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Spectroscopic Properties of the Photoproducts of Pyridoxal-5′-P Irradiation: Catalytic Site Recognition of Ribonuclease A

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Photoproducts of pyridoxal-5′-P, i.e., 4-pyridoxic-5′-P and bis-pyridoxal-5′-P, have been studied by spectroscopic methods. The spectroscopic properties of bis-pyridoxal-5′-P (bis-PLP) resemble those of pyridoxal-5′-P (PLP) under similar experimental conditions. The coupling of methylen hydrogens to the phosphorus atom has been shown by NMR spectroscopy. The singlet in the ^{31}P-NMR spectra and the triplet in ^{1}H-undecoupled experiments confirm the presence of the phosphate group in the 5′ position of the structure of the vitamin. The effect of pH and solvent composition on the relative distribution of species of bis-pyridoxine-5′-P (bis-PNP) has been investigated by absorption and fluorescence spectroscopy. The acid–base dissociation of the phosphate group is easily detected by emission spectroscopy. Bis-PNP and bis-PLP bind to the enzyme RNase A and they behave as competitive inhibitors with respect to the substrate cytidine-2′-3′-cyclic phosphate. The natural forms of vitamin B_6, pyridoxine, and pyridoxine-5′-P have no effect on the catalytic activity of the protein. Experimental evidence derived from fluorescence and inhibition experiments is consistent with the hypothesis that bis-PNP recognizes the catalytic site of RNase A.

KEY WORDS: Pyridoxal-5′-P; photoproducts; irradiation; ribonuclease A; catalytic site.

INTRODUCTION

Irradiation of aqueous solutions of pyridoxal-5′-P (PLP) results in a decrease in the concentration of the starting compounds accompanied by the appearance of photoproducts [1,2]. Formation of the new compounds depends on the experimental conditions used in the photochemical reactions, i.e., initial concentration of PLP and the presence or absence of O_2 in the reaction medium [3].

When pyridoxal-5′-P at a concentration of 0.2 m*M* is irradiated in the absence of O_2, two major photoproducts, i.e., 4-pyridoxic-5′-P (PPA) and 2,2′-dimethyl-3,3′-dihydroxy-5,5′-diphosphohydroxymethyl-4,4′-pyridyl (bis-PLP), are obtained.

The fluorescent properties of PPA attracted attention earlier, and several studies concerning its binding to proteins were reported [4,5]. Bis-PLP does not show fluorescence emission upon excitation with light, but the reduction product of bis-PLP, 2,2′-dimethyl-3,3′-dihydroxy-5,5′-diphosphohydroxymethyl-4,4′-pyridoxine (bis-PLP) is a compound of potential application as a probe in protein binding studies because of its fluorescent properties.

This work deals with characterization of the photoproducts (bis-PLP, bis-PNP, and PPA) by means of absorption, fluorescence, and nuclear magnetic reso-

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nance spectroscopies. We also report the binding of bis-PNP to RNase A and the spectroscopic changes effected by the binding process.

EXPERIMENTAL

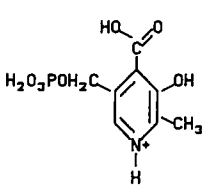
Materials

Pyridoxal-5'-P, pyridoxic acid, and RNase A were purchased from Sigma. The resin Dowex I-X-8 (50–100 mesh) was obtained from Bio-Rad and Sephadex LH-20 was purchased from Pharmacia. All other reagents were analytical grade.

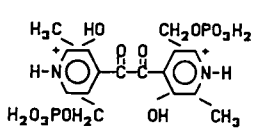
PPA, bis-PLP, and bis-PNP were synthesized as described below.

PLP was irradiated with light of wavelengths longer than 300 nm in the presence of oxygen [1]. After purification by chromatography on Dowex 1-X-8 resin, two major products were isolated: PPA and bis-PLP (Scheme I). The purity of the compounds was checked by thin-layer chromatography (TLC) on cellulose plates (polygram cel 300) by using mixtures of water:acetone:t-butanol:triethylamine (20:35:40:5) and propanol:NH_4OH:water (36:47:10) as eluants.

