The Pyridoxal 5′-Phosphate Site in Rabbit Skeletal Muscle Glycogen Phosphorylase *b*: An Ultraviolet and ^1H and ^{31}P Nuclear Magnetic Resonance Spectroscopic Study†

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ABSTRACT: ^1H NMR spectra of the 3-*O*-methylpyridoxal 5′-phosphate–*n*-butylamine reaction product indicated that this analogue forms a Schiff base in aprotic solvent. The uv spectral properties of 3-*O*-methylpyridoxal-5′-phosphate phosphorylase *b* correspond to those of the *n*-butylamine Schiff base derivative in dimethyl sulfoxide. On the basis of that and auxiliary uv and ^1H NMR spectra of pyridoxal and pyridoxal 5′-phosphate and the corresponding Schiff base derivatives we have verified that pyridoxal 5′-phosphate is also bound as a Schiff base to phosphorylase and not as an aldamine. Since 3-*O*-methylpyridoxal-5′-phosphate phosphorylase is active, a proton shuttle between the 3-hydroxyl group and the pyridine nitrogen is excluded. This directs attention to the 5′-phosphate group of the cofactor as a candidate for a catalytic function. ^{31}P NMR spectra of pyridoxal 5′-phosphate in phosphorylase *b* indicated that deprotonation of the 5′-phosphate group was unresponsive to external pH. Interaction of phosphorylase *b* with adenosine 5′-monophosphate, the allosteric effector required for activity, and arsenate, which substitutes for phosphate as substrate, triggered a conformational change which resulted in deprotonation of the 5′-phosphate group of pyridoxal 5′-phosphate at pH 7.6. It now behaved like in the pyridoxal 5′-phosphate–ε-aminocaproate Schiff base in aqueous buffer, where the diionized form is dominant at this pH. Differences of line widths of the adenosine 5′-monophosphate signal point to different life times of the allosteric effector–enzyme complexes in the presence and absence of substrate (arsenate).

We and others have searched in the past few years for a possible catalytic role of the phosphate group of pyridoxal 5′-phosphate in glycogen phosphorylase (EC 2.4.1.1) (Cortijo and Shaltiel, 1970; Pfeuffer et al., 1972a,b; Weisshaar and Palm, 1972; Feldmann et al., 1972, 1974; Vidgoff et al., 1974). At the same time several reports have appeared on the structure of pyridoxal-5′-P in phosphorylase (Johnson et al., 1970; Shaltiel and Cortijo, 1970; Honikel and Madsen, 1972). Honikel and Madsen (1972) have revived the original proposal of Kent et al. (1958) according to which pyridoxal-5′-P is bound to glycogen phosphorylase as a substituted aldimine (aldamine), whereas Johnson et al. (1970) have ascribed the uv spectral and fluorescent properties of pyridoxal-5′-P in glycogen phosphorylase to the protonation of the 3-hydroxyl group of an imine which, according to Shaltiel and Cortijo (1970), is in a highly hydrophobic environment. The available evidence, insofar as it is based on a comparison of the uv spectral and fluorescence properties of the phosphorylase-bound cofactor and pyridoxal-5′-P Schiff base derivatives in aprotic solvents, does not suffice to decide unequivocally between these different but plausible modes of binding. But the structure of pyridoxal-5′-P in glycogen phosphorylase must be known before one can profitably speculate on the catalytic role of protonatable sidechains of pyridoxal-5′-P (Bresler and Firsov, 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972b; Feldmann et al., 1972, 1974; see: Graves and Wang, 1972).

In the preceding paper (Feldmann et al., 1974) we have made use of 3-*O*-methylpyridoxamine-5′-P in reduced phosphorylase *b*, following up an observation originally made by Kent (1959) according to which an absorption band near 330 nm appears in reduced phosphorylase as the pH is lowered. Since the 3-OH group is blocked in the analogue-reconstituted phosphorylase, no absorbance band near 330 nm should be generated on acidification. This was the case, thus confirming the proposal of Johnson et al. (1970) that the 330-nm absorbance band results from the formation of the zwitterionic form with an unprotonated oxygen in position 3. This encouraged us to use 3-*O*-methylpyridoxal-5′-P for the study of the structure of pyridoxal-5′-P in phosphorylase because it allows exclusion of the phenolate form.[1] This analogue reconstitutes with apophosphorylase *b* a partially active enzyme (Shaltiel et al., 1969). ^1H NMR was used to distinguish sp^2 and sp^3 hybrid states of the hydrogen on the carbon bonded to C-4 of free pyridoxal-5′-P and analogues. We could verify with the aid of 3-*O*-methylpyridoxal-5′-P the proposal of Johnson et al. (1970) and Shaltiel and Cortijo (1970) according to which pyridoxal-5′-P is bound as a Schiff base to an ε-aminolysyl residue in a hydrophobic site in skeletal muscle phosphorylase *b* (see also Veinberg et al., 1974). This would hypothetically leave the 5′-phosphate group of the cofactor either confined to the hydrophobic binding domain or free to interact with the solvent. We have recently emphasized that this information is crucial to a role of protonation–deprotonation of the 5′-phosphate group in catalysis (Feldmann et al., 1974). We have therefore carried out ^{31}P NMR measurements of pyridoxal-5′-P bound to phosphorylase *b* with a new Bruker WH-180 WB NMR spectrometer.

