A Micellar Model for the Pyridoxal 5′-Phosphate Site of Glycogen Phosphorylase†

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ABSTRACT: Mixed micelles composed of hexadecyltrimethylammonium bromide and dodecylamine hydrochloride were found to be good models for the site accommodating pyridoxal 5′-phosphate (pyridoxal-5′-P) in glycogen phosphorylase. At neutral pH, pyridoxal-5′-P becomes entrapped by such micelles (as shown by gel filtration) and reacts in stoichiometric ratios with the primary amine to yield a Schiff base derivative embedded in a hydrophobic microenvironment. The resulting pyridoxal-5′-P micelles simulate the enzyme in their absorption spectrum (maxima at 333 nm (major) and at ~415 nm (minor)) and in their fluorescence characteristics (excitation maxima at 335 and ~415 nm, emission maximum at ~550 nm). These pyridoxal-5′-P micelles can be reduced with $NaBH_4$ with concomitant disappearance of the 333-nm band to yield a product with an absorption maximum at 288 nm similar to the absorption of $NaBH_4$-reduced phosphorylase at neutral pH. Removal of pyridoxal-5′-P from these micelles ("resolution") can be achieved by reaction with cysteine and gel filtration. This process resembles the resolution of "deformed" phosphorylase in that it takes place through an intermediate step whereby cysteine penetrates the micelle and reacts within the micelle with the entrapped pyridoxal-5′-P Schiff base. Unlike previous models for the vitamin B_6 site of phosphorylase, which consisted of Schiff base derivatives of the cofactor in nonaqueous solvents, the micellar model described here (like the functioning enzyme molecule) puts the pyridoxal-5′-P Schiff base in a hydrophobic microenvironment within an aqueous milieu, which can accommodate the water-soluble natural substrates of the enzyme.

One of the most intriguing structural features of glycogen phosphorylase lies in the fact that the enzyme, as isolated from a large variety of sources, contains stoichiometric amounts of pyridoxal 5′-phosphate (pyridoxal-5′-P) (one molecule per enzyme protomer; Baranowski et al., 1957; Illingworth et al., 1958; for reviews see Fischer et al., 1971; Graves & Wang, 1972). Several observations made since the discovery of this cofactor as a constituent of the enzyme strongly imply that pyridoxal-5′-P has a key physiological assignment in phosphorylase. These observations include the following. (a) Pyridoxal-5′-P is indispensable for the catalytic activity of the enzyme (as if it were a coenzyme) since if it is removed, even under very mild, fully reversible conditions, the resulting apoenzyme is devoid of catalytic activity (Shaltiel et al., 1966; Hedrick et al., 1966). (b) Pyridoxal-5′-P is recognized by apophosphorylase with a remarkable specificity, to the extent that it cannot be replaced by any of the naturally occurring forms of vitamin B_6, and that even among the synthetic analogues of pyridoxal-5′-P only a few are capable of endowing the enzyme with catalytic activity (Shaltiel et al., 1969b; Pfeuffer et al., 1972; Vidgoff et al., 1974). (c) There are very strong interactions between the pyridoxal-5′-P site and all the physiological regulatory sites of the enzyme (Shaltiel et al., 1966, 1972; Hedrick et al., 1969). (d) The process by which the cofactor can be made to leave or reenter the enzyme ("resolution" or "reconstitution") displays an astonishing stereospecificity, which is usually found in physiological processes (Shaltiel et al., 1966, 1969a; Hedrick et al., 1966, 1969). (e) Difference absorption spectroscopy (Bresler & Firsov, 1968) and fluorescence studies (Cortijo & Shaltiel, 1970, 1972) implicate pyridoxal-5′-P as being either part of the active site or intimately associated with it. (f) Pyridoxal-5′-P has been persistently preserved as part of the phosphorylase molecule throughout evolution.

Although in principle each one of the above-mentioned observations could be accounted for also if we assign a central structural role to pyridoxal-5′-P, the combination of these observations lead us to believe that it is not merely a structural building block in phosphorylase but is either directly involved in the catalytic event, or transfers a regulatory signal to or from the enzyme. We have thus a considerable amount of circumstantial evidence for such involvement, and although this would be the simplest explanation for the presence of pyridoxal-5′-P in phosphorylase, there is not yet any unequivocal direct evidence for such a physiological assignment.

Several attempts to demonstrate by means of model studies the direct participation of pyridoxal-5′-P in catalysis have failed so far (Fischer et al., 1963; Hedrick & Fischer, 1965) in spite of the fact that similar studies have been very successful in the case of other pyridoxal-5′-P enzymes (Snell, 1958; Braunstein, 1960; Snell & DiMari, 1970).

