Fluorescence Changes Induced by Binding of 4-Pyridoxic Acid 5'-Phosphate to Proteins*

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SUMMARY

The affinity constant ($K_a$) and stoichiometry of binding of 4-pyridoxic acid 5'-phosphate (4-pyridoxic-5'-P) complexed to the protein bovine serum albumin and aspartate aminotransferase were investigated by fluorometric methods.

Whereas bovine serum albumin binds 1 mole of 4-pyridoxic-5'-P per mole of protein with a $K_a = 4.6 \times 10^6$ M$^{-1}$, the enzyme, aspartate aminotransferase, binds 2 moles of 4-pyridoxic-5'-P per mole of protein with a $K_a = 1.3 \times 10^6$ M$^{-1}$ at neutral pH. The spectroscopic properties (absorption and fluorescence spectra) of the complexes were examined over a wide range of pH values. At neutral pH, the formation of the 4-pyridoxic-5'-P bovine serum albumin complex causes the following spectroscopic changes in the ligand: (a) a red shift of 15 nm in the band position of the emission spectrum, (b) an increase in the degree of polarization of fluorescence ($P = 0.32$), (c) an enhancement of the fluorescence lifetime ($r = 12$ ns), and (d) a small blue shift of approximately 2 nm in the band position of the absorption spectrum.

In contrast to the protein bovine serum albumin, the apoenzyme causes a blue shift of approximately 10 nm in the band position of the emission spectrum and a concurrent decrease in the fluorescence quantum yield of the ligand ($q = 0.05$).

No change in the band position of the absorption spectrum of the complexed ligand was detected. The fluorescence changes observed in 4-pyridoxic-5'-P bound to the aminotransferase can be correlated with the deprotonation of the pyridine nitrogen atom as a result of its interaction with an amino acid residue at the binding site.

EXPERIMENTAL PROCEDURE

Methods—Fluorescence emission spectra were obtained with the use of a spectrofluorimeter designed in our laboratory (3). The sample in a 1-cm cuvette was illuminated by monochromatic light obtained by passing the output of a xenon lamp through a Bausch and Lomb monochromator. The light emitted at right angles to the exciting light source was passed through a second Bausch and Lomb monochromator. An EMI phototube (6256s) was used as the fluorescence detector. Calibration of the exciting light source was carried out with solutions of Rhodamine B in ethylene glycol as described by Melhuish (4). The detector system was calibrated according to the method of White et al. (5). A bandwidth of 1 nm was used in the fluorometric measurements. Quantum yield of fluorescence ($q$) was calculated according to the method of Parker and Rees (6) with standards of known quantum yield. Two standards, quinine sulfate ($q = 0.46$) and 2-aminopyridine ($q = 0.60$), were chosen for these experiments (7).

Polarization of fluorescence measurements were performed in an apparatus similar to that described by Weber (8). Illumination was provided by a xenon lamp (200 watts) with wave lengths selected by a quartz prism monochromator. The bandwidth for excitation at 320 nm was 5 nm. Fluorescence polarized light was passed through a combination of Corning C-S-3-75 and C-S-5-58 glass filters. The detector system consisted of an EMI 9502B photomultiplier and a digital voltmeter. The

It was shown in earlier publications of our laboratory (1) that it is convenient to make use of the fluorescence properties of pyridoxamine-5-P to investigate the mechanism of binding of this cofactor to the catalytic site of the enzyme aspartate aminotransferase. Subsequently, it was reported that the technique of polarization of fluorescence provides valuable information about the degree of mobility of the cofactor interacting with the enzyme (2).

It is the purpose of this paper to examine the spectroscopic properties of 4-pyridoxic-5'-P and to investigate the possibility of using this chromophore as a fluorescence probe of the binding sites of aminotransferases.

It is shown that 4-pyridoxic-5'-P, a photoprotein of pyridoxal-5-P, has the ability of forming stable complexes with the proteins, bovine serum albumin and aspartate aminotransferase. The spectral changes detected when 4-pyridoxic-5'-P is bound to the aminotransferase cannot be interpreted in terms of the "hydrophobicity" of the binding site. However, these spectral changes can be correlated with the deprotonation of the pyridine nitrogen atom in the excited state as result of its interaction with an amino acid residue of the binding site.

