MICELLES OF PYRIDOXAL-5'-PHOSPHATE SCHIFF BASES - AN IMPROVED MODEL FOR THE B_6 SITE OF GLYCOGEN PHOSPHORYLASE

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SUMMARY

The spectral properties of Schiff bases obtained by reaction of pyridoxal-5'-P with n-alkylamines (C_nNH_2) at neutral pH depend upon the length of the hydrocarbon chain of the amine. While short-chain amines (e.g. n = 4,8) yield a product with absorption maxima at 405 and 273 nm (similar to those reported for pyridoxal-5'-P Schiff bases in an aqueous medium), higher members in the n-alkylamine series (e.g. n = 12), which form micelles under the conditions of the experiment, yield a product with absorption maxima at 335 and 252 nm, similar to those of Schiff bases in apolar solvents. Mixed micelles composed of hexadecyltrimethylammonium bromide and n-dodecylamine hydrochloride were found to entrap stoichiometric amounts of pyridoxal-5'-P (one mole per mole of the primary amine) and to yield a Schiff base. The resulting micelles simulate several absorption, fluorescence, and chemical properties of phosphorylase at neutral pH. This micellar model (like the functioning enzyme molecule) puts the pyridoxal-5'-P Schiff base in a hydrophobic microenvironment within an aqueous medium.

Glycogen phosphorylase, as isolated from a variety of sources, contains stoichiometric amounts (one molecule per protomer) of pyridoxal-5'-P (1). Several pieces of evidence suggest that this indispensable constituent of the enzyme (2) is not merely a building block but may directly participate in the catalytic event, or may transfer a regulatory signal to or from the enzyme (3). Attempts to demonstrate the direct involvement of pyridoxal-5'-P in catalysis by means of model reactions in an aqueous medium have failed so far (4,5). However, in recent years it was shown (6-8) that pyridoxal-5'-P in phosphorylase is embedded in a hydrophobic microenvironment while the physiological substrates of the enzyme (glycogen; P_i; glucose-1-P) are water soluble. An adequate model system for studying a possible coenzyme function of pyridoxal-5'-P in phosphorylase should therefore place the cofactor in a hydrophobic microenvironment within an aqueous medium to accomodate the substrates. Such a model system would not only put the cofactor in the unique ionization state or reactivity endowed by the hydrophobic milieu, but would also provide an abrupt microenvironmental transition in the

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Abbreviations used are: C_nNH_2, a normal alkylamine n-carbon-atoms long; CTAB, hexadecyltrimethylammonium bromide; P-Pxy $\overset{\varepsilon}{—}$ Lys, an N^6-(phosphopyridoxyl)-L-lysyl residue.

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vicinity of the cofactor, which by itself might be of great importance for catalysis. We wish to report here the preparation of a micellar model of pyridoxal-5'-P which resembles the B_6 site of phosphorylase in the mode by which the cofactor is covalently bound to the protein (Schiff base), in providing a hydrophobic microenvironment to the cofactor within an aqueous medium, and in several of its absorption, fluorescence and chemical characteristics.

MATERIALS AND METHODS

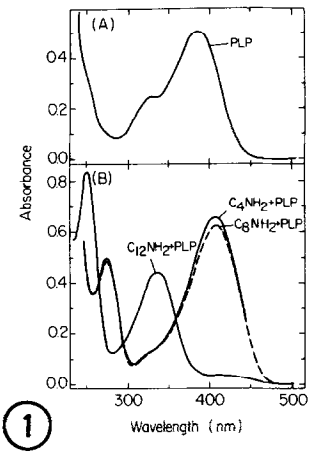
Chemicals. Pyridoxal-5'-P was obtained from Sigma, CTAB from Fluka, n-dodecylamine from Ega Chemie and Sephadex G-50 (coarse) from Pharmacia. The hydrochloride of $C_{12}{\cdot}NH_2$ was prepared by dissolving the free base in ether and adding concentrated HCl followed by two recrystallizations from ethanol. Imidazole was purchased from Fluka and recrystallized three times from ethyl-acetate before use. All other chemicals were the best available grade from commercial sources.