Materials and Methods

Enzymes. Phosphorylase *b* was prepared from frozen rabbit skeletal muscle by the procedure of Fischer and Krebs (1958),

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[1] The phenolate form refers to the 3-O^- form and the phenolic form to the 3-OH form of pyridoxal-5′-P.

as modified by Krebs et al. (1964), and recrystallized three times. Molar concentrations are given as monomer concentrations based on a molecular weight of 100 000/monomer (Cohen et al., 1971). There is one specific binding site for pyridoxal-5′-P and 5′-AMP per phosphorylase *b* monomer (Kastenschmidt et al., 1968a,b; Cohen et al., 1971). Phosphorylase *b* was freed of 5′-AMP either by passage over activated charcoal or Sephadex G-25 fine grade. The A_{260}:A_{280} ratio of the 5′-AMP free enzyme at pH 7 was ≤0.53. Apophosphorylase *b* was prepared by the procedure of Shaltiel et al. (1966) and reconstituted with 3-*O*-methylpyridoxal-5′-P for 120 min at 25 °C in 50 mM 2-glycero-P, 40 mM 2-mercaptoethanol buffer, pH 7.0. Reconstitution was complete with 0.093 mM apophosphorylase *b* at a 1:1 molar ratio. Addition of pyridoxal-5′-P did not result in further activation, making certain that reconstitution was completed. This was made possible because we had previously found that 3-*O*-methylpyridoxal-5′-P bound to phosphorylase *b* does not exchange with free pyridoxal-5′-P in 25 h at room temperature in glycero-P buffer, pH 6.8 (Pfeuffer et al., 1972b).

Activity Measurements. Initial velocities were measured in the direction of glycogen synthesis at 30 °C. Liberated P_i[2] was analyzed according to Fiske and Subbarow (1925). The reaction was started by enzyme addition. Activity assays were carried out in 100 mM sodium maleate, 20 mM 2-mercaptoethanol buffer, pH 6.5, containing 1% glycogen (corresponding to 5×10^{-3} M terminal nonreducing glucose residues), 100 mM glucose 1-phosphate, and 1 mM 5′-AMP. Phosphorylase *b* and 3-*O*-methylpyridoxal-5′-P phosphorylase *b* had under assay conditions specific activities of 85 and 36 μmol of P_i mg^{-1} min^{-1}, respectively. Protein concentrations were determined by absorbance measurements at 280 nm using an absorbance index, $E^{1\%}_{280}$ cm^{-1} = 13.2 (Kastenschmidt et al., 1968a).

Uv absorbance measurements were carried out with a Zeiss PMQ III or with a Cary 15 spectrophotometer. Temperature was kept constant by using jacketted thermostated cuvettes from Hellma. Protein samples were centrifuged at 30 000*g* for 20 min at 15 °C and kept dust free. Carefully matched and covered cuvettes were used. For comparing uv and ^{1}H NMR spectra concentrations were equalized by using 1-mm quartz cuvettes with inserted quartz plates to reduce the optical path to about 50 μm or less.

NMR Spectroscopy. ^{1}H NMR measurements were carried out either in D_2O or deuterated Me_2SO. The Schiff base derivatives of pyridoxal-5′-P and 3-*O*-methylpyridoxal-5′-P were prepared directly in the spectrometer tubes by adding 20 μl of freshly distilled *n*-butylamine to 10^{-1}–10^{-2} M pyridoxal-5′-P or 3-*O*-methylpyridoxal-5′-P solutions. Measurements were carried out with a Varian A-60 using tetramethylsilane as an internal standard.

^{31}P NMR spectra were recorded at 72.8 MHz with a Bruker WH-180 FT wide-bore superconducting NMR spectrometer. The measurements were carried out with 12 ml samples containing $3–5 \times 10^{-4}$ M enzyme in NMR tubes with a 20-mm outer diameter, and a concentric 5 mm insert containing 99% D_2O for the deuterium lock and in some cases triethyl phosphate as reference. For noise-modulated ^{1}H decoupling a narrow bandwidth (about 200 Hz) at 0.5 W was used. Data were acquired with the BNC-12 computer system, using quadrature detection and autoscan mode. The exponential multiplication (EM routine) uses time constants (TC) which are negative with decreasing exponential decay time. The decay time of exponential multiplication (sensitivity enhancement) is $T_{SE} = -AT/TC$. AT is the acquisition time. Line broadening is $\Delta\nu_{SE} = -TC/(\pi AT)$. Phosphorylase spectra were usually seen in each of the two channels at 1800 Hz spectral width and 1K data points. Acquisition time was 0.34 s, pulse width 30 μs (60° pulse angle), and line broadening 17 Hz. Transients (30 000–100 000) were collected for each spectrum requiring 3–9 h. Phosphorylase activity decayed less than 10% after 9 h of measurement. The spectra of the cofactor not bound to phosphorylase were recorded at a spectral width of 600 Hz and with 4K data points. Acquisition time was 6.8 s and pulse width 20 μs (40°); 16–25 transients were required for 2 mM concentrations of cofactor. Temperature was maintained at 30 ± 1 °C by air flow through the probe head and around the shim coils.

Materials. Pyridoxal and pyridoxal-5′-P were products of E. Merck AG. Pyridoxal-5′-P monomethyl ester was prepared according to Pfeuffer et al. (1972a). 3-*O*-Methylpyridoxal-5′-P was prepared from pyridoxal-5′-P by an unpublished four-step procedure (M. Viehhauser and K. Feldmann, 1974). 5′-AMP and glucose-1-P were products of Boehringer and Sons. Oyster glycogen was purchased from Merck. It was further purified and freed of nucleotides as described by Helmreich et al. (1967). Maleate, 2-glycero-P, ε-aminocaproic acid, and *n*-butylamine were obtained from Merck, Sephadex was from Pharmacia, and activated charcoal was from Merck; the latter was further purified by treatment with HCl and EDTA. N^{α}-Acetyllysine and polylysine were from Sigma, and morpholinopropanesulfonic acid and 2-mercaptoethanol were from Serva. D_2O, dimethyl-d_6 sulfoxide, and Me_4Si were from Roth, Karlsruhe. All other chemicals were of the highest grade commercially available. Doubly quartz distilled water was used throughout.