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1 The abbreviation used is: 4-pyridoxic-5'-P, 4-pyridoxic acid 5'-phosphate.
degree of polarization of fluorescence was measured with a precision of ±0.01.

Fluorescence decay time measurements were conducted in a TRW nanosecond decay time fluorimeter equipped with a model 32A decay time computer and a Tektronix 556 dual beam oscilloscope (9).

Exciting light was provided by a deuterium lamp. A Baird Atomic interference filter (maximum of transmission at 322 nm) was used for excitation and a Corning C-S-3-73 cut off filter for emission.

Materials—Aspartate aminotransferase from pig heart (l-aspartate-2-oxoglutarate aminotransferase, E-C-2-11) was prepared by the method of Jenkins et al. (10) with the modification of Martinez Carrion et al. (11). The cytoplasmic fraction (α) was used throughout these studies. Resolution of the holoenzyme into apoenzyme and free cofactor was performed according to the method of Scordi et al. (12). The resulting apoenzyme had less than 1% of the original aminotransferase activity.

Enzymatic assays of aspartate aminotransferase were conducted in the Cary model 15 spectrophotometer as described in a previous paper (1).

The protein bovine serum albumin was purchased from Sigma and used without further purification. The reagents, l-aspartic acid, α-ketoglutarate, and pyridoxal-5-P, were purchased from Sigma. NADH and the enzyme, malate dehydrogenase, were conducted in the Cary model 15 spectrophotometer as described in a previous paper (1). The cytoplasmic fraction (α) was used throughout these studies. Resolution of the holoenzyme into apoenzyme and free cofactor was performed according to the method of Scordi et al. (12). The resulting apoenzyme had less than 1% of the original aminotransferase activity.

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4-Pyridoxic-5'-P was prepared by the method of Morrison and Long (13) by irradiating pyridoxal-5-P with light having wave lengths longer than 300 nm in the presence of oxygen. The principal product of the reaction, 4-pyridoxic-5'-P, was isolated after purification by chromatography on a carboxylate ion exchange resin (Amberlite X-E-64). The product, M.P. 203° with decomposition, shows infrared and ultraviolet spectra characteristic of 4-pyridoxic-5'-P (14). Pyridoxic acid was commercially obtained, were of the highest purity available.

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RESULTS

The effect of pH on the relative distribution of 4-pyridoxic-5'-P structures in solution was examined by fluorescence spectroscopy. At neutral pH, the tripositive ion-phosphate dianion form of 4-pyridoxic-5'-P (II) displays an emission band centered at 425 nm. At pH values lower than 6, the protonation of the carboxyl group (pK = 5.5) brings about a blue shift of approximately 5 nm in the emission spectrum. The dipolar ion-phosphate monanion form (III) shows very low fluorescence yield (Table I). A decrease in the polarity of the environment by addition of the solvent, dioxane, changes the absorption and fluorescence characteristics of 4-pyridonic-5'-P and pyridoxic acid. As shown in Table I, the emission spectrum of 4-pyridoxic-5'-P undergoes a blue shift of approximately 10 nm when examined in the solvent system, water-dioxane (2:98, v/v). This shift in the band position of the emission spectrum is accompanied by a red shift in the absorption spectrum (Table I). Another fluorometric parameter, the fluorescence lifetime, was examined over the pH range 3 to 8. Despite the increase in fluorescence yield induced by protonation of the carboxyl group (pK 5.5) 4-pyridoxic-5'-P has a fluorescence lifetime of 9 ns at either pH 7.4 or pH 3. The fluorescence lifetime of 4-pyridoxic-5'-P in solvent mixtures fluctuates between 9 and 11 ns, the latter value being observed in the solvent mixture containing dioxane.

