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Role of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase. II. Mode of Binding of Pyridoxal 5′-Phosphate and Analogs of Pyridoxal 5′-Phosphate to Apophosphorylase *b* and the Aggregation State of the Reconstituted Phosphorylase Proteins†

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ABSTRACT: The inactive pyridoxal phosphate monomethyl ester is, like pyridoxal-P, nearly irreversibly bound to apophosphorylase *b*. Inactive pyridoxal phosphate monomethyl ester and active 3′-*O*-methylpyridoxal phosphate (and pyridoxal phosphate) bound to apophosphorylase *b* do not exchange with pyridoxal phosphate (or [4′-^{3}H]pyridoxal phosphate). Enzyme-bound 3′-*O*-methylpyridoxal phosphate *N*-oxide and pyridoxal are slowly displaced by pyridoxal phosphate. Differences in the reactivity of the C_4–azomethine bond which links pyridoxal phosphate to a lysyl residue in the phosphorylase protein were found in the case of pyridoxal phosphate analogs. At neutral pH and in the absence of deforming agents such as imidazole citrate the inactive pyridoxal phosphate monomethyl ester and pyridoxal phosphate bound to phosphorylase *b* were least attacked by $NaBH_4$ and L-cysteine. Pyridoxal, 3′-*O*-methylpyridoxal phosphate, pyridoxal phosphate *N*-oxide, and 3′-*O*-methylpyridoxal phosphate *N*-oxide were more reactive. The apparent p*K* values of the protonatable groups of pyridoxal and pyridoxal phosphate respectively were determined in a milieu approximating that of the hydrophobic pyridoxal phosphate binding site in phosphorylase. The apparent pK_2 value of the 5′-phosphate group of pyridoxal phosphate increased from 6.2 in water to 9.3 in 80% dioxane–water. Therefore, the 5′-phosphate group (pK_2) of pyridoxal phosphate in phosphorylase must project to a more polar environment, if it is to function as proton donor–acceptor group in phosphorylase catalysis. The aggregation state of holophosphorylase reconstituted from apophosphorylase *b* with pyridoxal phosphate and both active and inactive analogs of pyridoxal phosphate was compared by polyacrylamide gel electrophoresis either with or without prior reaction with dimethyl suberimidate. The pyridoxal phosphate monomethyl ester maintained (up to 30°) a quaternary structure which was indistinguishable by these criteria from that of active holophosphorylase *b*. At still higher temperatures (35°), the pyridoxal phosphate monomethyl ester phosphorylase *b* was less stable than pyridoxal phosphate phosphorylase *b*. The amount of cross-linked oligomers formed with dimethyl suberimidate indicates a high degree of structural homology and complementarity of the contact surfaces of the subunits of phosphorylase *b*.

Nature may have found it advantageous to utilize protonatable groups of a vitamin rather than those of amino acid side chains to facilitate catalysis. If pyridoxal-P[1] should have such a function in glycogen phosphorylase one would expect its position in the catalytic site to be as strictly determined as that of a particular amino acid residue in the covalent structure of the protein. The optimum topography of the catalytic region in an enzyme is preserved by a genetic translation mechanism which operates with a high degree of fidelity. X-Ray crystallography of lysozyme, and of other enzymes, has taught us that the exact arrangement of protonatable groups at hy-

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[1] Abbreviations of vitamin B_6 analogs follow IUPAC–IUB rules (1966, 1970) (see Pfeuffer *et al.*, 1972); FDNB, fluoro-2,4-dinitrobenzene; glycogen phosphorylase (EC 2.4.1.1), α-1,4-glucan:orthophosphate glucosyltransferase (EC 2.4.1.1).

drophobic substrate binding regions is the clue to the high efficiency of enzyme catalysis (Phillips, 1967; *cf.* Dickerson and Geis, 1969). Large parts of the protein apparently only help to stabilize and maintain the conformation of a relatively small active site. Thus, the fit of a prosthetic group to an enzyme and the resulting conformational stabilization are prerequisites for its catalytic function. Therefore, if one wishes to substantiate experimentally the idea that pyridoxal-P actually participates as a proton shuttle in the breaking or forming of α-1,4-glycosidic bonds (the reaction catalyzed by all α-glucan phosphorylases) one must convincingly show that inactive analogs of PLP with at least one of the protonatable groups blocked still fit into the specific site exactly like the active cofactor. With this in mind, the experiments reported in this paper were undertaken.