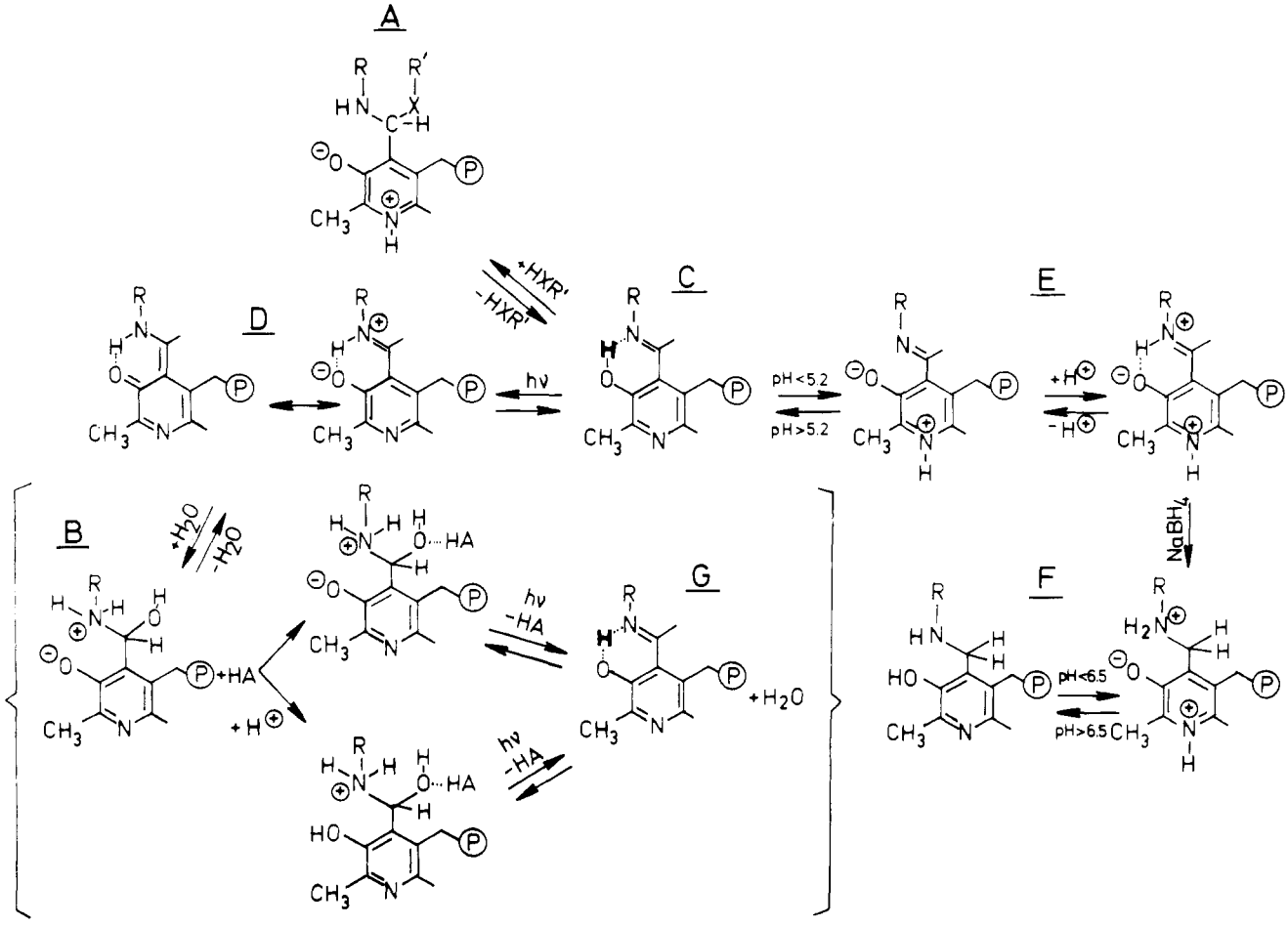
Results

Structure of Pyridoxal-5′-P in Phosphorylase. In Scheme I are summarized the structures of pyridoxal-5′-P under discussion. The absorbancies of various structures are in Table I. In the original proposal of Kent et al. (1958) pyridoxal-5′-P is bound to phosphorylase as an aldimine linked to an unknown nucleophilic group X(A). This group could also be a hydroxyl group of water forming a carbinolamine (B) as pointed out by Honikel and Madsen (1972). Johnson et al. (1970), Shaltiel and Cortijo (1970), and Cortijo et al. (1971) proposed that the catalytically active form is the Schiff base (C) absorbing at 333 nm and forming the tautomeric imines (D). Typical Schiff base absorbance at 415 nm is generated on perturbation (i.e.: low pH, NaCl in the cold, etc.) of the phosphorylase structure which makes bound pyridoxal-5′-P accessible to aqueous solvent (C → E). The exposed azomethine linkage can now be reduced with sodium borohydride (F) (Fischer et al., 1958; Graves et al., 1965; Strausbauch et al., 1967). The carbinolamines (B) form on excitation and removal of water Schiff bases (G) which absorb at 425 nm (Honikel and Madsen, 1972). The different location of the chelating proton (H) in C and G reflects different views on the energy potentials (C is Johnson et al., 1970, and G is Honikel and Madsen, 1972) (cf. Daltrozzo et al., 1973).

3-*O*-Methylpyridoxal-5′-P bound to phosphorylase does not absorb at neutral pH at 333 nm where the natural cofactor pyridoxal-5′-P with a protonated 3-hydroxyl group would be expected to absorb; instead, the absorbance of the 3-*O*-methyl analogue is hidden under the protein absorbance and appears at 297 nm when it was set off against apophosphorylase *b*

[2] Abbreviations used are: AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-O-monothiophosphate; P_i, inorganic phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

SCHEME I: Proposed Structures for Phosphorylase-Bound Pyridoxal-5′-P and Pyridoxamine-5′-P.



(Figure 1). The skewed absorption peak of the bound cofactor analogue could reflect a conformational difference between apophosphorylase and reconstituted phosphorylase (see Figure 1). Bartlett and Graves (1970) have reported spectral differences between phosphorylases *b* and *a*. The absorption band was not skewed in the case of 3-*O*-methylpyridoxal-5′-P bound to serum albumin.