In recent years, physicochemical studies suggested that at neutral pH pyridoxal-5′-P is bound to phosphorylase through a Schiff base structure and is embedded in a hydrophobic microenvironment (Shaltiel & Cortijo, 1970; Johnson et al., 1970). This suggestion has now been supported by a variety of studies carried out in several laboratories (Jones & Cowgill, 1971; Arrio-Dupont, 1971; Cortijo et al., 1971, 1976; Forrey et al., 1971; Cortijo & Shaltiel, 1972; Pfeuffer et al., 1972; Shaltiel et al., 1972; Veinberg et al., 1974, 1976; Feldmann et al., 1974; Feldmann & Helmreich, 1976; Jimenez, 1976). On the other hand, 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

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medium to accommodate 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 (hydrophobic → hydrophilic) transition in the vicinity of the cofactor, which by itself might be of great importance for catalysis (Phillips, 1966; Imoto et al., 1972; Murakami & Kondo, 1975). We wish to report here the preparation of a micellar model for the vitamin B_6 site in phosphorylase which represents an improved model for the enzyme along these lines.

Materials and Methods

Chemicals. Pyridoxal-5′-P was obtained from Sigma, $CetMe_3NBr$[1] from Fluka, *n*-dodecylamine from Ega Chemie, and Sephadex G-50 (coarse) from Pharmacia. The hydrochloride of C_{12}·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 Model 15 spectrophotometer. 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 & Rees (1960) using quinine sulfate in 0.1 N H_2SO_4 as a reference substance, and taking 0.55 as its quantum yield (Melhuish, 1961; Dawson & Windsor, 1968).

Results and Discussion

Effect of Hydrocarbon Chain Length on the Spectral Properties of Schiff Bases Formed between Pyridoxal-5′-P and n-Alkylamines. In an attempt to establish conditions for introducing pyridoxal-5′-P Schiff bases into micellar structures, we compared the spectral properties of the Schiff bases formed between pyridoxal-5′-P and various *n*-alkylamines under conditions where the *n*-alkylamines with larger hydrocarbon chains are known to form micelles (Tanford, 1973; Fendler & Fendler, 1975). It was found that 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 3×10^{-2} M), there is in all cases a disappearance of the spectral bands characteristic of free pyridoxal-5′-P under these conditions (absorption maximum at 385 nm and a shoulder at ~330 nm) with concomitant appearance of new absorption bands (Kupfer et al., 1977b). 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 an aqueous environment (Metzler, 1957; Christensen, 1958). However, in the case of *n*-dodecylamine, which is known to form micelles under such conditions (critical micelle concentration in water 1.3×10^{-2} M; Vies and Hoerr, 1960), the spectral properties of the resulting product are entirely different (absorption maxima at 335 and 252 nm) and resemble those obtained for pyridoxal-5′-P Schiff bases in apolar solvents (Heinert & Martell, 1963; Matsushima & Martell, 1967; Shaltiel & Cortijo, 1970; Feldmann & Helmreich, 1976).

[1] Abbreviations used are: C_nNH_2, a normal alkylamine *n* carbon atoms long; $CetMe_3NBr$, hexadecyltrimethylammonium bromide; P-Pxy-Lys, an N^6-(phosphopyridoxyl)-L-lysyl residue (according to the nomenclature suggested by the Commission on Biochemical Nomenclature (1970) *Biochemistry 9*, 4019); P_i, inorganic phosphate.