The binding of pyridoxal-P and analogs of pyridoxal-P to phosphorylase *b* was studied by competitive inhibition of reactivation and exchange of enzyme-bound cofactor. In order to learn more about the mode of binding of pyridoxal-P and pyridoxal-P analogs with blocked protonatable groups, the reactivity of the C_4–azomethine bond which links pyridoxal-P to an ϵ-aminolysyl group in the protein was determined. This bond was attacked by nucleophilic reagents such as $NaBH_4$ and L-cysteine under conditions that did not perturb the protein conformation.

Since pyridoxal-P appears to be bound to a highly hydrophobic region in phosphorylase (Shaltiel and Cortijo, 1970; Fischer *et al.*, 1970), the change in ionization of the 3-OH, the pyridinium N, and the 5′-phosphate moiety of pyridoxal and pyridoxal-P respectively on transfer from aqueous to dioxane–water solutions was determined. This information bears on the role that the protonatable groups of pyridoxal-P may play in catalysis by glycogen phosphorylases.

The aggregation state of the phosphorylase proteins carrying inactive pyridoxal-P analogs was compared by polyacrylamide gel electrophoresis with that of active phosphorylases. Finally oligomeric phosphorylases reconstituted from apophosphorylase *b* and active and inactive pyridoxal-P analogs were reacted with the bivalent reagent, dimethyl suberimidate, according to Davies and Stark (1970). The quantities of cross-linked subunits were compared as a measure of structural homology.

Materials and Methods

The source of the enzymes, coenzymes, and other materials is given in the preceding paper (Pfeuffer *et al.*, 1972). Enzyme molarity was calculated on the basis of a molecular weight of 200,000 for phosphorylase dimer (Cohen *et al.*, 1971).

Reagents. [^{3}H]$NaBH_4$ was a product of the Radiochemical Centre, Amersham, England (specific activity 110 Ci/mole). [4′-^{3}H]Pyridoxal-P was prepared following the procedure of Stock *et al.* (1966) and crystallized from water–acetone. The yield was 185 mg (37%). The specific activity was 5.5 Ci/mole. Dioxane was freshly distilled and freed from peroxides by passage over a short column of basic Al_2O_3. Dimethyl suberimidate was prepared according to Davies and Stark (1970). All other reagents were of the purest commercially available grade. Doubly distilled water was used throughout.

Inhibition by Analogs of Pyridoxal-P of the Reactivation of Apophosphorylase b with Either Pyridoxal-P or 3′-O-Methylpyridoxal-P. To apophosphorylase *b* at a final concentration of about 1 mg/ml (5×10^{-6} M), pyridoxal-P or 3′-*O*-methylpyridoxal-P was added at the molar ratios indicated in Figure 1. (There are two binding sites for pyridoxal-P per phosphorylase dimer of mol wt 200,000.) The inactive analogs, pyridoxal-P monomethyl ester or 3′-*O*-methylpyridoxal-P *N*-oxide, were added simultaneously with the active pyridoxal-P or 3′-*O*-methylpyridoxal-P at the concentrations indicated. Reconstitution was carried out in 50 mM glycerophosphate–50 mM 2-mercaptoethanol–2 mM EDTA buffer (pH 6.8) for 30 min at 30°. For activity measurements, the reconstituted enzyme was diluted with buffer to a final concentration of 50 μg/ml in the case of pyridoxal-P phosphorylase *b* and of 200 μg/ml in the case of 3′-*O*-methylpyridoxal-P phosphorylase *b* and assayed immediately in direction of glycogen synthesis (see Methods in Pfeuffer *et al.*, 1972). Incidentally, optimal reactivation of apophosphorylase *b* by 3′-*O*-methylpyridoxal-P at 30° and 60 min of incubation already occurred with equimolar concentrations. No further increase in specific activity resulted from reconstitution with an up to 50-fold molar excess of 3′-*O*-methylpyridoxal-P. This was unexpected (*cf.* Shaltiel *et al.*, 1969b).