A shift of the thiazolidine derivative of 3-*O*-methylpyridoxal-5′-P to shorter wavelength is apparent in Figure 1. The hydrated and the hemimercaptal forms of 3-*O*-methylpyridoxal-5′-P absorb at 278 nm (see Table I). This is in contrast to the pyridoxal-5′-P-thiazolidine derivative and native phosphorylase *b* which both absorb at 333 and 325 nm, respectively. The absorption of this derivative was assumed to support an aldamine structure of pyridoxal-5′-P in phosphorylase. Furthermore, there are differences in the binding of 3-*O*-methylpyridoxal-5′-P and pyridoxal-5′-P to phosphorylases. Whereas pyridoxal-5′-P remains bound to phosphorylase even below pH 5 as yellow Schiff base absorbing at 415 nm, 3-*O*-methylpyridoxal-5′-P is readily detached from the enzyme at acid pH or on precipitation with $(NH_4)_2SO_4$. Moreover, 3-*O*-methylpyridoxal-5′-P does not bind to aspartate aminotransferase (EC 2.6.1.1) (Furbish et al., 1969) and to D-serine dehydratase (EC 4.2.1.14) (Dowhan and Snell, 1970). This suggested that 3-*O*-methylpyridoxal-5′-P only binds to a hydrophobic site. Dempsey and Christensen (1962) had shown that bovine serum albumin, a rather hydrophobic protein with many ε-aminolysyl groups, binds pyridoxal-5′-P at neutral pH first as a typical Schiff base absorbing at 415 nm. After a few minutes the 415 nm band disappeared and a new band at 333 nm appeared. In contrast to pyridoxal-5′-P the 3-*O*-methyl derivative binds to bovine serum albumin only in one form which absorbs at 300 nm. Furthermore, 3-*O*-methylpyridoxal-5′-P can barely form Schiff bases with lysyl amino groups in a polar aqueous medium at neutral pH: a difference spectrum was taken with 7×10^{-4} M concentrations of 3-*O*-methylpyridoxal-5′-P and 2×10^{-4} M poly-L-lysine of mol. wt. 15 000 in phosphate buffer at pH 7.0, which indicated that less than 1 mol of analogue was bound per 15 000 g. Its absorbance maximum was assigned on the basis of the difference spectrum to 282 nm suggesting that 3-*O*-methylpyridoxal-5′-P was bound rather as carbinolamine than as a Schiff base. 3-*O*-Methylpyridoxal-5′-P bound barely measurably to the ε-amino group of N^{α}-acetyllysine or to *n*-butylamine in phosphate buffer, pH 7.0. Thus, 3-*O*-methylpyridoxal-5′-P at reasonable concentrations cannot form Schiff bases with solvent-exposed ε-aminolysyl side chains. An apolar environment should favor Schiff base formation with pyridoxal-5′-P even more than with 3-*O*-methylpyridoxal-5′-P because alkylation of the 3-hydroxyl group would be expected to hinder Schiff base formation in the planar geometry.

The data summarized in Table I show how difficult an unequivocal structural assignment of the 330-nm absorbance of pyridoxal-5′-P bound to phosphorylase is by comparison with pyridoxal-5′-P-*n*-alkylamine Schiff base absorbancies: although the absorbance of the model Schiff bases can be assigned to the sp^2 hybrid and the phenolic form, the 330-nm absorbance of phosphorylase could either be that of the sp^3 phenolate form, as in reduced phosphorylase, or of the sp^2 phenolic form. The former would correspond to an aldamine

and the latter to a Schiff base in the phenolic form. With the 3-*O*-Me analogue the phenolate form can be eliminated. But it becomes evident that a spectral distinction between sp^2 and sp^3 hybrids of the phenolic forms is ambiguous because of the similar (290 nm) absorbance of the sp^3 phenolic form of reduced phosphorylase. sp^2 and sp^3 hybrid states are, however, readily distinguished by proton resonance spectroscopy.

In Figure 2A are compared the uv absorption spectra of the phenolate forms of free pyridoxal-5′-P and its *n*-butylamine Schiff base with that part of the proton resonance spectrum which contains the signal for the hydrogen on the carbon bonded to C-4. The hydrogen is shown in bold face type. On Schiff base formation in D_2O resonance shifts to 8,9 ppm. This may be compared with measurements of a pyridine-4-carboxaldehyde Schiff base derivative and the corresponding adduct with 1,3-diaminopropane (O'Leary, 1971). In Me_2SO the cofactor is in the phenolic form and, accordingly, the absorption maxima of pyridoxal-5′-P and its reaction product with *n*-butylamine shift to shorter wavelengths (~340 nm and below 333 nm), but the chemical shift of the proton resonance for the Schiff base derivative is the same in Me_2SO as in D_2O, indicating that the phenolic form of pyridoxal-5′-P can also form a Schiff base (compare Figure 2A with 2B). This interpretation is strengthened by the data in Figure 2C, where the uv and ^1H NMR spectra of the hemiacetal form of free pyridoxal and of the corresponding *n*-butylamine Schiff base in Me_2SO are compared: The free pyridoxal hemiacetal absorbs at 291 nm (see also Metzler et al., 1973). According to Morozov et al. (1967) the hydrated form of the phenolic pyridoxal-5′-P absorbs at 295 nm. The proton signal for the sp^3 hybrid of the pyridoxal hemiacetal is observed at 6, 7 ppm. In the carbinolamine it would be expected below 6 ppm. The pyridoxal-*n*-butylamine Schiff base, however, has the same uv absorbance and proton resonance around 9 ppm as the Schiff base with pyridoxal-5′-P (compare Figure 2C with 2B). This indicates that pyridoxal likewise formed a Schiff base and not a carbinolamine. Uv- and ^1H NMR spectra of 3-*O*-methylpyridoxal-5′-P and the *n*-butylamine Schiff base in Me_2SO are shown in Figure 2D. A comparison with the pyridoxal-5′-P Schiff base in Figure 2B reveals a large spectral shift to shorter wavelengths (340 nm → 295 nm). Since the proton resonance spectra show the same hydrogen shift, the phenolic form of 3-*O*-methylpyridoxal-5′-P had likewise formed a Schiff base. Thus, the comparison of uv absorbance spectra with ^1H NMR spectra proves the existence of the phenolic forms of pyridoxal, pyridoxal-5′-P, and 3-*O*-methylpyridoxal-5′-P Schiff bases in Me_2SO.