Interaction of 4-Pyridoxic-6'-P with Serum Albumin—The effect of bovine serum albumin on the fluorescence properties of 4-pyridoxic-5'-P in solution was examined by fluorescence spectroscopy. At neutral pH, the tripositive ion-phosphate dianion form of 4-pyridoxic-5'-P (II) displays an emission band centered at 425 nm. At pH values lower than 6, the protonation of the carboxyl group (pK = 5.5) brings about a blue shift of approximately 5 nm in the emission spectrum. The dipolar ion-phosphate monanion form (III) shows very low fluorescence yield (Table I). A decrease in the polarity of the environment by addition of the solvent, dioxane, changes the absorption and fluorescence characteristics of 4-pyridonic-5'-P and pyridoxic acid. As shown in Table I, the emission spectrum of 4-pyridoxic-5'-P undergoes a blue shift of approximately 10 nm when examined in the solvent system, water-dioxane (2:98, v/v). This shift in the band position of the emission spectrum is accompanied by a red shift in the absorption spectrum (Table I). Another fluorometric parameter, the fluorescence lifetime, was examined over the pH range 3 to 8. Despite the increase in fluorescence yield induced by protonation of the carboxyl group (pK 5.5) 4-pyridoxic-5'-P has a fluorescence lifetime of 9 ns at either pH 7.4 or pH 3. The fluorescence lifetime of 4-pyridoxic-5'-P in solvent mixtures fluctuates between 9 and 11 ns, the latter value being observed in the solvent mixture containing dioxane.

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Interaction of 4-Pyridoxic-6'-P with Serum Albumin—The effect of bovine serum albumin on the fluorescence properties

![Scheme 1. Structure of pyridoxic-5'-P in solution.](https://example.com/scheme1.png)

![Figure 1. Decrease in the fluorescence intensity at 415 nm (excitation 307 nm) of 4-pyridoxic-5'-P as a function of pH.](https://example.com/figure1.png)
of 4-pyridoxic-5'-P was examined at several pH values. In these experiments protein solutions of approximately 3 mg per ml were used as solvents; and in order to maximize the binding of the ligand the molar ratio of protein to 4-pyridoxic-5'-P was of the order of 3:1. The fluorescence spectrum of 4-pyridoxic-5'-P in the presence of bovine serum albumin was then recorded and compared with the spectrum of free 4-pyridoxic-5'-P. It was found that over the pH range from 6 to 8 the protein bovine serum albumin promotes a red shift of 15 nm in the fluorescence emitted by the ligand (Table II and Fig. 2). This shift in the band position of the emission spectrum is accompanied by a small change in the band position of the absorption spectrum (Table II), and by an increase in the fluorescence lifetime (12 ns). Fig. 3 shows the change in the polarized fluorescence emitted by 4-pyridoxic-5'-P as a result of the addition of increasing concentrations of protein to a fixed concentration of 4-pyridoxic-5'-P (1.5 × 10⁻⁵ M). The measured polarization of fluorescence reflects the relative contribution of both free and bound ligand to the fluorescence of the system. When 4-pyridoxic-5'-P is the only component of the system, the polarization ($P_0 = 0.05$) is related to the rotational motion of free 4-pyridoxic-5'-P between excitation and emission of light. As soon as the protein is added to the system and the 4-pyridoxic-5'-P protein complex formed, the bound 4-pyridoxic-5'-P molecules are no longer able to rotate extensively during the lifetime of the excited state (12 ns), consequently the polarization, $P$, of the system is increased in the manner depicted in Fig. 3.

When the protein concentration is such that the ligand is complexed, the polarization of fluorescence approaches the value $P = 0.32$.

The increase in polarization of fluorescence associated with complex formation can be used to determine the affinity constant

![Graph](image)

**Fig. 2.** Corrected emission spectra for 4-pyridoxic-5'-P (■). 4-Pyridoxic-5'-P complexed to bovine serum albumin, mixing ratio, protein:ligand (3:1) (○). 4-Pyridoxic-5'-P complexed to apotransaminase, mixing ratio, protein:ligand (2:1) (●) obtained at pH 7.4 in 0.05 M Tris-acetate buffer. The optical density of the samples at the exciting wave length (317 nm) was 0.1 for 1-cm cuvettes. Areas beneath the curves are proportional to the fluorescence quantum yields.

**Fig. 3.** Changes in polarization of fluorescence ($P$) of 4-pyridoxic 5'-P (1.5 × 10⁻⁵ M) on titration with increasing concentration of bovine serum albumin at pH 7.4 in 0.05 M Tris-acetate buffer. Experiments conducted at 25° with polarized incident light of 360 nm.