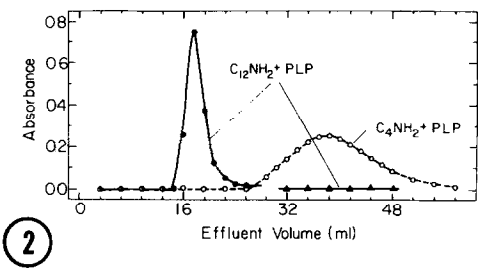
Absorption and Fluorescence Measurements. Absorption measurements were taken with a Cary spectrophotometer model 15. Fluorescence measurements (corrected excitation and emission spectra) were carried out with a Perkin-Elmer MPF 3L spectrofluorimeter. Quantum yields were determined as described by Parker and Rees (9) using quinine sulfate in 0.1 N H_2SO_4 as a reference substance, and taking 0.55 as its quantum yield (10,11).

RESULTS AND DISCUSSION

When pyridoxal-5'-P (10^{-4} M) is allowed to react at neutral pH with an excess of n-butylamine, n-octylamine or n-dodecylamine (all at a concentration of 3 x 10^{-2} M), there is in all cases a disappearance of the spectral bands characteristic of pyridoxal-5'-P under these conditions (Figure 1A) with concomitant appearance of new absorption bands (Figure 1B). In the case of n-butylamine (or n-octylamine) the product formed has two absorption bands, at 405 and 273 nm, very similar to those reported for pyridoxal-5'-P Schiff bases in aqueous environment (12). However, in the case of n-dodecylamine, which is known to form micelles under such conditions (critical micelle concentration in water 1.3 x 10^{-2} M (13)), the spectral properties of the resulting product (absorption maxima at 335 and 252 nm, Figure 1B) resemble those obtained for pyridoxal-5'-P Schiff bases in apolar solvents (14,15,6).

The fact that the pyridoxal-5'-P conjugate with n-dodecylamine is indeed entrapped in a high molecular weight micellar structure was demonstrated by applying the products in each case on a molecular sieve (Sephadex G-50, coarse). As seen in Figure 2, while the Schiff base product formed between n-butylamine and pyridoxal-5'-P was retarded on the column, emerging at a position characteristic of low-molecular-weight compounds, the conjugate formed between n-dodecylamine and pyridoxal-5'-P was excluded at the front, where the high-molecular-weight particles emerge. It should also be noted that the fractions originating from the reaction mixture of n-butylamine and pyridoxal-5'-P (o, Figure 2) had an absorption maximum at 405 nm, while those originating from the reaction mixture





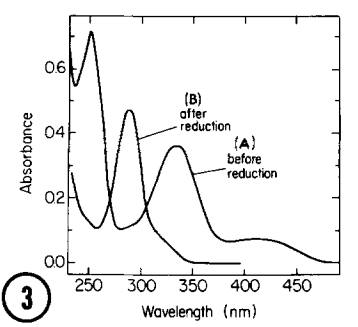


FIGURE 1: Absorption spectrum (A) of pyridoxal-5'-P (PLP, 10^{-4}M) and (B) of Schiff bases formed between pyridoxal-5'-P (10^{-4} M) and the indicated n-alkylamine (0.03 M). In all cases the solvent was an imidazole buffer (0.1 M) adjusted to pH 6.8 with HCl. The spectra of the reaction mixtures in (B) were monitored after standing in the dark for ∿ 1 h (20°).