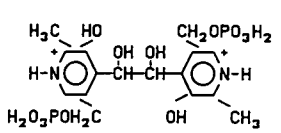
Bis-PNP was obtained by reduction of bis-PLP with $NaBH_4$ [1]. To a solution of bis-PLP (pH 6), a stoichiometry concentration of $NaBH_4$ was added and allowed to react for 1 h in an ice bath. The solution was freed of borate by evaporation with methanol and concentrated under vacuum. Further purification by chromatography on Sephadex LH-20 yielded a pure compound as shown by TLC.



PPA



Bis-PLP



Bis-PNP

Scheme I

Methods

UV–visible spectroscopy was carried out using a Shimadzu UV-160 spectrophotometer. The path length was 10 mm.

Fluorescence experiments were conducted in a precision fluorimeter equipped with two Bausch and Lomb monochromators and a light source xenon lamp (150 W).

Fluorescence decay measurements were made using the monophotonic technique with an Ortec nanosecond spectrophotometer. Excitation was set at 340 nm and the emission filtered through a Corning filter (C-S-3-72). The excitation source was a free-running flash lamp operating in air at 1 atm. The decay function was accurately fitted to either a mono- or biexponential decay using nonlinear least-squares analysis [6]. All computations were performed with an IBM Personal Computer Model 60.

Emission anisotropy measurements were done in a SLM Polarization apparatus. Illumination was provided by a xenon lamp (150 W) with wavelengths selected by a grating monochromator. Fluorescence light emitted by the samples was passed through a Corning filter (C-S-3-72). Polarization values were measured with a precision of 0.005.

^{1}H-NMR spectra were recorded on a Brucker AMX 400. Sixty-four transients were collected using a 12-s delay to obtain fully relaxed spectra.

^{31}P-NMR spectra were recorded on a Brucker AMX 400 with a ^{31}P Larmor frequency of 160 MHz. The pH of the sample was measured with a Beckman pH meter and combined electrode and adjusted to the desired value by adding ^{2}HCl or NaO^2H. All pH measurements were by direct meter reading. The samples were prepared by repeated freeze-drying from ^2H_2O (99.96 isotopical purity) CIL. Five-millimeter tubes were employed for NMR experiments and the chemical shifts are reported as parts per million (ppm) downfield for the internal reference 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS).

RESULTS

Luminescence of Bis-PNP

Bis-PNP is the reduction product of bis-PLP by $NaBH_4$ (see Experimental) which has not been previously characterized by spectroscopic methods.

The absorption spectra show three bands, at 295, 330, and 310 nm, in the whole pH range (Fig. 1). From the changes in the absorbance and wavelength with the pH, two inflection points, at pH 4.9 and 10.0, are ob-

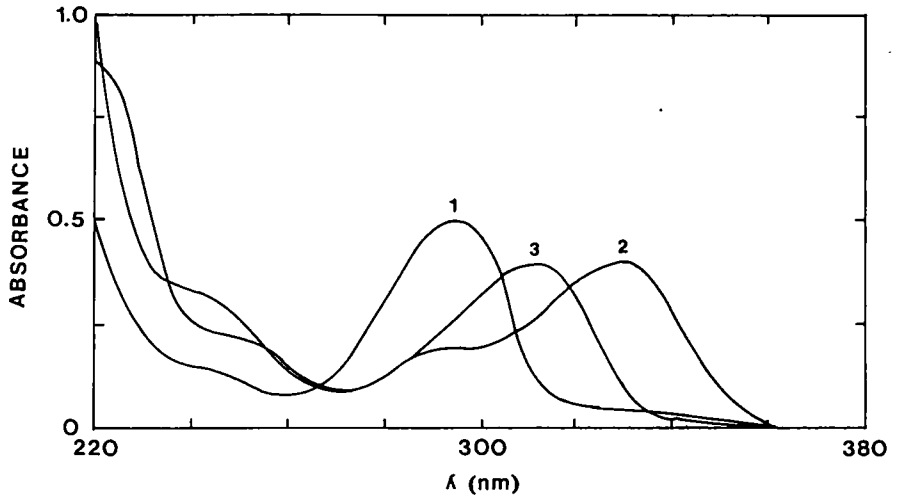


Fig. 1. Absorption spectra of bis-PNP ($1 \cdot 10^{-4}$ *M*). (1) pH 3.0; (2) pH 7.0; (3) pH 12.0.