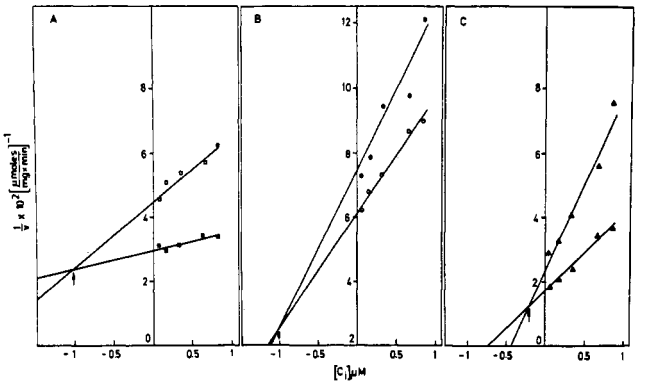


FIGURE 1: Competitive inhibition of reactivation of apophosphorylase *b* by 3′-*O*-methylpyridoxal-P *N*-oxide and pyridoxal-P monomethyl ester. Apophosphorylase *b* was reactivated by pyridoxal-P (A,C) or 3′-*O*-methylpyridoxal-P (B). The ordinate gives reciprocal specific activities. The abscissae in A and B give the concentration of 3′-*O*-methylpyridoxal-P *N*-oxide and in C the concentration of pyridoxal-P monomethyl ester. The inactive analogs were added in a 1- to 25-fold molar excess over binding sites. In B the 3′-*O*-methylpyridoxal-P which on reactivation gave an uninhibited specific activity of 21 μmoles of $P_i \times min^{-1} \times mg^{-1}$ was added at the following ratios of cofactor to binding sites: (●) 0.65 and (○) 0.75. In A and C the pyridoxal-P which on reactivation gave an uninhibited specific activity of 53 (A) and 57 (C) μmoles of $P_i \times min^{-1} \times mg^{-1}$ was added at the following ratios of cofactor to binding sites: In part A: (□) 0.45 and (■) 0.70; in C: (△) 0.75 and (▲) 1.0. The best fit to the straight lines was drawn by the method of least squares.

Exchange of Enzyme-Bound Analogs by [4′-^{3}H]Pyridoxal-P. Apophosphorylase *b* was reconstituted with a molar excess over binding sites with analogs of pyridoxal-P for 1 hr at 30° as described in (Pfeuffer *et al.*, 1972; Hedrick *et al.*, 1966). Unbound cofactors were removed by chromatography on Sephadex G-25 (fine grade) and enzyme-bound cofactor was determined by the method of Wada and Snell (1961). Although the analyses confirmed that the stoichiometrically expected amount of inactive cofactor was bound to the enzyme, in most cases a small and variable reactivation was observed within 5 min on addition of [4′-^{3}H]pyridoxal-P. The results given in Figure 2 are corrected for the amount of incompletely reconstituted apophosphorylase *b*. Exchange of inactive analogs by [4′-^{3}H]pyridoxal-P was followed at room temperature in sodium glycerophosphate–2-mercaptoethanol