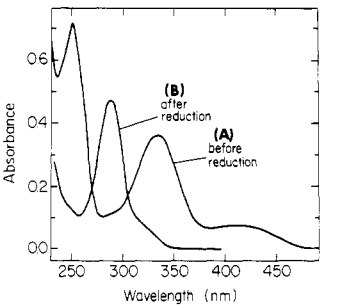
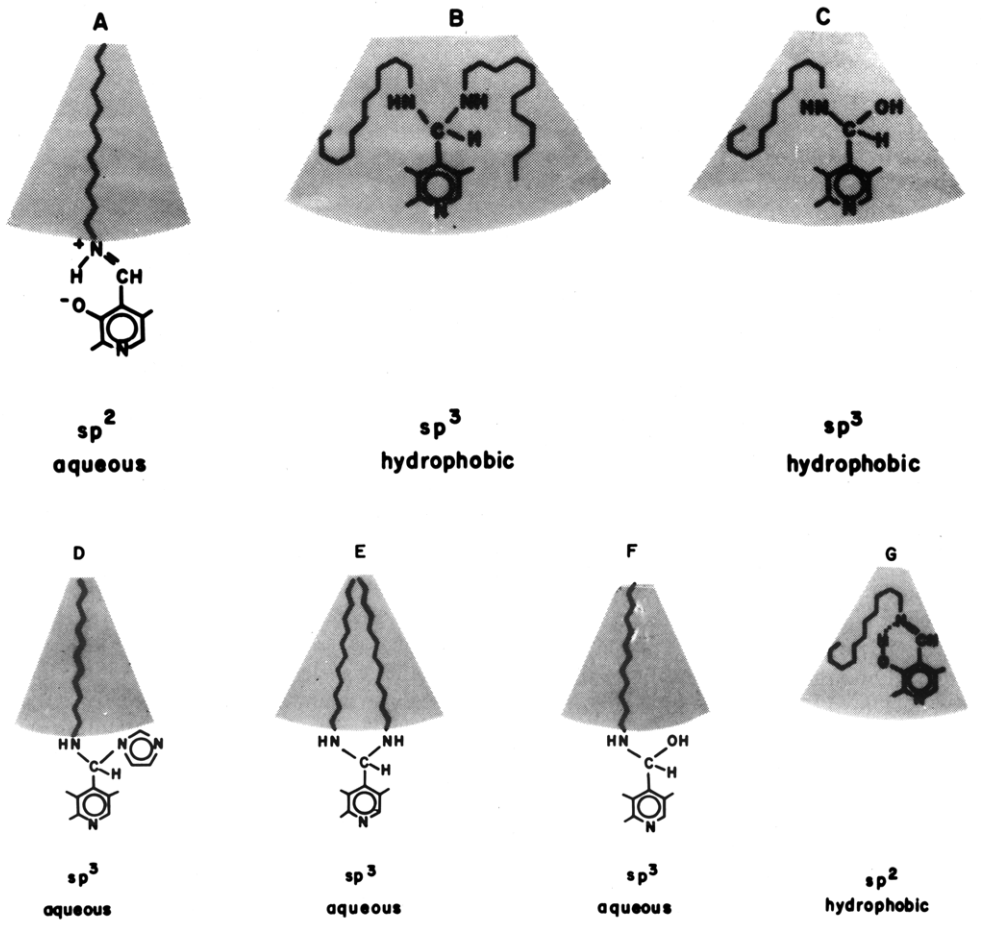


FIGURE 1: (A) Absorption spectrum of the Schiff base formed by reaction of equimolar concentrations of pyridoxal-5′-P and $C_{12}NH_2$·HCl (both 10^{-4} M) in the presence of $CetMe_3NBr$ (10^{-3} M). The reaction was allowed to proceed in a sodium phosphate buffer (10^{-3} M) (pH 6.4) and the spectrum of the product was monitored after standing in the dark for ~1 h (20 °C). (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.

Structures Which Could Account for the Spectral Properties of the n-Dodecylamine–Pyridoxal-5′-P Micelles. In view of the fact that these micelles have absorption maxima at 335 and 252 nm, it seems unlikely that they could have any of the structures A, B, or C (Chart I) since compounds with analogous structures are known to have absorption maxima at other wavelengths (see footnotes to Chart I). However, since the experiment described above was carried out in 0.1 M 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 structures D to F (Chart I), all of which could have an absorption maximum at ~335 nm. C-4′ in structures D, E, and F has an sp^3 hybridization and the 3-hydroxypyridine ring in D–F is immersed in an aqueous milieu. The absorption of such compounds would therefore have a maximum at ~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 (Buell & Hansen, 1960; Tobias & Kallen, 1975). On the other hand, C-4′ in structure G has an sp^2 hybridization, but since its 3-hydroxypyridine ring is immersed in a hydrophobic milieu, it would have an absorption maximum at ~335 nm by analogy to the absorption spectrum of the Schiff base product formed between a primary alkylamine and pyridoxal-5′-P in an organic solvent (Heinert & Martell, 1963; Matsushima & Martell, 1967; Shaltiel & Cortijo, 1970; Shaltiel et al., 1972; Feldmann & Helmreich, 1976). 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.