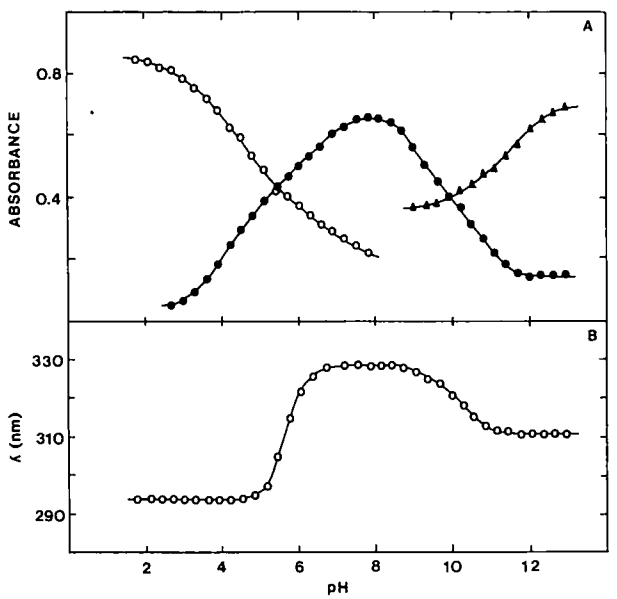


Fig. 2. Absorption spectroscopy of bis-PNP ($1 \cdot 10^{-4}$ *M*). (A) Variation of absorbances with pH: (○) λ = 295 nm; (●) λ = 330 nm; (▲) λ = 310 nm. (B) Variation of maximum wavelength with pH.

tained (Fig. 2). According to these results protonation of the *o*-hydroxyl group in the ground state produces a shift from 330 to 295 nm, while dissociation of the nitrogen heterocyclic proton brings about a change from 330 to 310 nm.

At neutral pH, bis-PNP displays a symmetrical emission band centered at about 398 nm by excitation at 330 nm. The band position of the emission spectra is significantly affected by decreasing the pH of the solution to pH 4.5, where an emission at 420 nm is obtained. In this pH interval the variation of the emission wavelength shows an inflection around pH 5.7 (Fig. 3). No further changes were observed in acidic solutions above pH 1.

In strong acid solutions, a significant blue shift in the emission spectrum corresponding to protonation of the phenolic group was observed upon excitation at the maximum of absorption of 295 nm. Behavior of this kind suggests that the phenolic group in the excited state becomes protonated at extremely low pH values. Accordingly, the pK^* of the excited state was calculated with the aid of Weller's equation and found to be −4.9.

In basic media, a blue shift is observed in the emission spectra corresponding to dissociation of the proton from the heterocyclic nitrogen in the ground state (310 nm) as illustrated in Table I. Thus, there is no significant difference between the pK values in the ground and those in the excited states of this group.

Similar spectral changes were observed when the emission and absorption properties of pyridoxine-5'-P (PNP) were examined at increasing concentrations of sulfuric acid and at alkaline pH values, respectively (Table I). Morozov [3] reported a small blue shift in the emission spectra of PNP at neutral pH. They assigned it to the dissociation of the phosphate group because this is the only difference observed when it is compared with pyridoxine (PN). We have reproduced this small variation to shorter wavelengths (ca. 4 nm) in the pH interval 4–9 with an inflection around pH 6.4. Since the emission