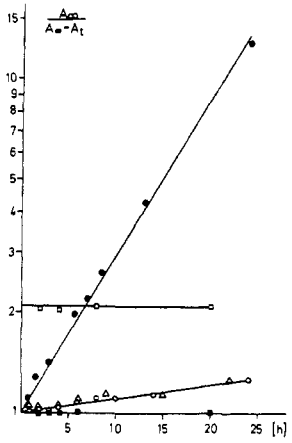


FIGURE 2: Exchange of enzyme-bound pyridoxal-P analogs by pyridoxal-P. A refers to the specific activity 57 μmoles of $P_i \times min^{-1} \times mg^{-1}$ of uninhibited pyridoxal-P phosphorylase *b*. (●) 3′-*O*-Methylpyridoxal-P *N*-oxide phosphorylase *b* (1.15×10^{-5} M); (○) pyridoxal phosphorylase *b* (1.68×10^{-5} M); (□) 3′-*O*-methylpyridoxal-P phosphorylase *b* (2.5×10^{-5} M); (■) pyridoxal-P monomethyl ester phosphorylase *b* (2.5×10^{-5} M). The concentration of pyridoxal-P was in each case twice that of the bound analog. Pyridoxal was also exchanged with [4′-^{3}H]pyridoxal-P (△) and radioactivity incorporation was determined. The time of incubation with pyridoxal-P is stated in the abscissa. The best fit to the straight lines was drawn by the method of least squares.

buffer (pH 6.8). At the times indicated in Figure 2, an aliquot (0.5 ml) was removed from the incubation mixture and passed over a Sephadex G-25 column (5 × 2 cm). Enzymatic activity and radioactivity were measured in aliquots of the eluate as described in Pfeuffer *et al.* (1972).

Reducibility of Pyridoxal-P Analogs Bound to Phosphorylase b by $NaBH_4$. Apophosphorylase *b* (3×10^{-5} M) was reconstituted in glycerophosphate buffer (pH 6.8) with 6.3×10^{-5} M pyridoxal-P or pyridoxal-P *N*-oxide and with 1.8×10^{-4} M pyridoxal-P monomethyl ester for 20 min at 30° as described in Pfeuffer *et al.* (1972). The concentrations of 3′-*O*-methylpyridoxal-P, 3′-*O*-methylpyridoxal-P *N*-oxide, and pyridoxal were 3.6×10^{-4} M and the time of reconstitution was 60 min. Unbound coenzyme was removed by gel filtration with Sephadex G-25. Reduction of the phosphorylases (3×10^{-5} M) with $NaBH_4$ was carried out in the same glycerophosphate buffer (pH 6.8) at 0°. To 1.5 ml of enzyme solution, six portions (50 μl each) of a [^{3}H]$NaBH_4$ solution in ice-cold water (0.6 mg of [^{3}H]$NaBH_4$/ml) were added stepwise under gentle stirring over a period of 3 hr. The reduced enzymes were again passed over a Sephadex G-25 column. The extent of reduction was checked in three independent ways. (1) Unreduced cofactor was determined by the method of Wada and Snell (1961). The values before reduction correspond to the total amount of enzyme-bound cofactor. (2) The extent of reactivation of the apoenzyme with pyridoxal-P was measured after removal of unreduced coenzyme by the method of Shaltiel *et al.* (1966). Enzymatic activity was measured in the routine assay (*cf.* Pfeuffer *et al.*, 1972). (3) The reduced enzyme-bound cofactor was determined from the protein-bound radioactivity.

Reactivity of Enzyme-Bound Pyridoxal-P and Analogs of Pyridoxal-P toward L-*Cysteine.* Apophosphorylase *b* was reconstituted with pyridoxal-P or analogs of pyridoxal-P as described in the preceding section. The reconstituted phosphorylases (0.8 ml containing 5–6 mg/ml of enzyme) were dialyzed in 100 ml of 100 mM L-cysteine–50 mM glycerophosphate–2 mM EDTA buffer (pH 6.8) for 4 hr at 4°. Dialysis was then continued for another 8 hr in 200 ml of the same buffer in which cysteine was replaced by 5 mM 2-mercaptoethanol. Note that no imidazole citrate was present in the solutions! (See Shaltiel *et al.*, 1966.) For comparison, enzymes were dialyzed for the same times and in the same volumes of the 2-mercaptoethanol buffer. Residual enzyme-bound cofactor was determined by the method of Wada and Snell (1961).