The "abnormal" 333-nm absorbance of the pyridoxal-5′-P Schiff base in phosphorylase is therefore explained by the hydrophobic binding domain where the phenolic form dominates. In the case of 3-*O*-methylpyridoxal-5′-P binding to phosphorylase additional points are pertinent: the shift to still shorter wavelengths is probably in part due to the unprotonated pyridine nitrogen. This follows from the ionization scheme of 3-hydroxypyridines of Metzler and Snell (1955); see also Pocker and Fischer (1969). However, since a shift was also seen with the *o*-methoxybenzaldehyde Schiff base with n-hexylamine when compared with the corresponding salicylaldehyde Schiff base (Veinberg et al., 1974), the 3-*O*-methyl group probably distorts the azomethine linkage, thus affecting the resonance system. This distortion could also give rise to the syn form assumed by Veinberg et al. (1974).

Protonation of the 5′-Phosphate Group of Pyridoxal-5′-P. 3-*O*-Methylpyridoxal-5′-P phosphorylase is active, although the 3-OH group is unprotonatable. This excludes the attractive possibility of a proton shuttle between the 3-hydroxyl group and the pyridine nitrogen of pyridoxal-5′-P (cf. Ahrens et al., 1970) and directs attention to a role of the 5′-phosphate group of the cofactor in catalysis. The phosphate group pK_2 is blocked in the pyridoxal-5′-P monomethyl ester. This analogue forms with apophosphorylase *b* an inactive holoenzyme with 5′-AMP controlled quaternary and tertiary structures like active phosphorylase (Pfeuffer et al., 1972a,b; Weisshaar and Palm, 1972; Feldmann et al., 1972, 1974; Feldmann, unpublished experiments, 1975). Thus, the 5′-phosphate group may not only serve as a structural ligand but its protonation–deprotonation may be necessary for catalysis.

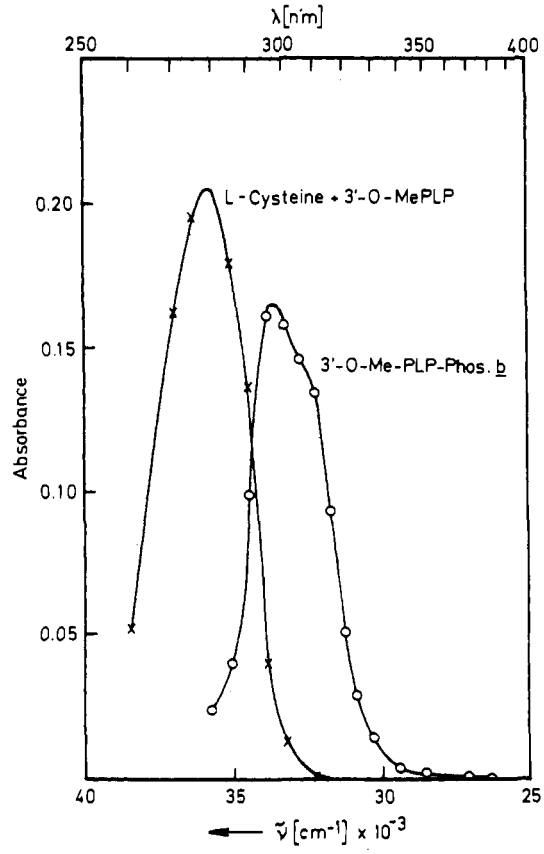


FIGURE 1: Absorption spectra of 3-*O*-methylpyridoxal-5′-P-phosphorylase *b* and the thiazolidine derivative of 3-*O*-methylpyridoxal-5′-P. Measurements with the phosphorylase *b* derivative in 50 mM 2-glycero-P, 40 mM 2-mercaptoethanol buffer, pH 7.0, at room temperature. The thiazolidine derivative was formed with 100 mM cysteine and 0.42 mM 3-*O*-methylpyridoxal-5′-P in 50 mM glycero-P, pH 7, and measured in a 1 mm cuvette.

A titration curve of the 5′-phosphate of pyridoxal-5′-P in 100 mM *N*-morpholino-3-propanesulfonic acid, 2 mM EDTA-Na buffer was derived from ^{31}P NMR measurements and is given in Figure 3. The pK_2 of pyridoxal-5′-P and the ε-aminocaproate Schiff base is 6.2, as expected (cf. Pfeuffer et al., 1972b). Differences between free pyridoxal-5′-P and the Schiff base derivative presumably reflect the equilibrium between hydrated and unhydrated pyridoxal-5′-P. As control, the ^{31}P resonance of the monomethyl ester of pyridoxal-5′-P was measured and a few measurements were made with that analogue bound to phosphorylase *b* which are not included in Figure 3. The measurements showed an upfield shift, but as in the case of the free analogue the protonation of the phosphate did not change in the pH range studied.

In Figures 4A–D we compare the ^{31}P NMR spectra of pyridoxal-5′-P in phosphorylase *b* in the absence of ligands (A),

Table I: U V ABSORPTION MAXIMA OF PYRIDOXAL - 5'-P DERIVATIVES

The reaction products are in parentheses. Measurements in aqueous solutions unless specified

COFACTOR	COFACTOR REACTED WITH						
	L-cysteine (Thiazolidine)	2-mercapto-ethanol (Hemimercaptal)	H_2O (Hydrate)	Apophos. b and reduced with $NaBH_4$ (Red. Phos. b. Secondary amine)	n-alkylamine (Schiff's base)	Bov. serum albumin	Apophos b (Native Phos.b)
	[nm]	[nm]	[nm]	[nm]	[nm]	[nm]	[nm]
Pyridoxal-5'-P as phenolate	325 a)	322 b)	325 c)	333 d)	415 e)	415 f)	415 g)
Pyridoxal-5'-P in the phenolic form	297 h)	288 h)	295 c)	290	333 h) 335 e)	332 f)	333 g)
3-CH_3-O-pyridoxal-5'-P	278 h)	277 h)	278 i)	—	295 h)	300 i)	297 i)

a) Buell and Hansen (1960); b) Schuster and Winkler (1970); c) Morazov et al. (1967), Metzler et al. (1973); d) cf.: Feldmann et al. (1974); e) Shaltiel and Cortijo (1970), Arnio-Dupont (1971); f) Dempsey and Christensen (1962); g) Kent et al. (1958); h) in dimethylsulfoxide; Measurements a-c, f, i at pH 7 and d, g at pH 5 - pH 7. Measurements e in dioxane-water.