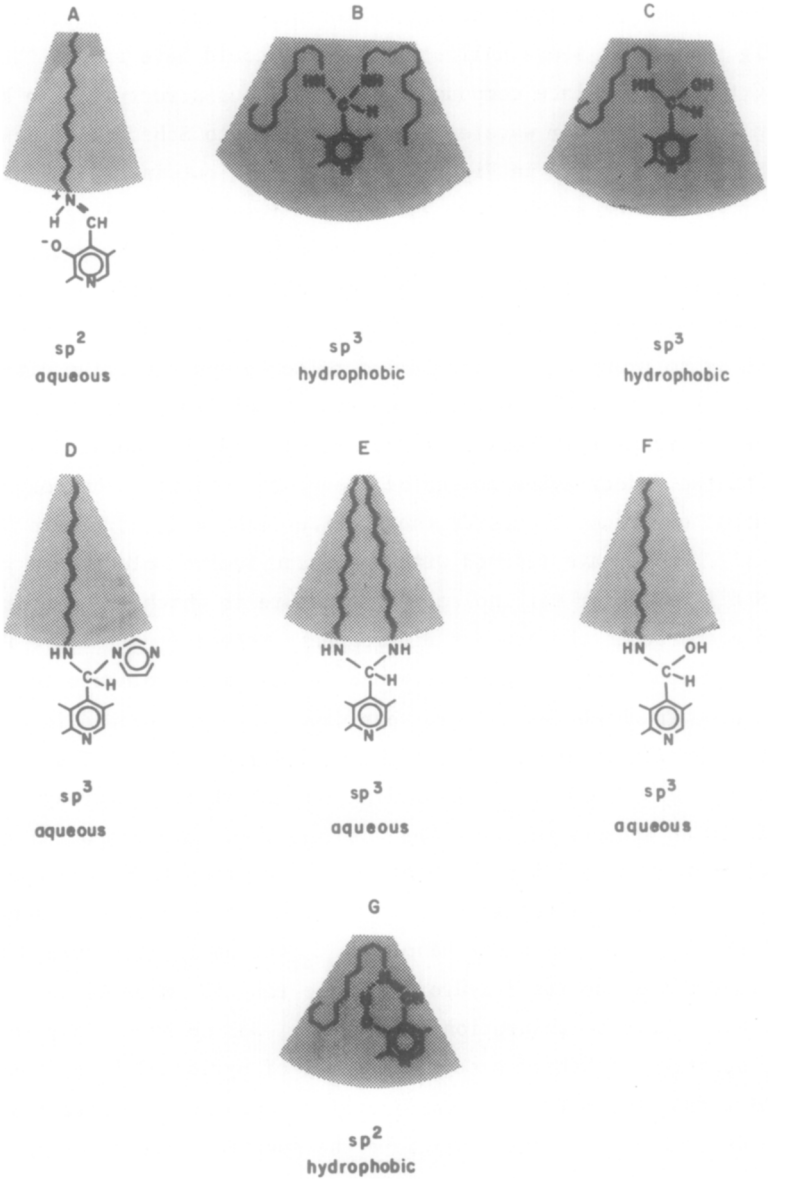
FIGURE 2: Gel filtration of the Schiff bases formed between pyridoxal-5'-P (PLP) and C_4NH_2 or $C_{12}NH_2$. Pyridoxal-5'-P (5 x 10^{-4} M) and the indicated alkylamine (0.03 M) were allowed to react (∿ 1 h at 20°) in an imidazole buffer (0.1 M) adjusted to pH 6.8 with HCl. A sample (1.2 ml) of each of the two reaction mixtures was applied (separately) on a Sephadex G-50 column (35 x 1.5 cm) equilibrated and run with a solution of the appropriate alkylamine (0.03 M) in the same imidazole buffer. Fractions of 1.6 ml were collected and their absorbance at 405 nm (o), 335 nm (●) and 385 nm (▲) were monitored. These wavelengths correspond (respectively) to the absorption maxima of C_4NH_2 + pyridoxal-5'-P, $C_{12}NH_2$ + pyridoxal-5'-P and of free pyridoxal-5'-P (cf. Figure 1).

FIGURE 3: (A) Absorption spectrum of the Schiff base formed by reaction of equimolar concentrations of pyridoxal-5'-P and $C_{12}NH_2$·HCl (both 10^{-4} M) in the presence of CTAB (10^{-3} M). The reaction was allowed to proceed in a sodium phosphate buffer (10^{-3} M), pH 6.4, and the spectrum of the product was monitored after standing in the dark for ∿ 1 h (20°). (B) Absorption spectrum of the reduced Schiff base formed after addition of 50 µl of a 0.1 M $NaBH_4$ solution (in the same phosphate buffer) to 10 ml of solution (A).

between n-dodecylamine and pyridoxal-5'-P (●, Figure 2) had an absorption maximum at ∿ 330 nm. Furthermore, under the conditions of this latter experiment all the pyridoxal-5'-P was bound to the micelles, since there was no excess of free pyridoxal-5'-P (absorption maximum at 385 nm) which, if present, would have been retarded on this column (cf. the absorption of the fractions denoted by ▲ in Figure 2).