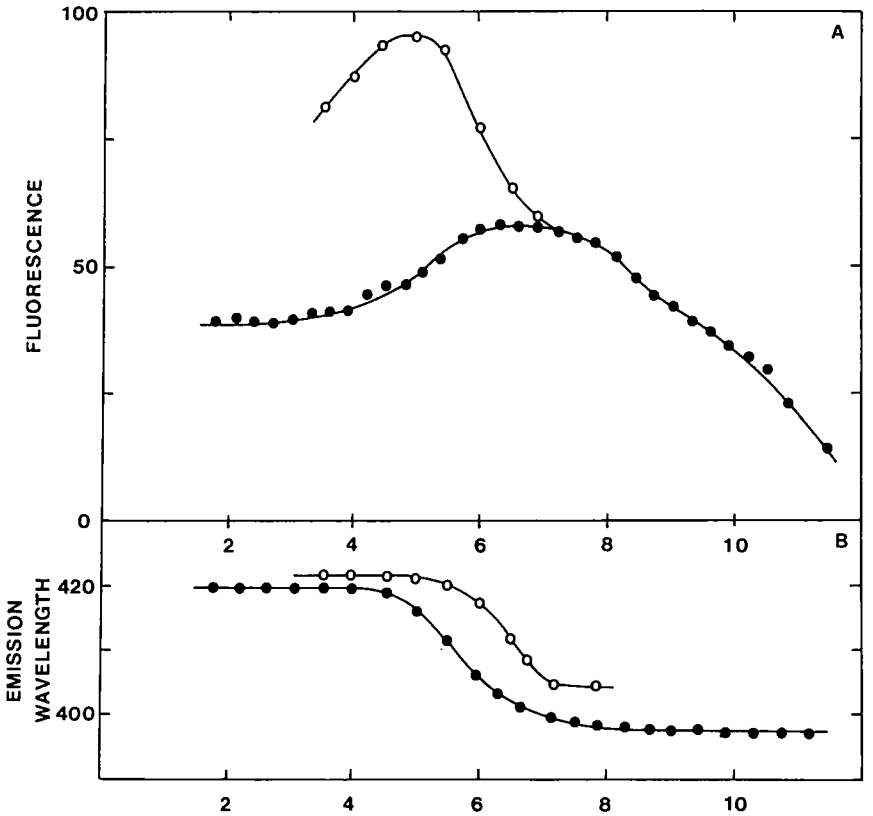


Fig. 3. Fluorescence spectroscopy of bis-PNP ($1 \cdot 10^{-5}$ *M*). Changes in the fluorescence intensity (A) and emission wavelength (B) with pH in the absence (•) and in the presence (○) of RNAse A (150 μ*M*). The excitation wavelength was 330 nm.

spectrum of bis-PNP at neutral pH values also displays a blue shift, it seems reasonable to assume that the inflection at pH 5.7 is an estimate of the p*K* value of the phosphate group when the molecule is in the excited state (Fig. 3).

The emission of bis-PNP decays in a biexponential manner with a lifetime of 2.5 ns, which is similar to that of PNP but shorter than the lifetime of PPA ($\tau = 9$ ns).

The effect of the solvent composition on the relative distribution of bis-PNP species in solution was also examined with spectroscopic methods. The band position of the recorded emission spectrum is affected by the presence of increasing concentrations of dioxane. Hence, at a 20:60 ratio of water:dioxane the emission maximum is shifted to 375 nm. The excitation spectrum reveals that the maximum excitation wavelength is shifted to 310 nm. Similar spectral changes were observed when the emission and absorption properties were examined in aqueous solution at alkaline pH values due to dissociation of the proton of the pyridine ring. The results suggest that at a low dielectric constant the unprotonated species of the heterocycle of bis-PNP are favored. The effects have been also studied with PNP, and in parallel behavior, the properties of the molecule bearing an unprotonated ring ($\lambda_{exc} = 310$ and $\lambda_{em} = 375$) are reached in 20:80 water:dioxane.

The spectroscopic properties of PPA, a photoproduct of PLP, were also evaluated over a wide range of pH values. The dissociation of a proton from the phosphate group of PPA results in a red shift in the emission spectrum of approximately 10 nm at neutral pH. In the pH interval 6–8 a parallel decrease in the fluorescence intensity is observed with an inflection at around pH 6.9. This variation is not observed in pyridoxic acid.

Hence, emission spectroscopy can be used to detect the deprotonation of the phosphoryl groups of PPA.

Bis-PNP/Borate Complex

Like pyridoxine [7], bis-PNP reacts with boric acid to form a stable complex in aqueous solution. The resulting complex exhibits a maximum of absorption at

