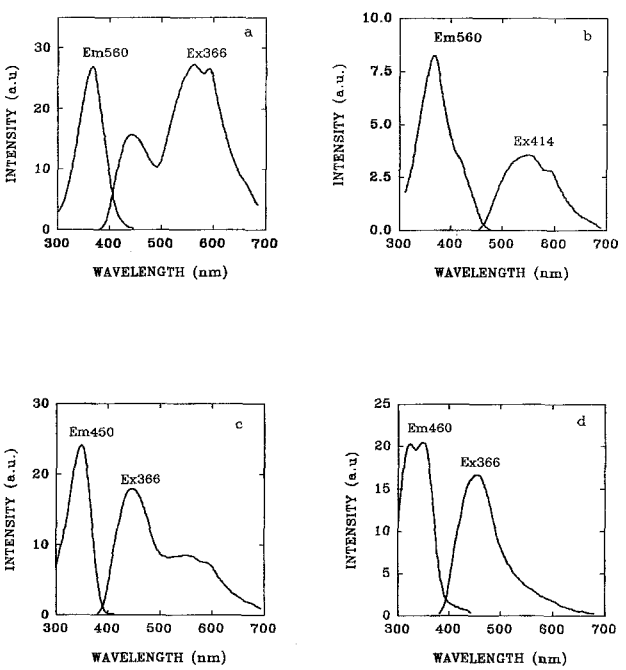



**Fig. 4.** Excitation and emission spectra of the PL–Ile Schiff bases. (a) pH 12; (b) pH 10; (c) pH 10; (d) pH 8. The emission and excitation wavelengths are indicated in each excitation and emission spectrum, respectively.

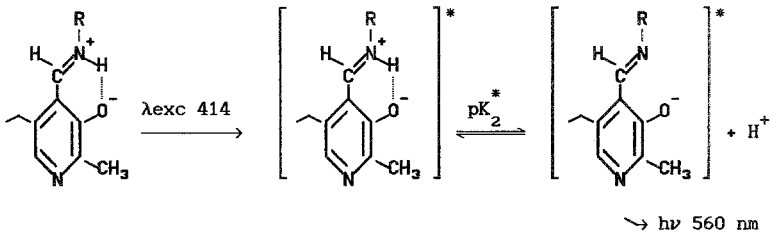
Scheme 1 shows imine species in neutral and alkaline solutions. According to UV–visible results, imonium–phenolate (di- and monoprotonated forms) together with imine free base predominates in aqueous solutions. However, small amounts of other tautomeric forms, i.e., enolimine and dipolar forms, may also be present according to the general behavior of PLP Schiff bases.[1,2]

In strong alkaline solutions PL–Ile shows an emission maximum at 560 nm upon excitation at 366 nm. The excitation spectrum, showing a maximum at 366 nm, confirms that the emission is caused by the free imine existing in solution at pH > p$K_2$ (Table I) (Fig. 4a).

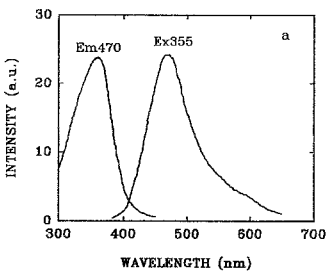
At pH 10 (note that the p$K_2$ value is about 10.6), emission at 560 nm is also observed upon excitation at 414 nm. However, the excitation spectrum reveals that free ($\lambda_{exc}$ 365 nm) and monoprotonated imines ($\lambda_{exc}$ 414 nm) contribute to the emission spectrum (Fig. 4b). A study of the emission upon excitation at 414 nm revealed a shift in the emission maximum from 500 nm (at pH 7) to 560 nm (at pH 10) with an apparent p$K_2^*$ value around 8.5 (Fig. 2b).

Scheme 2 explains the above behavior. The monoprotonated imine displays an emission maximum around 500 nm upon excitation 414 nm with a quantum yield about 0.001 i.e., almost 20-fold lower than diprotonated species. The pH dependence of the emission maximum in the pH range p$K_1$ < pH < p$K_2$ may be related to a hydrogen bond breaking in the monoprotonated species. Therefore, the emission at 560 nm suggests that formation of the unprotonated imine in the excited state (p$K_2^* \simeq 8.5$) is reached at a lower pH than in the ground state (p$K_2 \simeq 10.6$).

A pH 10 the emission spectrum shows bands at 450–460 and 560 nm upon excitation at 366 nm. However, the excitation spectrum reveals that emission at 450 nm is due only to species absorbing at 365 nm (Fig. 4c). At pH 8, a similar behavior is observed upon excitation




Scheme 2



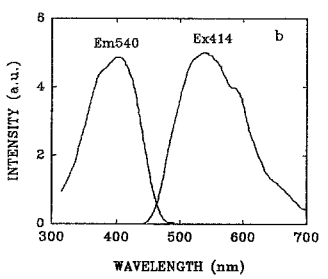




**Fig. 5.** Excitation and emission spectra of the PLP–Ile Schiff bases. (a) pH 13.6; (b) pH 12. The emission and excitation wavelengths are indicated in each excitation and emission spectrum, respectively.

at 366 nm, with a lower emission at 560 nm (Fig. 4d). However, the excitation spectrum shows absorption occurring at 325 and 365 nm that can be assigned to the hemiacetal of PL and dipolar form of the Schiff base, respectively (Fig. 4d) (Scheme 1).