Determination of Apparent pK's of Pyridoxal-P, Pyridoxal, and Other Compounds in Dioxane–Water. The apparent p*K* values of the phosphate groups of pyridoxal-P, of orthophosphoric acid, and for comparison of benzoic acid and of morpholine (in the same concentrations) were potentiometrically determined. The titration curves were graphically evaluated. Measurements in dioxane–water mixtures were carried out at 25° with a Beckman pH-meter Model G 8508 using a special electrode, Model HTA, Schott a. Gen. Mainz, Germany. According to the manufacturer's specification this electrode is not affected by KOH up to pH 13 or by mixtures containing up to 80% (v/v) of organic solvents. The electrode was soaked in 3.5 M KCl prior to use. The titration of 5×10^{-3} M H_3PO_4 was performed with 0.01 N KOH. Pyridoxal-P, benzoic acid, and morpholine (2×10^{-3} M) were first adjusted to pH 13 with 1 N KOH and then titrated with 0.01 N HCl. Pyridoxal-P and H_3PO_4 solutions containing more than 70% dioxane–water began to separate into two phases at pH >9. The values obtained at high dioxane concentrations and pH >9 are therefore less reliable and not as reproducible as the other measurements. The apparent p*K* values of pyridoxal were determined by absorbancy and fluorescence measurements. The pH of the pyridoxal solutions (2×10^{-5} M) was adjusted by titrating the same solution of pH 13 against another solution of pH 1, containing the same concentration of pyridoxal in order to avoid concentration errors. As already noticed by Metzler and Snell (1955) the ultraviolet (uv) absorption spectra of pyridoxal in neutral dioxane–water and in water differ markedly. Near neutrality, the dipolar species predominates in aqueous solution, whereas in hydrophobic solvents the uncharged species mainly exists. Since the absorption maxima at 288 nm of the protonated and at 280 nm of the neutral forms of pyridoxal are rather similar, one cannot follow accurately enough the change in protonation by absorbancy measurements. We measured, therefore, changes in the fluorescence of pyridoxal as a function of pH. The measurements had to be carried out at high (80%) dioxane concentrations since at lower concentrations (*i.e.*, 50% dioxane) the decay of the unpolar form is no longer proportional to the decrease in fluorescence intensity at 320 nm. This results from the differences of the apparent p*K* values in the ground and in the excited state (Förster, 1950; Weller, 1952, 1954). Fluorescence measurements were carried out with the equipment built in our workshop. This instrument is described in detail elsewhere (K. Feldmann *et al.*, 1972).[2]

Polyacrylamide Gel Electrophoresis. Disc electrophoresis in polyacrylamide gel was carried out according to Maurer (1968). The temperature of the gel was kept at the desired level by circulating water from a constant-temperature bath through the cooling chamber. The same concentration of gel and buffer were used as described by Davis *et al.* (1967)

[2] Manuscript in preparation.

and by Hedrick and Smith (1968). Enzyme solutions were diluted with 50% (v/v) glycerol in 0.034 M asparagine–Tris buffer (pH 7.3) 30–50 μl containing 18–30 μg of protein was applied to the gel. Electrophoresis was carried out for 3 hr at the temperatures indicated. At the start, 20 mA was applied and then increased after 10 min to 40 mA. Each gel block was charged with 200 V. The gel was stained with a 0.5% solution of Amido Black in 7% acetic acid and decolorized electrophoretically at 50 mA.