Table I. Spectroscopic Properties of Bis-PNP and Related Compounds

Sample	Solvent	λ_a absorption	λ_f fluorescence
Bis-PNP	Sulfuric acid (45%)	295	345
Bis-PNP	Water, pH 4.0	330	420
Bis-PNP	Water, pH 7.8	330	398
Bis-PNP	Water, pH 12.0	312	375
Bis-PNP	Dioxane:water, 20:80	330	400
Bis-PNP	Dioxane:water, 60:20	310	375
Bis-PNP	Borate (0.1 *M*), pH 7.5	290	398
Bis-PNP	Borate (0.1 *M*), pH 11.0	286	348
PNP	Sulfuric acid (45%)	292	338
PNP	Water, pH 5.4	324	401
PNP	Water, pH 8.1	324	397
PNP	Water, pH 12.0	309	381
PNP	Dioxane:water, 20:80	330	400
PNP	Dioxane:water, 60:20	310	375
PPA	Water, pH 6.0	316	427
PPA	Water, pH 9.0	318	438

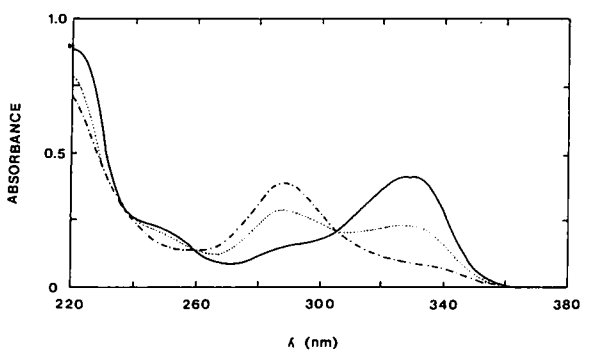


Fig. 4. Absorption spectra of bis-PNP ($1 \cdot 10^{-4}$ *M*) in the absence (——) and in the presence of different concentration of boric acid at pH 7.5. (····) $1.2 \cdot 10^{-4}$ *M*); (—·—·) $1 \cdot 10^{-3}$ *M*.

290 nm, which resembles the absorption band of bis-PNP when the phenolic group is fully protonated in the ground state. Spectrophotometric titrations of a fixed concentration of bis-PNP at pH 7.5 with increasing concentrations of boric acid reveal an increase in the absorption band at 290 nm together with a decrease in the absorption band centered at 330 nm (Fig. 4).

These results allow us to determine the concentration of bis-PNP/borate complex and to calculate the equilibrium constant using Eqs. (1) and (2):

$$\frac{1}{n} = \frac{K_D}{[B]} + 1 \qquad (1)$$

$$n = 1 - \frac{[F]}{[T]} \qquad (2)$$

where $[F]$ is the concentration of free bis-PNP, $[T]$ the total concentration of bis-PNP, and $[B]$ the concentration of boric acid.

A linear plot of $1/n$ against $1/[B]$ yields a dissociation constant $K_D = 1.2 \times 10^{-4}$ *M* for the complex at 25°C. A dissociation constant $K_D = 2.2 \times 10^{-3}$ *M* was obtained for the pyridoxine/borate complex at pH 7.7. The magnitude of the dissociation constant indicates that in the presence of an excess of boric acid (0.1 *M*), most of the molecules of bis-PNP are complexed to the ligand.

Although absorption spectroscopy confirms the presence of molecular species absorbing at 290 nm, it was expected that upon excitation at this absorption wavelength, the complex would display an emission band centered at 345 nm. Contrary to our expectations, the emission band of bis-PNP in the presence of boric acid (0.1 *M*) exhibits a maximum at 398 nm, coinciding with the emission of free bis-PNP. This apparent discrepancy between absorption and fluorescence measurements can be explained if it is assumed that the bis-PNP/borate complex undergoes a rapid process of dissociation upon illumination with light of 290 nm. This interpretation of the results implies that the complex in the excited state is less stable than in the ground state.

In basic media, at pH values higher than the dissociation p*K* of the bis-PNP pyridine ring nitrogen, the borate complex yields an absorption spectrum centered at 286 nm, showing a small blue shift with respect to the absorption maximum at neutral pH. The spectrum also resembles the absorption band of bis-PNP when the phenolic group is fully protonated in the ground state but, in this case, bearing an unprotonated ring nitrogen. With an increasing concentration of borate and a fixed concentration of bis-PNP at pH 11, an increase in the absorption band at 286 nm together with a decrease in the absorption at 310 nm is observed. Since absorption spectroscopy confirms the presence of molecular species absorbing at 286 nm, the complex should display an emission band centered at 345 nm. In agreement with this expectation, the emission band of bis-PNP in the presence of boric acid (0.1 *M*) exhibits a weak maximum centered at 348 nm (Table I). Parallel behavior is also observed in pyridoxine (PN) and PNP, where absorption bands at 287 and 286 nm are obtained for the borate complex in basic medium instead of the 292 and 290 nm at neutral pH. Upon excitation at these wavelengths, emission spectra centered at 336 nm are obtained in both compounds, coinciding with the emission of free PN and PNP in strong acid solutions. Finally, in all cases upon excitation at 310 nm (absorption corresponding to the unprotonated ring nitrogen), emission of free PN and

PNP shows a weak emission at longer wavelengths, corresponding to the free ligand.