A study of the emission maximum upon excitation at 365 nm leads to a gradual change from 460 nm (pH 8) to 560 nm (pH >12) (data not shown), with an inflection around pH 10.5. This value is an estimate of the apparent $pK^*_{DU}$ for the dissociation of dipolar form to unprotonated imine.

Schemes 1 and 2 are also valid for PLP–Ile with $pK^*_2 = 8.5$, $pK^*_{DU} > 13.6$, and an emission maximum for the unprotonated imine at 540 nm. In this case, even in strong alkaline solutions, an emission maximum at 470 nm appears upon excitation at 355 nm, (Fig. 5a). Also, excitation at 414 nm leads to the emission at 540 nm of the unprotonated imine (Scheme 2) (Fig. 5b). These results suggest that the emission of unprotonated species is observed only after conversion from monoprotonated imonium–phenolate in the excited state (Scheme 2), but not upon direct excitation of the unprotonated imine in solution. The high basicity of the ring nitrogen may favor dipolar species in the excited state, whose emission is then observed (Fig. 5a).

## DISCUSSION

The present paper aimed to study fluorescence properties of the Schiff bases of PLP and PL with Ile in aqueous solutions. A study of the absorption spectra under conditions favoring formation of the Schiff base confirms the presence of ketoenamine species (imonium–phenolate), as di- and monoprotonated forms together with the unprotonated imine in strong basic solutions.

According to a pioneer study[9] on the fluorescence of PLP Schiff bases with amino acids and primary amines, our results at neutral pH show fluorescence emission centered around 500 nm, upon excitation at 415 nm, for PL and PLP Schiff bases. Moreover, a bell-shaped variation of the fluorescence intensity in weakly acid media is interpreted as being caused for the hydrolysis of the imine. A quantum yield of 0.021 is estimated for diprotonated species, in good agreement with reported values.[9] No significant differences are found for the dissociation $pK_a$ of the ring nitrogen in the ground and excited state for these Ile–Schiff bases. A quantum yield 20-fold lower is obtained for the monoprotonated form compared to the diprotonated form.

In strong alkaline solutions, the free base of the imine with a similar low quantum yield shows an emission maximum centered at 560 or 540 nm for PL or PLP Schiff bases. Then considering the absorption maximum for the unprotonated species, 365 nm, a shift of around 200 nm is observed. This finding may be compared to the Stokes shift observed in the enzyme glycogen phosphorylase. A study of the Schiff base of PLP–hexylam-

ine in organic solvents was reported as a model of the coenzyme binding site attending to its spectroscopic properties.[7,8]

One interesting aspect of our work is that the long shift is observed only to the unprotonated form of PL–Ile. In PLP–Ile, we were unable to measure this shift even in strong alkaline solutions. This fact suggests a high basicity for the ring nitrogen of the unprotonated imine in the excited state.

In earlier studies,[9] an emission at 430 nm, upon excitation at 340–360 nm was associated with the emission of the unprotonated species, contrary to our observation (emission maximum at 560 nm). This fact would explain the failure in applying Weller's prediction dealing with acid and basic forms related through $pK_2$ in these studies.

The present study brings about evidences of the influence of the intramolecular hydrogen bond on PLP Schiff bases. The $pK_2$ value of the imine appears more than 2 pH units above that of Ile ($pK_a = 9.7$). In general, Schiff bases have $pK_a$ values lower than the mother amine.[2] In our study a $pK_2^*$ value around 8.5 is reached, suggesting that the imine group behaves as it would in the absence of a hydrogen bond. Therefore, assuming no significant differences between excited and ground states, the $pK_2^*$ value can be considered an approximation to the imine dissociation that would follow the general rule. With a simple Weller's diagram, values of about 2.5 and 5.0 kcal/mol can be calculated for the intramolecular interaction in PL and PLP Schiff bases, respectively. These estimates are within the experimental values found for hydrogen bonding.[17]

PL–Ile shows the same $pK_2^*$ value. However, the difference of 2.0 pH units in the $pK_2$ value (ground state) when these imines are compared suggests a protective role of the phosphate group. Moreover, in PLP–imines a hindered electron-drain effect caused by this group would explain the higher basicity of the imine nitrogen compared to PL derivative. In the absence of a hydrogen bond, the ring nitrogen would likely be sensitive to this effect (the $pK_{DU}^*$ for PLP–Ile seems to be 3 pH units higher, at least). Therefore, the phosphate group may be responsible for an enhancement of the ring and imine nitrogen basicity which could be regulated by protonation of the phosphate group itself.

In conclusion, the fluorescence of the Schiff bases seems to be determined by fast proton transfers in the excited state, in which the acid–base centers of the molecule are involved. Therefore, knowledge of the effects that regulate these reactions would help in understanding the fluorescent properties of PLP in enzymatic environments.

## ACKNOWLEDGMENTS

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