Reaction of Phosphorylase Proteins with Dimethyl Suberimidate. Reaction of phosphorylase proteins ($1–5 \times 10^{-5}$ M) with dimethyl suberimidate ($1–5 \times 10^{-3}$ M) was carried out in 0.2 M triethanolamine buffer (pH 8.5) for 3 hr at room temperature as described by Davies and Stark (1970). Solutions of dimethyl suberimidate were freshly made before use. The pH of the reaction mixture was 8.1–8.2. All enzymes were free of AMP except where stated otherwise. After reaction with dimethyl suberimidate the proteins were incubated with 1% sodium dodecyl sulfate–1% 2-mercaptoethanol in the same buffer for 2 hr at 37° according to Davies and Stark (1970). The amount of oligomers which withstand this treatment is a measure of the extent of cross-linking. Phosphorylase proteins not treated with dimethyl suberimidate are completely dissociated into monomers by dodecyl sulfate treatment. Holophosphorylase *b*, apophosphorylase *b*, and the analog reconstituted phosphorylases were stable in triethanolamine buffer for days. Apophosphorylase *b* stored in triethanolamine buffer and then transferred to 50 mM sodium glycerophosphate–50 mM 2-mercaptoethanol buffer (pH 6.8) had, on reconstitution with pyridoxal-P the same specific activity (*i.e.*, 60 μmoles of $P_i \times min^{-1} \times mg^{-1}$ in the routine assay) as the same apophosphorylase preparation stored in glycerophosphate buffer. Apophosphorylase *b* (2.5×10^{-5} M) was reconstituted for 1 hr at 30° as described. The concentrations of pyridoxal-P, 3′-*O*-methylpyridoxal-P, pyridoxal-5′-P monomethyl ester, and pyridoxal were 1×10^{-4} M. Unbound cofactors were removed by chromatography on Sephadex G-25 (fine grade). The column was preequilibrated with triethanolamine buffer. Polyacrylamide gel electrophoresis in dodecyl sulfate was carried out at 30° for 2.5 hr at 80 mA and 50 V under the conditions of Davies and Stark (1970).

Attempts to resolve pyridoxal-P from dimethyl suberimidate cross-linked phosphorylase *b* involved incubation of the enzymes, free of AMP, for 1 hr in an ice bath in 0.8 M imidazole–0.2 M L-cysteine adjusted with citrate to pH 6.2 according to Shaltiel *et al.* (1966). The protein was then precipitated by addition of a saturated $(NH_4)_2SO_4$ solution (pH 6.2) to 50% saturation, redissolved in glycerophosphate–2-mercaptoethanol buffer (pH 6.8), and dialyzed in the same buffer in the cold. Protein-bound cofactor was determined by the method of Wada and Snell (1961). Preincubation of holophosphorylase with 0.5–1% glycogen (and/or 1×10^{-3} M AMP) increased its solubility and prevented precipitation on reaction with more than 5×10^{-3} M dimethyl suberimidate. Apophosphorylase *b* was even less soluble, since precipitation occurred with more than 5×10^{-4} M dimethyl suberimidate.

Results

Competitive Inhibition of Reactivation of Apophosphorylase b by Inactive Pyridoxal-P Analogs. When free and enzyme-bound pyridoxal-P and analog are at equilibrium, and pyridoxal-P and analog bind to the same site, the binding of pyridoxal-P and analog to apophosphorylase can be treated according to Dixon and Webb (1964) using

$$\frac{1}{v} = \frac{K_a}{V_{max}[S]} + \frac{1}{V_{max}} + \frac{K_a}{V_{max}[S]}\frac{[I]}{K_i}$$