Absorption Properties of Bis-PLP

Bis-PLP is a major product in the photochemical reaction of PLP in desoxygenated solutions. Although absorption spectroscopy have been employed to detect its presence in solution, the influence of pH on the relative population of different species in solution has not been investigated. This PLP photoproduct does not show fluorescence, therefore the effect of pH on the relative distribution of bis-PLP species in solution could be examined by absorption spectroscopy.

Absorption spectra show three main bands, at 295, 287, and 390 nm, over the whole pH range. The absorption at 295 nm predominates in acid solutions and decreases with pH, with an inflection centered around pH 3.3, while a blue shift is observed to 287 nm. This absorption and a weak band at 390 nm remain nearly constant at neutral pH values. In basic solution, the 390-nm band increases with pH, while the absorption at 287 nm decreases with an inflection to pH 8.6 (Fig. 5). According to its structure and taking into account the spectroscopic properties of PLP, the shift in acid medium is produced by the protonation of the *o*-hydroxyl group. The apparent p*K* value of 8.6 corresponds to the dissociation of the proton from the heterocyclic nitrogen. These changes resemble the effect of pH on the distribution of pyridoxal-5′-P species in solution [8].

NMR Studies

Table II summarizes the proton and phosphorus NMR properties of bis-PNP and bis-PLP. The variation of the chemical shift in solutions of different p*D* values shows that proton signals in acidic solutions appear more downfield than in basic solutions. The ^{1}H-NMR properties of pyridoxic-5′-P are also included in Table II. These spectra are recorded at different pH values, where bipolar ion, tripolar ion, and dianion species are present in solution. The proton signals correspond to 2-CH_3, 5-CH_2, and 6-H since phenolic and alcoholic protons are immediately exchanged for deuterium.

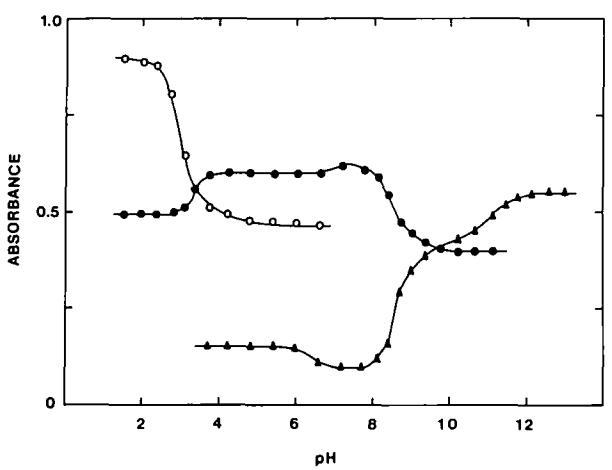


Fig. 5. Absorption spectroscopy of bis-PLP ($1 \cdot 10^{-4}$ *M*). Variation of absorbance with pH: (○)λ = 295 nm; (●)λ = 287 nm; (▲) λ = 390 nm.

Table II.(a) Proton Chemical Shifts of Pyridoxic Acid and Related Compounds in D_2O (δ); (b) Phosphorus Chemical Shifts in D_2O Relative to 85% Phosphoric Acid

a

Compound	p*D*	Group 2-CH_3	Group 5-CH_2	Group 6-H
PA	2.0	2.65	5.06	8.15
PA	5.1	2.59	4.92	8.08
PA	5.6	2.60	4.92	8.08
PA	7.4	2.50	4.85	7.90
PA	9.4	2.45	4.72	7.80
PA	12.0	2.32	4.56	7.55
PPA	5.9	2.59	5.27[a]	8.16
PPA	12.0	2.45	4.86[a]	7.89
Bis-PLP	2.6	2.70	5.40[a]	8.50
Bis-PLP	5.9	2.45	5.25[a]	8.30
Bis-PNP	8.0	2.50	5.30	7.87

b

Compound	p*D*	δ
PPA	9.6	4.5
Bis-PLP	2.6	0.5
Bis-PNP	8.0	2.5

[a] Doublet peaks. The rest are singlet.