In view of the fact that these micelles have absorption maxima at 335 nm and 252 nm (Figure 1B), it seems unlikely that they could have any of the structures A, B and C (Scheme I) since compounds with analogous structures are known to have absorption maxima at other wavelengths (see legend to Scheme I). However, since the experiment illustrated in Figure 1B was carried out in a 0.1M imidazole buffer (for reasons of solubility) and in the presence of a large excess of amine over pyridoxal-5'-P (300 fold), the absorption maximum of the product (at 335 nm) could in principle be due to any one of the following structures, all of which could have an absorption maximum at $\sim$ 335 nm: (1) Pyridoxal-5'-P could have reacted with the amino group of a dodecylamine molecule and the imino nitrogen of an imidazole, leaving the 3-hydroxypyridine ring to protrude out of the micelle into the aqueous environment (Scheme I, structure D); (2) Pyridoxal-5'-P could have reacted with two dodecylamine molecules, leaving again its 3-hydroxypyridine ring in the aqueous environment outside the micelle (Scheme I, structure E); (3) Pyridoxal-5'-P could have reacted with one dodecylamine molecule to yield a hydrated Schiff base (or carbinolamine) structure in which the 3-hydroxypyridine ring would again be in the aqueous environment (Scheme I, structure F); (4) Pyridoxal-5'-P could have reacted with one dodecylamine molecule to form a Schiff base structure, in which the 3-hydroxypyridine ring is embedded in a hydrophobic microenvironment within the micelle (Scheme I, structure G). The C_4' carbon in structures D, E and F has an $\underline{sp}^3$ hybridization and their 3-hydroxypyridine ring is immersed in an aqueous milieu. The absorption of such compounds would therefore have a maximum at $\sim$ 335 nm by analogy to the products formed upon reaction of pyridoxal-5'-P with aminothiols, geminal diamines, etc., in an aqueous medium at neutral pH (16). On the other hand, the C_4' carbon in structure G has an $\underline{sp}^2$ hybridization, but since its 3-hydroxypyridine ring is immersed in a hydrophobic milieu, it would have an absorption maximum at $\sim$ 335 nm by analogy to the absorption spectrum of Schiff base derivatives of pyridoxal-5'-P dissolved in organic solvents (6,14-17). It was therefore necessary to obtain an improved micellar model which would still simulate the spectral properties of glycogen phosphorylase, and whose covalent structure as well as the microenvironment of the 3-hydroxypyridine ring could be established unequivocally.

An improved model for the pyridoxal-5'-P site in phosphorylase was obtained by using mixed micelles composed of CTAB (10^{-3} M) and n-dodecylamine (10^{-4} M) in the absence of imidazole. As seen in Figure 3A, pyridoxal-5'-P binds in stoichiometric amounts to such micelles (one molecule of pyridoxal-5'-P per molecule of n-dodecylamine) to yield a product with absorption maxima at 333 nm and 250 nm. These absorption characteristics exclude structures A, B and C in Scheme I. The fact that these model micelles are prepared in the absence of imidazole excludes structure D, and the stoichiometric binding of pyridoxal-5'-P



Scheme I: Possible structures for the pyridoxal-5'-P micelles indicating the mode of covalent binding of the cofactor to the micelle, the hybridization of its C_4' atom and the microenvironment of its 3-hydroxypyridine ring (shadowed areas indicate a hydrophobic milieu). Structure A should have an absorption maximum at 405-415 nm by analogy to that reported for pyridoxal-5'-P Schiff bases in an aqueous environment (6, 12). Structures B and C should have an absorption maximum at 288-297 nm by analogy to that of thiazolidine or hemimercaptal derivatives of pyridoxal-5'-P (respectively) when dissolved in organic solvents (3,6,22). Structures D - G should all have an absorption maximum at 325 - 335 nm,as indicated in the text.

to the micelles, together with fact that on the average each n-dodecylamine molecule is probably surrounded by CTAB molecules (which cannot bind covalently to pyridoxal-5'-P) makes it unlikely that structure E represents the mode of binding of the cofactor to the micelles.