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**Table II**

*Fluorescence properties of 4-pyridoxic-5'-P protein complexes*

| Sample                                      | Solvent                        | Absorption | Fluorescence | Quantum Yield | Lifetime | Polarization | $K_A$    |
|---------------------------------------------|--------------------------------|------------|--------------|---------------|----------|--------------|----------|
| Bovine serum albumin-4-pyridoxic-5'-P       | Tris-acetate buffer (pH 7.4)    | 315        | 440          | 0.18          | 12       | 0.32         | 4.6 × 10⁴ M⁻¹ |
| Bovine serum albumin-4-pyridoxic-5'-P       | Phosphate buffer (pH 7.4)       | 315        | 440          | 0.18          | 12       | 0.32         | 4.6 × 10⁵ M⁻¹ |
| Bovine serum albumin-4-pyridoxic-5'-P       | (pH 7.4) + NaCl ($I = 0.5$)     | 317        | 425          | 0.15          | 9        | 0.06         |          |
| Bovine serum albumin-pyridoxic acid         | Tris acetate buffer (pH 7.4)    | 315        | 410          | 0.32          | —        | 0.28         | 10⁴ M⁻¹     |
| Apotransaminase-4-pyridoxic-5'-P            | Tris-acetate buffer (pH 7.4)    | 317        | 415          | 0.05          | —        | 0.22         | 1.3 × 10⁴ M⁻¹ |
| Apotransaminase 4 pyridoxic-5'-P            | Phosphate buffer (pH 7.4)       | 317        | 425          | 0.15          | —        | 0.06         |          |
| Apotransaminase-4-pyridoxic-5'-P            | (pH 7.4) + NaCl ($I = 0.5$)     | 317        | 415          | 0.04          | —        | 0.21         | 2 × 10⁴ M⁻¹  |
| Apotransaminase-pyridoxic acid              | Tris-acetate buffer (pH 7.4)    | 317        | 415          | 0.30          | —        | 0.05         |          |

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FIG. 4. Changes in polarization of fluorescence (P) upon addition of varying concentrations of 4-pyridoxic-5'-P to a fixed concentration of bovine serum albumin (1.75 × 10^{-6} M) in 0.05 M Tris-acetate buffer (pH 7.4). Experiments conducted at 25° with polarized incident light of 320 nm.

The polarization of fluorescence of free (PF) and bound (PB) 4-pyridoxic-5'-P as well as the polarization of fluorescence values (P) recorded when both free and bound 4-pyridoxic 5'-P are in equilibrium were used to calculate the fraction (α) of the ligand bound (Equation 1) (16, 17).

\[
α = \frac{F/P_{F}}{[P/F_{F}] - 1 + [1 - (P_{F}/P_{B})]}
\]

The fluorescence intensity of free 4-pyridoxic-5'-P (F₀) and the fluorescence emitted when both free and bound ligand are in equilibrium (F) were recorded using unpolarized exciting light of 320 nm.

The results of these fluorescence measurements were analyzed by the method of Scatchard (18) (Equation 2).

\[
\frac{\bar{v}}{[L]} = K_{d}(n-\bar{v})
\]

where [L] is the concentration of free ligand, Kₐ, the association constant, n, number of binding sites, and \(\bar{v} = \alpha \cdot \frac{[L]_0}{[P]_0}\) is the average number of ligand molecules per mole of protein.

Since the results of the fluorometric titrations are adequately represented by Equation 2, it was possible to determine one binding site with an association constant of 4.6 × 10⁶ M⁻¹ at pH 7.4 in 0.05 M Tris-acetate (Fig. 5). Although an association constant of similar magnitude was determined in 0.05 M phosphate buffer (pH 7.4), it should be emphasized that the stability of the bovine serum albumin-4-pyridoxic-5'-P complex is affected by changes in the ionic strength of the medium (Table II).