Stoichiometric Binding of Pyridoxal-5′-P to Mixed Micelles of n-Dodecylamine and $CetMe_3NBr$. An improved model for the pyridoxal-5′-P site in phosphorylase was obtained by using mixed micelles composed of $CetMe_3NBr$ (10^{-3} M) and *n*-dodecylamine (10^{-4} M) in the absence of imidazole. As seen in Figure 1A, pyridoxal-5′-P binds in equimolar ratios to such micelles (one molecule of pyridoxal-5′-P per molecule of *n*-dodecylamine) as implied by the disappearance of the absorption band characteristic of free pyridoxal-5′-P (maximum at 385 nm, under the conditions of the experiment) and by the formation of a product with absorption maxima at 333 and 250 nm (minor peak also at ~415 nm). These absorption characteristics exclude structures A, B, and C in Chart I. The fact that these model micelles are prepared in the absence of imidazole excludes structure D and the stoichiometric binding

CHART 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.[a]



[a] 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 (Metzler, 1957; Christensen, 1958; Shaltiel & Cortijo, 1970; Feldmann & Helmreich, 1976). 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 (Shaltiel & Cortijo, 1970; Shaltiel et al., 1972; Feldmann et al., 1974). Structures D–G should all have an absorption maximum at 325–335 nm as indicated in the text.

of pyridoxal-5′-P to the micelles, together with the fact that on the average each *n*-dodecylamine molecule is probably surrounded by $CetMe_3NBr$ 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, Chart I) or through a Schiff base structure in which the 3-hydroxypyridine ring is immersed in a hydrophobic milieu (G, Chart 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 ~289 nm (by analogy to the absorption properties of pyridoxamine-5′-P or of P-Pxy-$^{\epsilon}$-Lys in apolar solvents), while in the case of structure F it should absorb at ~333 nm, by analogy to the absorption properties of pyridoxamine-5′-P or of P-Pxy-$^{\epsilon}$-Lys in an aqueous medium at neutral pH (cf. Cortijo & Shaltiel, 1970, 1972). As seen in Figure 1B, the $NaBH_4$-reduced micelles have an absorption maximum at 288 nm, supporting the assignment of structure G (Chart I) to these micelles.

Binding of Pyridoxal-5′-P to the Mixed Micelles and the Types of Forces That Might be Involved. Under the conditions of the experiment depicted in Figure 1, equimolar concentrations of pyridoxal-5′-P and an alkylamine which would not be part of a micelle (e.g., ethylamine) would not yield a considerable extent of Schiff base formation, since under such conditions the equilibrium of the reaction would be extensively shifted in the direction of dissociation of the Schiff base into its constituents. Therefore, the fact that pyridoxal-5′-P binds stoichiometrically to the mixed micelles of $CetMe_3NBr$ and *n*-dodecylamine must be due to an additional contribution of the micellar structure to the entrapment of pyridoxal-5′-P by either ionic or hydrophobic interactions, or both. Indeed, when pyridoxal-5′-P is added to micelles of $CetMe_3NBr$ alone, the spectrum of the cofactor suggests a hydrophobic interaction with the micelles, as indicated by an ipsochromic displacement of the absorption maximum from 385 to 360 nm (not illustrated) which is reminiscent of that observed in the transfer of pyridoxal-5′-P from an aqueous medium to an organic solvent such as dimethyl sulfoxide (cf. Feldmann & Helmreich, 1976). Furthermore, if pyridoxal-5′-P is allowed to react with an equimolar concentration of an alkylamine in the presence of a constant excess of $CetMe_3NBr$ (at a concentration above

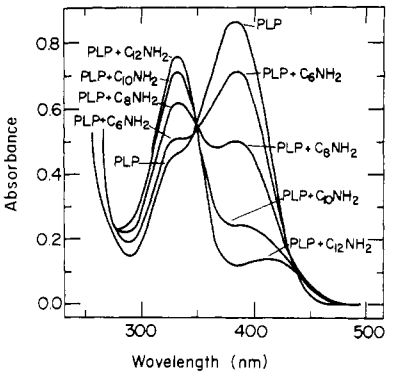


FIGURE 2: Absorption spectra of the Schiff bases formed between pyridoxal-5′-P (PLP) and *n*-alkylamines of various chain lengths in the presence of $CetMe_3NBr$. Each reaction mixture contained pyridoxal-5′-P (2×10^{-4} M), the indicated *n*-alkylamine hydrochloride (2×10^{-4} M), and $CetMe_3NBr$ (10^{-3} M) in a 10^{-2} M sodium phosphate buffer (pH 7.1). The spectra of the mixtures were monitored after standing in the dark for ~1 h (20 °C).