The results in the ^{31}P-NMR spectra indicate that in H-decoupled experiments, bis-PNP shows a singlet downfield of phosphoric acid (Table II). The H-undecoupled spectra, on the other hand, show the characteristic coupling of phosphorus with the two methylene protons in the 5-CH_2 substituent. The coupling constant, J_{HP}, is estimated as 2.5–0.2 Hz.

Binding of Bis-PNP to RNase A

In these experiments protein solutions of 150 μ*M* were used as solvents, and to maximize the binding of the ligand, the molar ratio of RNase A to bis-PNP was of the order of 15:1.

The fluorescence spectra of bis-PNP in the presence of protein at various pH values were then compared with

those of free bis-PNP. As shown by the results in Fig. 3 the binding of bis-PNP to the enzyme is markedly influenced by the pH of the medium. A maximum enhancement of the fluorescence emitted by bis-PNP was observed at pH 5.5.

As the pH of the medium is increased from 4.5 to 7.5, the fluorescence emitted by bis-PNP is changed in the manner illustrated in Fig. 3. Concomitantly, the maximum of emission is shifted from 422 to 405 nm.

As a result of its binding to RNase A, the fluorimetric titration curve of bis-PNP in the presence of protein shows a small change in the inflection point (0.7 U) compared to that of free bis-PNP. It is found that at pH 5.5 the protein promotes a red shift of 12 nm in the fluorescence emitted by the ligand (Fig. 6). This shift in the band position is accompanied by an increase in the emission anisotropy from 0.03 to 0.12.

To demonstrate that bis-PNP is bound to the catalytic site of RNase A, displacement experiments with the inhibitor cytidine-2'-monophosphate were performed [5, 8]. Figure 6 shows that the addition of cytidine-2'-monophosphate to the complex bis-PNP/RNase causes a decrease in the fluorescence emitted by bound bis-PNP. Displacement of bis-PNP by the inhibitor cytidine-2'-monophosphate was also demonstrated by a decrease in the emission anisotropy from 0.12 to 0.03 at saturating concentrations of the inhibitor.

The binding of bis-PNP to RNase A was also tested by enzymatic assays using cytidine-2'-3'-P as substrate in the reaction mixture [10]. It was found that bis-PNP acts as a competitive inhibitor with respect to the substrate, with a K_i = 10 μM at pH 6.

Thus, two independent measurements, i.e., direct titration of the enzyme with bis-PNP and enzymatic assays, reveal that bis-PNP recognize the catalytic binding site of RNase A. Bis-PLP also inhibits the enzyme RNase A by competing with cytidine-2'-3'-P. Its K_i is of 10 μM at pH 6. In marked contrast to bis-PNP and bis-PLP, pyridoxine-5'-P and pyridoxine have no effect on the catalytic activity of RNase A at concentrations of 1 mM.

DISCUSSION

The effect of pH and solvent composition on the relative distribution of species of bis-PNP in solution have been investigated in the present work. The spectroscopic properties of fully protonated and unprotonated bis-PNP species have been measured and differentiated from those existing at neutral pH values. pK values corresponding to the dissociation of hydroxy and pyridine nitrogen groups in the ground and excited states have been obtained by absorption and fluorescence spec-