In an attempt to establish whether pyridoxal-5'-P binds to such mixed micelles through a carbinolamine-type structure in which the 3-hydroxypyridine ring is embedded in an aqueous medium (F, Scheme I) or through a Schiff base structure in which the 3-hydroxypyridine ring is immersed in a hydrophobic milieu (G, Scheme I) we reduced the micelles with $NaBH_4$. If we assume that the reduction itself does not bring about a dislocation of the 3-hydroxypyridine ring from inside the micelle out (or vice versa), then the absorption properties of the $NaBH_4$-reduced micelles should allow us to establish whether the bound pyridoxal-5'-P had been in structure F or G before reduction. In the case of structure G, the reduced micelles should have an absorption maximum at $\sim$ 289 nm (by analogy to the absorption properties of pyridoxamine-5'-P or of P-Pxy $\overset{\varepsilon}{\text{—}}$ Lys in apolar solvents), while in the case of structure F it should absorb at $\sim$ 333 nm, by analogy to the absorption properties of pyridoxamine-5'-P or of P-Pxy $\overset{\varepsilon}{\text{—}}$ Lys in an aqueous medium at neutral pH (cf. (8,18)). As seen in Figure 3B, the $NaBH_4$-reduced micelles have an absorption maximum at 288 nm, supporting the assignment of structure G (Scheme I) to these micelles.

The Schiff base product formed between pyridoxal-5'-P (10^{-4} M) and mixed micelles composed of n-dodecylamine (10^{-4} M) and CTAB (10^{-3} M) simulates at neutral pH the fluorescence properties of the pyridoxal-5'-P site of glycogen phosphorylase. These micelles have a green fluorescence (excitation maxima at 335 nm and at $\sim$ 415 nm, emission maximum at $\sim$ 550 nm) similar to those reported for the enzyme at neutral pH (19,20). The quantum yield of this fluorescence was found to be (at 20°) 0.003 $\pm$ 0.002, which is lower than that of the enzyme at the same temperature ($\sim$ 0.015, cf. Figure 5, reference 20).

In conclusion, this model simulates the pyridoxal-5'-P site of glycogen phosphorylase in several of its features: in its absorption properties, in its fluorescence characteristics (excitation and emission maxima), in containing stoichiometric amounts of pyridoxal-5'-P bound to a primary amine through a Schiff base structure, and in the absorption properties of the product obtained after reduction with $NaBH_4$ (cf. 6, 8,21). The quantum yield of the green fluorescence of the pyridoxal-5'-P micelles described here is lower ($\sim$ 5 fold) than that of the cofactor site in phosphorylase. However, this could be due to the fact that the nature of the microenvironment in the micellar model is somewhat different from the enzyme in its hydrophobicity, polarity and close proximity of functional groups which might enhance or quench the fluorescence of the chromophore.

The most important feature of this model lies in the fact that it represents a closer approximation of the pyridoxal-5'-P site in phosphorylase than previously described models, in which the cofactor was simply allowed to react with a large excess of an alkylamine and dissolved in an organic solvent. Here, as in the case of the enzyme, the pyridoxal-5'-P is embedded in a hydrophobic microenvironment which lies within an aqueous medium. The development of pyridoxal-5'-P micellar models in which there is an abrupt drop in hydrophobicity in the vicinity of the cofactor makes it possible to study the interaction between the substrates of the enzyme which are water-soluble (phosphate, glycogen or glucose-1-phosphate) and the pyridoxal-5'-P moiety, which, if involved in catalysis, has to function with the ionization state and chemical reactivity dictated to the cofactor by the hydrophobic microenvironment. Such a model may thus provide us with a key for proving or disproving the direct involvement of pyridoxal-5'-P in the catalytic event.

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