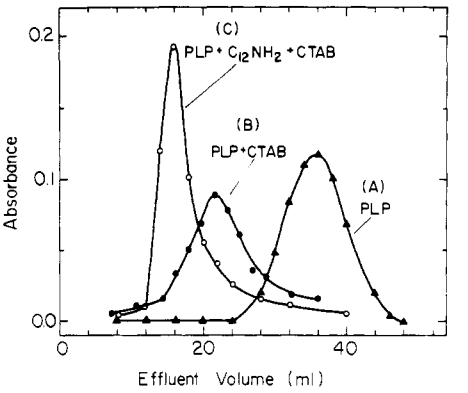


FIGURE 3: Binding of pyridoxal-5′-P (PLP) to micelles of $CetMe_3NBr$ (CTAB) and $C_{12}NH_2$. Three solutions were prepared: (A) of pyridoxal-5′-P (2×10^{-4} M in a 10^{-3} M sodium phosphate buffer, pH 7.1); (B) of pyridoxal-5′-P (2×10^{-4} M) and $CetMe_3NBr$ (10^{-3} M) in a 10^{-3} M sodium phosphate buffer (pH 6.9); (C) of pyridoxal-5′-P (2×10^{-4} M), $C_{12}NH_2$ (2×10^{-4} M), and $CetMe_3NBr$ (10^{-3} M) in a 10^{-3} M sodium phosphate buffer (pH 6.9). After standing in the dark for 1 h (20 °C) samples (1.5 mL) of each of the solutions A, B, and C were subjected to chromatography (20 °C) on three identical columns (Sephadex G-50, coarse, 35 × 1.5 cm) equilibrated and run (respectively) with solutions A′, B′, and C′ which were identical with A, B, and C but contained no pyridoxal-5′-P. The absorption spectra of the fractions were taken and the absorption values at their maxima were monitored. These maxima were at 385 nm (▲) for column (A); at 380 nm (●) for column (B), and at 335 nm (○) for column (C).

its critical micelle concentration) it can easily be shown that the extent of Schiff base formation (estimated by the ratio between the absorption at 333 nm and that at 385 nm) increases with increasing hydrocarbon chain length, which in itself determines of course whether the Schiff base conjugate will be entrapped within the micelle or not (Figure 2).

The fact that the pyridoxal-5′-P conjugate with the *n*-dodecylamine is indeed entrapped in a high molecular weight micellar structure was demonstrated by applying the product on a molecular sieve (Sephadex G-50). As seen in Figure 3C the product formed by equimolar concentrations of pyridoxal-5′-P and *n*-dodecylamine in the presence of $CetMe_3NBr$ is excluded in the front, where the high molecular weight particles emerge, while free pyridoxal-5′-P is retarded on the same column (Figure 3A). It should also be noted that the micelles formed by $CetMe_3NBr$ alone also bind the cofactor (Figure 3B) in agreement with the spectral data described above.

Simulation of the Fluorescence Properties. The Schiff base product formed between pyridoxal-5′-P (10^{-4} M) and mixed micelles composed of *n*-dodecylamine (10^{-4} M) and $CetMe_3NBr$ (10^{-3} M) simulate at neutral pH the fluorescence properties of the pyridoxal-5′-P site of glycogen phosphorylase. As seen in Figure 4, these micelles have a green fluorescence (excitation maxima at 335 nm and at ~415 nm, emission maximum at ~550 nm) similar to those reported for the enzyme at neutral pH (Shaltiel & Fischer, 1967; Cortijo et al., 1971). The quantum yield was found to be (at 20 °C) 0.003 ± 0.002, which is lower than that of the enzyme at the same temperature [~0.015 (cf. Figure 5, Cortijo et al., 1971) or 0.019 (Honikel & Madsen, 1972)].

"Resolution" of the Pyridoxal-5′-P Micelles with Aldehyde Reagents. Another interesting analogy between the pyridoxal-5′-P site of glycogen phosphorylase and the pyridoxal-5′-P micelles obtained by reaction of the cofactor with *n*-dodecylamine and $CetMe_3NBr$ was observed in studying the process by which pyridoxal-5′-P can be made to leave the micelles following reaction with aldehyde reagents. It was previously shown that removal of pyridoxal-5′-P from glycogen phosphorylase *b* ("resolution") can be achieved by first loosening the grip of the protein on pyridoxal-5′-P ("deformation") and then reacting the cofactor with a stereospecific aldehyde reagent such as L-cysteine (Shaltiel et al., 1966, 1967, 1969a; Hedrick et al., 1969). According to these studies, L-cysteine reacts with the pyridoxal-5′-P moiety while it is still bound to