This is illustrated by the experiments included in Fig. 6B, where polarization of fluorescence measurements were conducted on samples containing bovine serum albumin and 4-pyridoxic-5'-P (mixing molar ratio 3:1) at varying ionic strengths. As the ionic strength of the medium is increased from 0.1 to 0.5, the degree of polarized fluorescence emitted by 4-pyridoxic-5'-P is decreased from 0.32 to 0.1. Concomitantly, the maximum of emission is shifted from 440 to 425 nm as the concentration of NaCl is increased. At an ionic strength of 0.5, the fluorescence emitted by the system bovine albumin-4-pyridoxic-5'-P increases and the maximum of emission corresponds to that of free 4-pyridoxic-5'-P. The stability of the complex bovine serum albumin-4-pyridoxic-5'-P is also affected by changes in the pH of the solution. At pH values lower than 5.5, the polarization of fluorescence and the maximum of emission of 4-pyridoxic-5'-P remains essentially invariant upon the subsequent addition of increasing...
concentrations of bovine serum albumin (Fig. 6A). This is taken as an indication that protonation of the carboxyl group of 4-pyridoxic-5'-P prevents the binding of this ligand to the protein.

Interaction of 4-Pyridoxic-5'-P with Apotransaminase—The interaction of 4-pyridoxic-5'-P with the apoprotein of the enzyme aspartate aminotransferase induces several changes in the fluorescence properties of the ligand. As shown in Fig. 2, the addition of 4-pyridoxic-5'-P to the apoprotein (molar ratio 1:2) induces in parallel to diminution of fluorescence yield, a change in the band position of the emission spectrum. Thus the fluorescence yield of bound 4-pyridoxic-5'-P is lower than the corresponding yield of free ligand at pH 7.4, while the emission spectrum centered at around 415 nm is shifted towards shorter wavelength when compared with free 4-pyridoxic-5'-P (425 nm) in the same buffer.

The fluorescence quenching effect associated with complex formation was used to determine the affinity constant of 4-pyridoxic-5'-P for the apoenzyme. Fig. 7 shows how the fluorescence intensity, excited at 317 nm, changes with increasing concentrations of 4-pyridoxic-5'-P while keeping the protein concentration constant. The fluorescence intensity of free (F₀) and bound 4-pyridoxic-5'-P (Fₘ) as well as the fluorescence observed when both free and bound ligand are in equilibrium (F) were used to calculate the fraction (α) of ligand bound. Equation 3

\[
\alpha = \frac{(F₀ - F)}{(F₀ - Fₘ)}
\]

where \(F₀\) is the actual observed fluorescence when all the ligand is bound to the protein. \(Fₘ\) was determined directly by adding increasing concentrations of apoenzyme to a fixed concentration of 4-pyridoxic-5'-P. Maximum quenching (90%) was obtained at a molar ratio of apoprotein to 4-pyridoxic-5'-P of approximately 5:1.

The average number of ligand molecules bound per mole of apoenzyme (\(n\)) was calculated for points along the titration curve by means of Equation 4.

\[
\bar{v} = \alpha \left( \frac{L₀}{P₀} \right)
\]

where \(L₀\) and \(P₀\) are the total ligand and protein concentrations, respectively. The results of the fluorometric titrations yield a straight line when \(\bar{v}/L\) is plotted versus \(\bar{v}\) (Fig. 8). An association constant of \(1.3 \times 10^6\) M⁻¹ and \(n = 2\) is determined from this plot.

Since the addition of 4-pyridoxic-5'-P to the apoprotein of the enzyme aspartate aminotransferase inhibits the reconstitution of aminotransaminase activity, it was desirable to determine the affinity of the inhibitor for the apoprotein by a method based on enzymatic activity measurements. To this end, samples of apoenzyme (0.1 mg) were incubated with varying concentrations of 4-pyridoxic-5'-P in 1 ml of 0.05 M Tris-acetate buffer (pH 7.4) at 25°C for 1 hour. The solutions were then diluted with a solution of 4-pyridoxic-5'-P (10⁻⁴ M) and kept at 25°C for 1 hour. The solutions were then diluted with a solution of 4 pyridoxic-5’P (10⁻⁴ M) and kept at 25°C for 1 hour. Aliquots were removed and assayed for enzymatic activity. The results of the inhibition studies were analyzed by means of Equation 5.