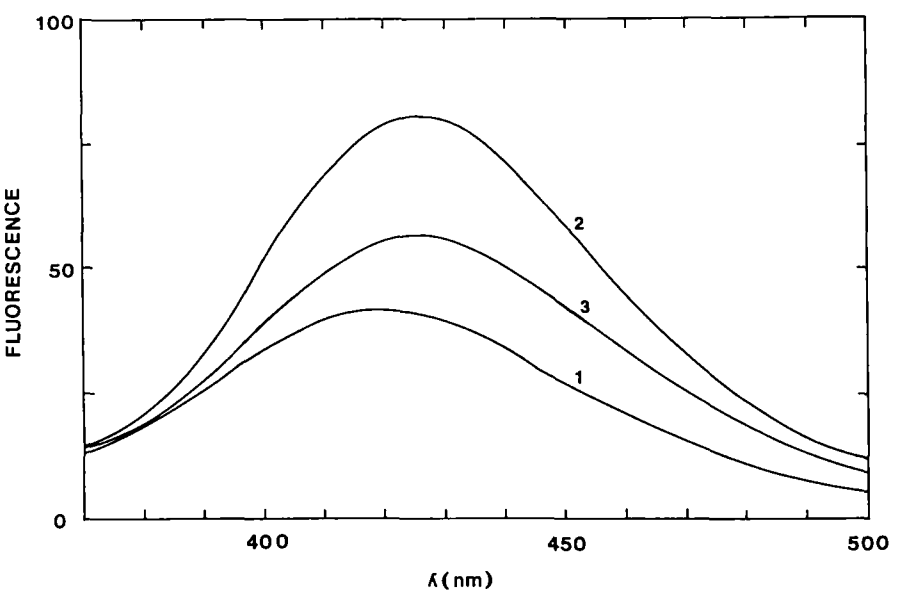


Fig. 6. Emission spectra of bis-PNP (10 μM) in the absence (curve 1) and presence (curve 2) of RNase A (150 μM) at pH 5.5. Effect of the addition of cytidine 2'-monophosphate (0.3 mM) (curve 3) on the fluorescence emitted by bis-PNP (10 μM) in the presence of RNase A (150 μM) at pH 5.5. Samples were incubated for 30 min prior to fluorescence measurements. Excitation was at 330 nm. Fluorescence intensity is given as arbitrary units.

troscopy, respectively. In addition, by varying the solvent composition, it has been shown that species bearing an unprotonated ring nitrogen are favored against zwitterion.

The acid–base p*K* values in the ground and excited states for bis-PNP are very similar to those obtained for PNP as would be expected from a comparison of their structures. In addition, the solvent effect on the relative distribution of species of PNP in solution is similar to that found with bis-PNP.

The acid–base dissociation of the phosphate group of bis-PNP is easily detected by emission spectroscopy. Thus, a blue shift in the emission spectrum together with a decrease in fluorescence intensity is detected upon excitation at 330 nm.

The effects of pH on the relative distribution of the species of bis-PLP in solution have been determined. The spectroscopic properties of the species with a protonated phenolate and an unprotonated ring nitrogen resemble those of PLP under similar experimental conditions.

The ^{1}H-NMR spectra show the presence of the groups of the vitamin B_6 ring, i.e., 2-CH_3, 5-CH_2, and 6-H, in addition to –CH(OH)–upfield (Table II). The methylene groups show coupling to the phosphorus atom as would be expected in a phosphorylated vitamin B_6 derivative. The spectrum of bis-PLP is nearly identical to that of PLP and also shows the methylene group as a doublet because of the ^{31}P coupling [11].

^{31}P-NMR spectroscopy of bis-PLP has shown a chemical shift downfield relative to the resonance of phosphoric acid. This chemical shift is similar to those already reported for pyridoxal-5′-P and pyridoxamine-5′-P [12]. Moreover, the chemical shift for bis-PNP corresponds to the phosphate dianion; and the presence of phosphate in the 5′ position of the structure of vitamin B_6 was confirmed by the ^{1}H-undecoupled experiment, where the singlet was transformed into a triplet.

An interesting feature of the present studies is the finding that bis-PNP recognizes a binding site of RNase A. Moreover, the results obtained by fluorescence and inhibition experiments are consistent with the hypothesis that bis-PNP recognizes the catalytic site of RNase A.

The pH dependence of the fluorescent properties of free bis-PNP are different from those observed when it is bound to RNase A. This finding might be interpreted for the enzyme-bound bis-PNP as variable steric constraint on the phosphate imposed by amino acid residues at the active site of RNase A. The phosphate monoanion appears to be stabilized by possible interactions with amino acid residues of the protein as revealed by an increase in its p*K* value compared to free bis-PNP in solution.

Since the ligand bis-PNP possesses several groups capable of interacting with amino acid residues of protein, it is worthwhile considering the use of this chromophore as a fluorescence probe of the binding sites of other enzymes.

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