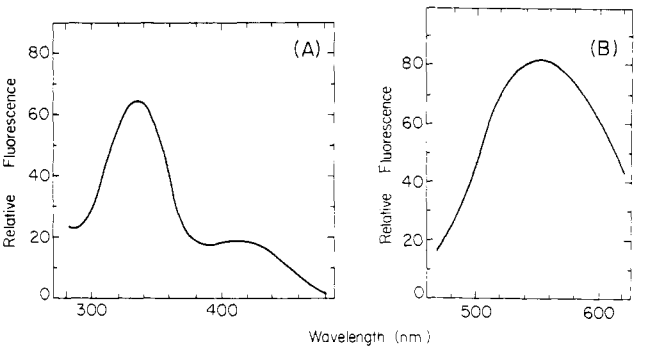
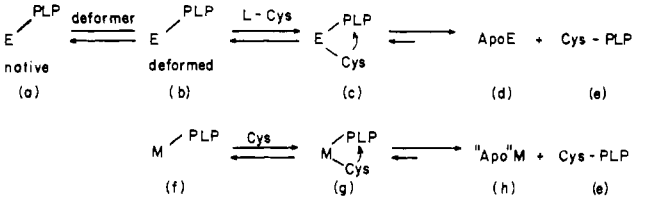


FIGURE 4: Fluorescence properties of the Schiff base formed between pyridoxal-5′-P (10^{-4} M), $C_{12}NH_2$·HCl (10^{-4} M), and $CetMe_3NBr$ (10^{-3} M) in a sodium phosphate buffer (10^{-3} M), pH 6.4. The reaction was allowed to proceed in the dark (1 h at 20 °C) and then the corrected spectra were recorded: (A) excitation spectrum taken (at 20 °C) with emission at 545 nm (instrument sensitivity × 100); (B) emission spectrum taken (at 20 °C) with excitation at 335 nm (instrument sensitivity × 30).

the "deformed" protein (b, Scheme I) and then the cofactor leaves the holoenzyme-L-cysteine complex (c) to yield the apoenzyme (d) and a thiazolidine derivative of pyridoxal-5′-P (e). Spectroscopic monitoring of the reaction between L-cys-

SCHEME I: Proposed Mechanism for Removal of Pyridoxal-5′-P ("Resolution") by Reaction with Cysteine (A) from the Enzyme (E-PLP); (B) from Mixed Micelles Composed of $C_{12}NH_2$, $CetMe_3NBr$, and Pyridoxal-5′-P (M-PLP).[a]



[a] Resolution yields an apoenzyme (Apo E) in the case of glycogen phosphorylase and micelles devoid of pyridoxal-5′-P ("Apo"M) (cf. Shaltiel et al., 1967; Hedrick et al., 1969).

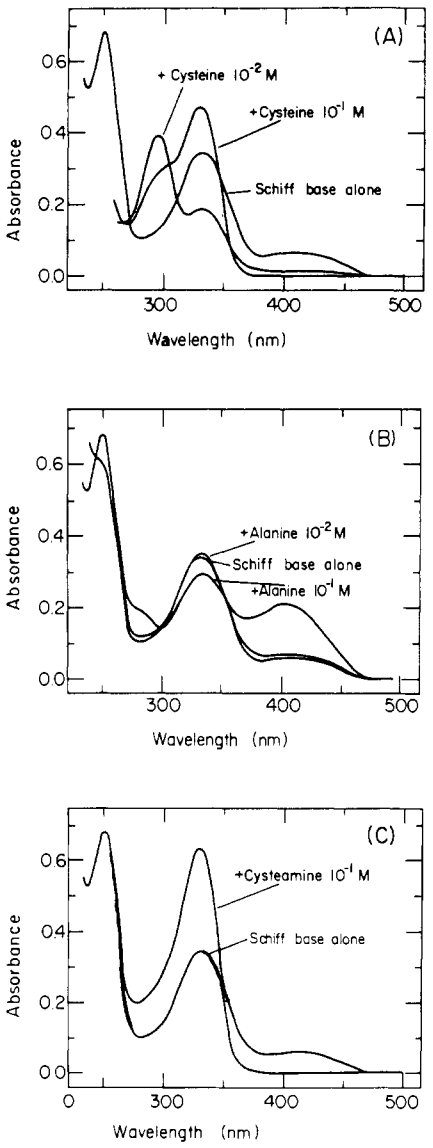


FIGURE 5: Removal of pyridoxal-5′-P from mixed micelles of $C_{12}NH_2$·HCl and $CetMe_3NBr$ monitored spectrophotometrically. Pyridoxal-5′-P (1.1×10^{-4} M) was reacted with mixed micelles composed of $C_{12}NH_2$·HCl (1.1×10^{-4} M) and $CetMe_3NBr$ (1.1×10^{-3} M) in a sodium phosphate buffer (10^{-3} M), pH 6.6. The reaction mixture was allowed to stand in the dark (1 h, 20 °C) and then "resolution" was attempted by addition of either L-cysteine·HCl (A); L-alanine + NaCl (at equimolar concentration to match the ionic strength of solution A)(B); or cysteamine·HCl (C) (all at pH 6.6 and the indicated final concentrations) and after 1 h (20 °C) the absorption spectra of the various samples were recorded. The final concentrations of the micelle constituents (after the dilution caused by addition of the neutralized aldehyde reagent (1 or 0.1 M)) were pyridoxal-5′-P (10^{-4} M), $C_{12}NH_2$·HCl (10^{-4} M), and $CetMe_3NBr$ (10^{-3} M).