in the presence of 100 mM arsenate (B), in the presence of 0.8 mM 5'-AMP and 100 mM arsenate (C), and with 100 mM NaCl and 0.88 mM 5'-AMP (D). Phosphorylase *b* activity has a nearly absolute dependency on 5'-AMP (Helmreich and Cori, 1964a,b). Arsenate was used as anionic substrate instead of phosphate because the latter bound to phosphorylase gives a strong signal which interferes with the phosphate signal of pyridoxal-5'-P. Arsenate can replace phosphate as substrate (Helmreich and Cori, 1964b). Arsenolysis cleaves α-1-4-glycosidic bonds in glycogen, yielding glucose 1-arsenate which is readily hydrolyzed to glucose and arsenate (Katz and Hassid, 1951). Arsenate has an affinity for phosphorylase *b* which is similar to that of phosphate, but V_{max} with arsenate and phosphorylase *b* is only about one-fifth that with phosphate under comparable conditions and at 50 mM concentrations of anionic substrates in the assay mixture described in Materials and Methods (but without glucose-1-P). The ^{31}P NMR measurements in Figure 4 were carried out in the pH range 7.2–7.6. Since the ionic strength varied considerably in the different experiments, control experiments with 100 mM NaCl were carried out. Addition of NaCl had a slight effect on the phosphor resonance spectra of phosphorylase *b* (Figure 4D). Comparing Figure 4A with 4B and 4D and Figure 3 shows that addition of either 5'-AMP (D) or arsenate (B) shifted the ^{31}P signal of pyridoxal-5'-P by about 30 Hz downfield. The accuracy of the measurements was about 10 Hz. Addition of both the allosteric effector and the anionic substrate shifted the phosphorus signal of phosphorylase-bound pyridoxal-5'-P about 200 Hz or more downfield (compare Figure 4C with 4A, B, and D). It now overlaps with the phosphate band of bound 5'-AMP which is broadened but cannot be resolved into individual components. (Compare Figure 4C and D). The sharp peak on top is that of free 5'-AMP (Figure 4C and D). We cannot exclude that the AMP peak in the experiments with phosphorylase *b* (Figure 3) and in the presence of 100 mM NaCl (Figure 4D) may include signals from the bound pyridoxal-5'-P in the diionized form. In more recent experiments this difficulty was overcome by using adenosine 5'-*O*-monothiophosphate (AMPS) which activates phosphorylase *b* more effectively than 5'-AMP (Murray and Atkinson (1968)). The thiophosphate signal is shifted by more than 3000 Hz downfield and hence does not overlap with the phosphate signal of pyridoxal-5'-P bound to phosphorylase *b* (Feldmann, K., and Hull, W. E. (1976), manuscript in preparation).

The chemical shifts observed with holophosphorylase *b* are interpreted to mean that the 5'-phosphate group of bound pyridoxal-5'-P is unresponsive to external pH because it is tucked away in the hydrophobic binding domain; but it is deprotonated at pH 7.6 on interaction with the allosteric effector, 5'-AMP, and the anionic substrate arsenate (compare Figure 4C with 4A, B, and D and Figure 3). We have not yet refined our measurements to quantitate this effect, but it would appear that 80% or more of the 5'-phosphate of the cofactor becomes deprotonated under these conditions. A quantitative study of the concentration dependence and the time relationship of the effects of allosteric effector and substrate is in progress; this should give interesting information on the chemical mechanism of allosteric activation of phosphorylase *b*.

Discussion

According to the available information in a recent comprehensive review of Davis and Metzler (1972) the majority of pyridoxal-5'-P containing enzymes absorb at 360 nm at