\[
\frac{V₀}{V} = 1 + K[I]
\]

where \(V₀\) is the velocity of the enzymatic reaction in the absence of inhibitor and \(V\) is the velocity in the presence of inhibitor. This method gives an inhibition constant of \(2 \times 10^6\) M⁻¹, which agrees well with the association constant determined using the fluorometric method previously described.
The aim of the studies reported in this paper was to investigate the fluorescence properties of 4-pyridoxic-5'-P bound to the proteins bovine serum albumin and aspartate aminotransferase. The results of the polarization of fluorescence titrations indicate that the presence of a carbonyl group in the chemical structure of 4-pyridoxic-5'-P and pyridoxic acid is essential for binding to the protein bovine serum albumin. The addition of 4-pyridoxic-5'-P to a solution of bovine serum albumin causes a red shift of approximately 15 nm in the band position of the emission spectrum and a concurrent increase in the fluorescence lifetime of the ligand. In order to understand the spectroscopic changes of 4-pyridoxic-5'-P complexed to the protein bovine serum albumin, it is important to realize that the red shift in emission is more pronounced and of opposite direction than the corresponding shift in the absorption spectrum (2 nm) Table II. According to accepted theories (19) dealing with solvent effect on electronic spectra, a behavior of this kind reflects the ability of the solvent molecules to reorient themselves during the lifetime of the excited state. Although relaxation effects are not well understood when the ligand molecules are strongly adsorbed to the protein surface, it is likely that emission shifts to lower energies are also influenced by strong interactions of the ligand with polar amino acid residues at the binding site.

In contrast to the 4-pyridoxic-5'-P-bovine serum albumin complex, the emission spectrum of 4-pyridoxic-5'-P bound to the apotransaminase displays a blue shift of approximately 10 nm when compared with free 4-pyridoxic-5'-P at neutral pH. This fluorescence shift to higher energy is accompanied by a pronounced decrease in the fluorescence quantum yield of the complexed ligand. The presence of a phosphate group in the chemical structure of 4-pyridoxic-5'-P is essential for binding and inhibition of aminotransferase activity, since pyridoxic acid failed to interact with the apoenzyme as judged by fluorescence and activity measurements. In order to explain the spectroscopic changes induced by binding of 4-pyridoxic-5'-P to the apoprotein, it is worthwhile to compare the spectroscopic properties of the complexed ligand with those of free 4-pyridoxic-5'-P in aqueous solutions of varying pH.

An analysis of the emission properties of 4-pyridoxic-5'-P reveals that the dipolar ion-phosphate dianion form (III), which is the predominant structure at alkaline pH, has fluorescence characteristics similar to that of 4-pyridoxic acid-5'-P complexed to the apotransaminase. Thus, a blue shift in the emission spectrum and a concurrent decrease in the fluorescence quantum yield are observed when 4-pyridoxic-5'-P forms a complex with the apoenzyme. In view of these considerations, it seems reasonable to propose that the transfer of a proton from the pyridine nitrogen atom to an acceptor amino acid residue on the protein is responsible for the fluorescence changes observed.

It should be noted that a blue shift in the emission spectrum is detected when 4-pyridoxic-5'-P is dissolved in solutions of lower polarizability than water; however, the absorption spectrum is shifted towards the red and the fluorescence yield is enhanced (Table I). The spectroscopic changes induced by interaction of a ligand with a protein may provide valuable information about the microenvironment of the binding site. Therefore, it is reasonable to ask whether the spectroscopic changes detected when the ligand is bound to the protein can be correlated with the results obtained when the ligand is dissolved in well defined solvent mixtures. Recent experiments by Brand and Gohlke have shown that one must be careful in the interpretation of emission changes induced by binding of the ligand N-arylaminonaphthalene sulfonate to the protein bovine serum albumin, since the fluorescence characteristics of the dye adsorbed to the macromolecule are influenced by factors other than the polarizability of the environment.

Some caution must also be exercised in drawing conclusions concerning the detailed characteristics of the binding site of 4-pyridoxic-5'-P. There are two aspects of the studies presented in this paper that are worthwhile considering in the interpretation of the fluorescence changes. (a) The fluorescence properties are affected by the nature of the protein to which the ligand is bound. (b) The ligand possesses several groups,
pyridine nitrogen atom, phosphate and carboxyl groups, capable of interacting with amino acid residues at the binding site. Whereas the carboxyl group in the structure of the ligand is essential for binding to bovine serum albumin, it was found that both phosphate group and the pyridine nitrogen atom play an important role in the formation of the 4-pyridoxic-5'-P-apo-transaminase complex.

Hence, it follows that knowledge of the specific interactions of the ligand groups with amino acid residues of the macromolecule is basic to an understanding of the spectroscopic changes observed.

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