teine and the mixed micelles of pyridoxal-5′-P shows that the process simulates the resolution of glycogen phosphorylase and occurs through a distinct intermediate step (g, Scheme I) in which a thiazolidine derivative of pyridoxal-5′-P is formed within the micelle, which then leaves it to yield an "apomicelle" (h) and a thiazolidine derivative of pyridoxal-5′-P (e).

This analogy is based on the following experimental evidence. When L-cysteine (final concentration 10^{-2} M) reacts with the pyridoxal-5′-P micelles there is a decrease in the absorption at 333 nm with a concomitant appearance of a peak at 295 nm (Figure 5A), similar to that of a thiazolidine derivative of pyridoxal-5′-P when dissolved in an organic solvent such as *N,N′*-dimethylformamide (Shaltiel & Cortijo, 1970; Shaltiel et al., 1972) or dimethyl sulfoxide (Feldmann & Helmreich, 1976). This spectral change could be accounted for if we assume that under these conditions an intermediate is formed, in which the thiazolidine derivative of pyridoxal-5′-P is still entrapped in the micelle. Furthermore, by increasing the concentration of L-cysteine up to 10^{-1} M there is an appearance of a peak at 330 nm (Figure 5A) which could be due to a shift in the equilibrium (Scheme I) and "resolution" of the micelles with concomitant release of the thiazolidine derivative of pyridoxal-5′-P into the aqueous environment.

L-Alanine, an analogue of L-cysteine which lacks the sulfhydryl group, cannot form a thiazolidine derivative with pyridoxal-5′-P and does not bring about resolution of phosphorylase in the presence of deforming agents (Shaltiel et al., 1966, 1969a; Hedrick et al., 1969). Unlike L-cysteine, this analogue does not significantly alter the spectrum of the pyridoxal-5′-P micelles when present at a final concentration of 10^{-2} M (compare panels A and B in Figure 5). However, at a higher concentration of L-alanine (10^{-1} M) there is a decrease in the peak at 333 nm with a concomitant increase in the absorption around 405 nm, which could be due to the removal of some pyridoxal-5′-P from the micelle out into the aqueous medium, in other words to a shift in the equilibrium between the pyridoxal-5′-P entrapped within the micelle and the pyridoxal-5′-P outside the micelle, mostly bound to L-alanine in a Schiff base structure.

In spite of the above, it should be emphasized that, unlike the case of the enzyme, the reaction between the micelles and pyridoxal-5′-P is not a stereospecific process. Under the conditions of the experiment depicted in Figure 5A, the spectral changes obtained with D-cysteine (not illustrated) are identical with those obtained with L-cysteine. Moreover, upon reaction of the pyridoxal-5′-P micelles with cysteamine (which does not cause resolution of "deformed" phosphorylase; Shaltiel et al., 1966, 1969a) there is an increase in absorption at 330 nm (Figure 5C) which is due to "resolution" of the micelles, as will be shown below.

The correlation between the spectral changes depicted in Figure 5 and the occurrence of "resolution" could be established unequivocally by gel filtration. As seen in Figure 6B, the pyridoxal-5′-P micelles are not resolved by L-alanine nor is pyridoxal-5′-P removed from the micelles by reaction with L-cysteine at a final concentration of 10^{-2} M (Figure 6C). But if the concentration of L-cysteine is increased up to 0.09 M "resolution" does occur (Figure 6D). These results support our suggestion that "resolution" of the micelles occurs indeed through an intermediate step in which cysteine forms a thiazolidine derivative with pyridoxal-5′-P within the micelle and that this derivative is then released into the aqueous medium (Scheme I). Gel filtration experiments also demonstrate the lack of stereospecificity in the "resolution" of the micelles, since the process takes place with cysteamine (Figure 6E) or D-cysteine (not illustrated).