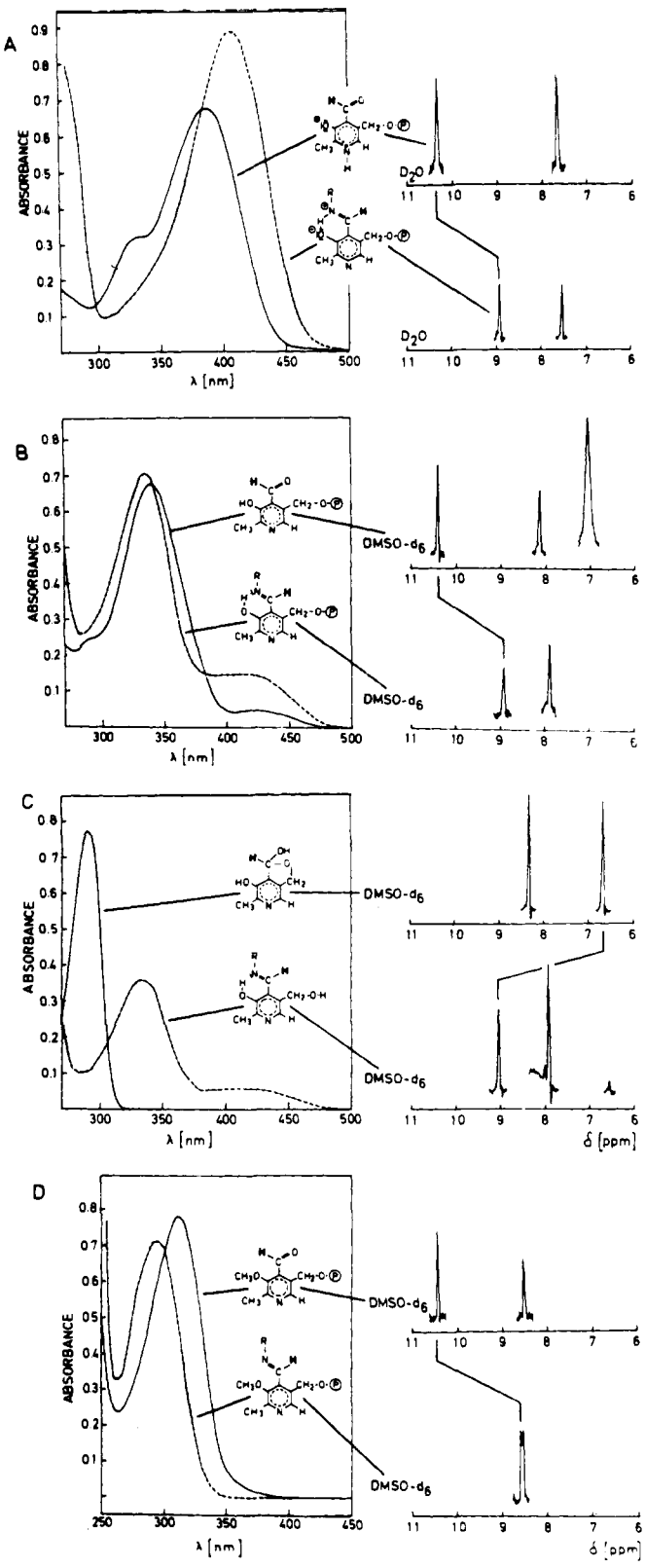


FIGURE 2: Uv and ^1H NMR spectra of free pyridoxal-5′-P and derivatives. Complete NMR spectra of pyridoxal-5′-P and pyridoxal in D_2O with the assignments of the protons are given by Korytnik and Ahrens (1970). Here are only marked the resonance bands for the C_4 bonded hydrogens (see text).

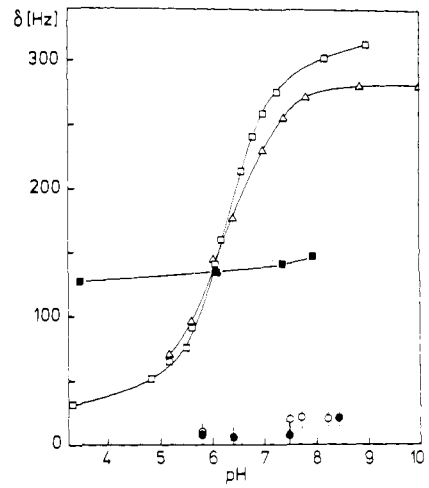


FIGURE 3: pH titration of pyridoxal-5′-P and derivatives derived from ^{31}P NMR measurements. -□—□-, free pyridoxal-5′-P (5 mM); -Δ—Δ-, pyridoxal-5′-P Schiff base with ϵ-aminocaproate: ϵ-aminocaproate (100 mM) was reacted with 2 mM pyridoxal-5′-P. -■—■-, pyridoxal-5′-P monomethyl ester (2 mM); -●—●-, pyridoxal-5′-P in phosphorylase *b*; -○—○-, pyridoxal-5′-P in phosphorylase *b* in the presence of 1 mM 5′-AMP (see text). All measurements at 30 °C in 100 mM *N*-morpholino-3-propanesulfonate 2 mM EDTA-Na buffer; pH was adjusted with NaOH or HCl. In the case of phosphorylase *b* 50 mM 2-mercaptoethanol was added to the buffer.

higher pH and at 420 nm at lower pH. Both forms are tautomers of Schiff bases and catalytically active. But there exist enzymes with the typical pyridoxal-5′-P Schiff base absorbance at 420 nm at low pH which absorb at shorter wavelengths, between 330 and 340 nm at higher pH. For some of these enzymes, aldamine structures have been proposed (O'Leary, 1971; Yonaha et al., 1975). But interestingly, in those pyridoxal-5′-P dependent enzymes where the 330–340 nm absorbing forms are active, the cofactor appears to be bound as a Schiff base. This is the case with glycogen phosphorylase and *E. coli* tryptophanase. With the latter enzyme Morino and Snell (1967) could demonstrate, at pH 8.0 and in the presence of K^+, a transition from an enzymatically inactive form with a λ_{max} of 420 nm to an active form with a λ_{max} of 337 nm. Since both forms were completely inactivated by borohydride reduction, the cofactor is bound in both cases by an azomethine linkage to apotryptophanase. Moreover, in tryptophanase a conformational change at the active site was made responsible for the formation of active and inactive enzyme. Snell (1975) suggested that the shift toward shorter wavelengths on deprotonation of tryptophanase could result from a hydrophobic and uncharged environment with retention of the hydrogen bond. This corresponds to the explanation given for the structure of pyridoxal-5′-P in phosphorylase.

The ^{31}P NMR titration of free pyridoxal-5′-P corresponds to that recently published by Martinez-Carrion (1975). The chemical shifts of the phosphorus nucleus of pyridoxal-5′-P bound to rabbit skeletal muscle phosphorylase *b* and that bound to cytoplasmic pig heart aspartate aminotransferase (Martinez-Carrion, 1975) are different. Although, in contrast to free pyridoxal-5′-P, the chemical shift of the bound cofactor was insensitive in both enzymes to pH changes in the stability range of the enzymes between pH 5.6 and 8.4, in the transaminase it corresponded to the diionized form but in phosphorylase to the protonated form of pyridoxal-5′-P. The unresponsiveness to external pH was interpreted in the case of the transaminase as shielding of the phosphate of the bound cofactor. Lysine or arginine residues, whose ionization constants are outside the pH range studied, were assumed to form with pyridoxal-5′-P highly stable complexes. No significant changes occurred in the chemical shifts of the ^{31}P resonance of transaminase-bound pyridoxal-5′-P on addition of the