Evaluation of the Micellar Model. The micellar model described here 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 $NaBH_4$ (for the spectral properties of $NaBH_4$-reduced phosphorylase cf. Kent et al., 1958; Fischer et al., 1958; Shaltiel & Cortijo, 1970; Johnson et al., 1970; Cortijo & Shaltiel, 1972). An interesting similarity is found in the mechanism of "resolution" of the micelles, i.e., the process by which an aldehyde reagent reacts with the pyri-

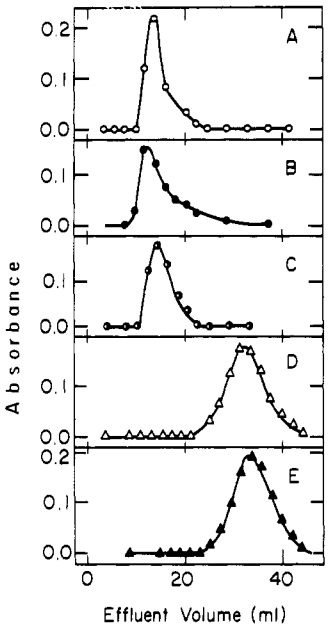


FIGURE 6: Removal of pyridoxal-5′-P from mixed micelles of $C_{12}NH_2$·HCl and $CetMe_3NBr$ shown by gel filtration. Five reaction mixtures were prepared, each containing pyridoxal-5′-P (2×10^{-4} M), $C_{12}NH_2$·HCl (2×10^{-4} M), $CetMe_3NBr$ (10^{-3} M), and a sodium phosphate buffer (10^{-3} M). These reaction mixtures were allowed to stand in the dark (1 h, 20 °C), and then "resolution" was attempted by reaction with various aldehyde reagents at the indicated final concentrations: (A) no addition (control); (B) L-Ala + NaCl (each at a final concentration of 0.09 M, the NaCl being added to match the ionic strength of solutions D and E); (C) L-Cys·HCl (10^{-2} M); (D) L-Cys·HCl (0.09 M); (E) cysteamine·HCl (0.09 M). The final concentrations of the Schiff base constituents (pyridoxal-5′-P and $C_{12}NH$·HCl) were adjusted in each case to 2×10^{-4} M. After standing in the dark (1 h, 20 °C) samples (1.5 mL) of the solutions A to E were applied each on a Sephadex G-50 (coarse) column (37 × 1.2 cm) equilibrated and run with solutions A′ to E′ (respectively) which were identical with A to E but contained no pyridoxal-5′-P. All the solutions A to E and A′ to E′ had a final pH of 6.9 ± 0.1. The absorbance of the effluent was monitored in each case at the absorption maximum of the sample applied: at 335 nm (○) for column A; at 335 nm (●) for column B; at 290 nm (◑) for column C; at 330 nm (△) for column D; and at 330 nm (▲) for column E.

doxal-5′-P of the micelle and brings about its release into the external aqueous environment. Although the resolution of the micelle does not exhibit the stereospecificity found in the enzyme, it does proceed through an analogous intermediate step, in which cysteine forms a thiazolidine derivative *within* the micelle (Scheme I).

It should be mentioned, however, that the quantum yield of the green fluorescence of the pyridoxal-5′-P micelles described here is lower (~fivefold) than that of the cofactor site in phosphorylase. 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. In this context it is interesting to note that upon deformation of phosphorylase there is a considerable quenching (46%) in the green fluorescence of the enzyme (Shaltiel & Fischer, 1967; Hedrick et al., 1969). It is possible, therefore, that the structure of the micellar model is closer to that of the "deformed" enzyme, which is in a conformation that can still restore full activity upon removal of the deforming agent.

The most important feature of this micellar model lies in the fact that it represents a closer approximation of the pyridoxal-5′-P site in phosphorylase. Unlike previous models which consisted simply of Schiff base derivatives of the cofactor in organic solvents, the model described here (like the functioning enzyme molecule) puts the pyridoxal-5′-P Schiff base in a hydrophobic environment within an aqueous medium. As such, it makes it possible to study interactions or reactions (if any) between the substrates of the enzyme, which are water soluble (phosphate, glycogen, or glucose-1-P), and the pyridoxal-5′-P moiety, which, if involved in catalysis, has to function with the ionization state and chemical reactivity endowed to it by the hydrophobic microenvironment. The fact that the model creates an abrupt microenvironmental (hydrophobic → hydrophilic) transition in the vicinity of the cofactor is an additional feature of the model which might per se be of importance for catalysis. Such models may therefore provide us with a key for proving or disproving a direct involvement of pyridoxal-5′-P in the catalytic event.

The binding of pyridoxal-5′-P to apophosphorylase is certainly an important step in the biosynthesis of the active holoenzyme, and the resolution of the enzyme may be important in its degradation (Katunuma, 1974). Resolution and reconstitution of phosphorylase may also prove to be a physiological means for regulating the activity of phosphorylase. In view of the mechanistic analogy demonstrated here between the "resolution" of the pyridoxal-5′-P micelles and that of the "deformed" enzyme, models of this type may allow us to gain a further insight into this process.

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