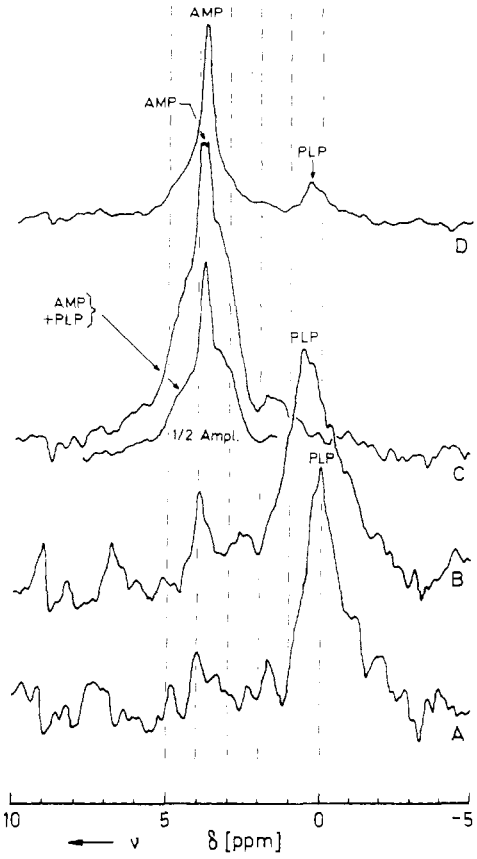


FIGURE 4: ^{31}P NMR spectra of phosphorylase *b*. Measurements carried out at 30 °C in *N*-morpholinopropanesulfonate buffer containing 2-mercaptoethanol as described in the legend to Figure 3. A; 0.44 mM phosphorylase *b*, pH 7.2, 30 000 transients (see Materials and Methods). B; 0.4 mM phosphorylase *b*; pH shifted to 7.6 on addition of 100 mM arsenate, 76 000 transients. C; 0.4 mM phosphorylase *b*, 100 mM arsenate and 0.8 mM 5′-AMP, pH 7.6, 40 000 transients. The amplification of the curve in the insert is reduced by a factor of 2. D; 0.44 mM phosphorylase *b*, 100 mM NaCl and 0.88 mM 5′-AMP, pH 7.3, 30 000 transients.

substrate glutamate and the substrate analogue 2-methylaspartate and of 2-oxoglutarate which forms abortive complexes with this enzyme. The participation of the phosphate group of pyridoxal-5′-P in transaminase-catalyzed reactions (Furbish et al., 1969) was thus considered unlikely (Martinez-Carrion, 1975). In phosphorylase *b*, in contrast to aspartate transaminase, interaction with specific ligands, 5′-AMP and an anionic substrate, caused deprotonation of the phosphate of pyridoxal-5′-P. The smaller effect observed with 5′-AMP alone might reflect the relatively weak binding of 5′-AMP to phosphorylase in the absence of substrates. The role of the anionic substrate would then be to increase the affinity of 5′-AMP to the enzyme which by a conformational change alters the ionization of the phosphate group of pyridoxal-5′-P. Although k_{on} and k_{off} of 5′-AMP and phosphorylase *b* have not been measured (see also Feldmann et al., 1974), 5′-AMP has an apparent K_a value for phosphorylase *b* activation of 3–5 $\times 10^{-4}$ M without phosphate and 4×10^{-5} M with phosphate (Helmreich and Cori, 1964a). A similar relationship would be expected for arsenate. But the smaller V_{max} of phosphorylase *b* with arsenate compared to phosphate commands caution in relating the effect of arsenate to its function as anionic substrate. Additional experiments are needed to see whether or not other multivalent anions, for example sulfate, which cannot substitute for phosphate as substrate, are equally effective (cf. Kastenschmidt et al., 1968b). The broadening of the 5′-AMP peak on addition of arsenate (compare Figure 4D with 4C) was correlated with deprotonation of the 5′-phosphate group of the coenzyme, but this does not yet prove that the conformational change on binding of allosteric effector and anionic substrate is essential for catalysis or that the proton removed from the phosphate group of pyridoxal-5′-P actually participates in catalysis. But Feldmann and Hull recently showed that the 5′-phosphate group of pyridoxal-5′-P in phosphorylase *a*, prepared with adenosine-5-O-γ-monothiotriphosphate (ATPγS) according to Gratecos and Fischer (1974), was deprotonated at pH 7.6 even in the absence of 5′-AMP. In contrast to phosphorylase *b* the thiophosphorylated form is active in the absence of the allosteric nucleotide (Gratecos and Fischer, 1974). Hence, this recent finding gives confidence that the change in the ionization of the 5′-phosphate group of the cofactor on binding of 5′-AMP and arsenate to phosphorylase *b* reported in this paper, is indeed significant (cf. Feldmann, K., and Hull, W. E. (1976), manuscript in preparation; Feldmann et al. (1976)).

As this work was in progress (cf. Feldmann et al., 1974; and paper presented by E. J. M. Helmreich at the joint US–USSR symposium on biological pyridoxal catalysis, Leningrad, Aug 16–23, 1974) a report by Busby et al. (1975) appeared. The Oxford group observed a signal arising from the phosphorus of pyridoxal-5′-P in phosphorylase *b* made up of more than one component. The spectra were recorded at 129 MHz and at 36.43 MHz. The width of the resonance band was ca. 900 Hz at 129 MHz and 150 Hz at 36.43 MHz. From these data a line width in between would have been expected in our measurements since they were carried out at 72.8 MHz, but the line width was between 45 and 70 Hz. This difference might be related to different experimental conditions and instrumentation: Busby et al. worked with up to 2 mM concentrations of phosphorylase *b* in 50 mM triethanolamine HCl, 100 mM KCl, 1 mM EDTA buffer, pH 7.78 at 20 °C whereas in our work only one-fifth to one-tenth of this concentration was used at 30 °C. Furthermore, we have taken special care to remove 5′-AMP from the enzyme. Busby et al. (1975) have tentatively assumed that the two components of the signal observed correspond to two ionization states of the phosphate group of pyridoxal-5′-P. Addition of 5′-AMP or the inhibitor glucose-6-P reduced the line width of the pyridoxal-5′-P signal. The major portion of the phosphorus resonance from pyridoxal-5′-P in the 5′-AMP-phosphorylase *b* complex was allocated to the monoanion frequency. Hence, it was assumed that the two signals represent an equilibrium between two slowly interchanging conformations which is shifted on addition of 5′-AMP and glucose-6-P. Since we have not seen a two-component signal of pyridoxal-5′-P in phosphorylase *b* regardless of whether or not ligands were bound (see Figure 4), although our resolution was about ten times greater than that of Busby et al. (1975), we cannot support this assumption. Additional experiments should resolve the discrepancies in experimental observations and consequent interpretations.

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tions. This paper is dedicated to F. Lynen on the occasion of his 65th birthday.

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