where [I] is the total inhibitor concentration, K_a is the apparent dissociation constant of the active cofactor (pyridoxal-P or 3′-*O*-methylpyridoxal-P), and K_i is that of the inhibitory analog. The data in Figure 1A–C show that 3′-*O*-methylpyridoxal-P *N*-oxide and pyridoxal monomethyl ester competitively inhibit reactivation of apophosphorylase *b* by pyridoxal-P or 3′-*O*-methylpyridoxal-P. In the course of these experiments less inhibition was observed at longer reconstitution times because pyridoxal-P slowly replaced the inactive 3′-*O*-methylpyridoxal-P *N*-oxide on the enzyme. Thus, the data in Figure 1A merely reflect differences in the rate of binding between pyridoxal-P and 3′-*O*-methylpyridoxal-P *N*-oxide. They do not allow one to calculate K_a or K_i because in the time allowed for reconstitution equilibrium was not reached. The data demonstrate, however, that the inactive analogs 3′-*O*-methylpyridoxal-P *N*-oxide, pyridoxal-P monomethyl ester, and pyridoxal all compete for the same site with pyridoxal-P or the active analog 3′-*O*-methylpyridoxal-P.

Exchange of Enzyme-Bound Analogs by Pyridoxal-P. If an enzyme-bound inactive cofactor exchanges with pyridoxal-P the protein should progressively become more active with time. Moreover, the extent of reactivation should be proportional to the amount of pyridoxal-P bound. Reactivation and incorporation of [4′-^{3}H]pyridoxal-P could be described by a pseudo-first-order progress curve (Figure 2). There also was good correlation in the experiments with pyridoxal and not shown here, with 3′-*O*-methylpyridoxal-P *N*-oxide phosphorylase *b* (up to a twofold molar excess of pyridoxal-P) between incorporated radioactivity and reactivation. The 3′-*O*-methylpyridoxal-P *N*-oxide and pyridoxal were replaced by pyridoxal-P with a half-time of exchange of approximately 7 hr for 3′-*O*-methylpyridoxal-P *N*-oxide and for pyridoxal, a half-time of *ca.* 80 hr was estimated from Figure 2. The 3′-*O*-methylpyridoxal-P and pyridoxal-P monomethyl ester bound to phosphorylase did not exchange with pyridoxal-P, even in 25 hr (Figure 2). Exchange, as one might expect, was concentration dependent. Therefore, activity assays were carried out immediately following dilution. There is little doubt, on the basis of the experiments in Figure 2, that the mode of binding of the inactive analogs 3′-*O*-methylpyridoxal-P *N*-oxide and pyridoxal differs from that of pyridoxal-P monomethyl ester. The latter analog bound to phosphorylase *b* could not be replaced by pyridoxal-P (or by 3′-*O*-methylpyridoxal-P, not shown in Figure 2, *cf.* Ehrlich, 1972). Thus in this respect, the inactive analog pyridoxal-P monomethyl ester behaved like active 3′-*O*-methylpyridoxal-P or pyridoxal-P, in that there was no exchange of [4′-^{3}H]pyridoxal-P in phosphorylase *b* by unlabeled pyridoxal-P either (*cf.* Ehrlich, 1972). Thus, pyridoxal-P, 3′-*O*-methylpyridoxal-P, and pyridoxal-P monomethyl ester are practically irreversibly bound to the phosphorylase protein.

Reactivity of Phosphorylase-Bound Pyridoxal-P Analogs against $NaBH_4$ and L-Cysteine. Recently Shaltiel and Cortijo (1970) (see also Johnson *et al.*, 1970) presented evidence that at neutral pH, pyridoxal-P is buried in a hydrophobic pocket in native phosphorylase. Thus, the azomethine bond linking pyridoxal-P to the protein cannot be reduced by $NaBH_4$, because as pointed out by Shaltiel and Cortijo (1970) the

TABLE I: Reduction of Phosphorylase-Bound Pyridoxal-P and Pyridoxal-P Analogs by $NaBH_4$.[a]

Cofactor Used for Reconstitution	Moles of Cofactor Bound in Azomethine Linkage per Mole of Monomer *b*		Moles of Bound Cofactor Reduced		
	Before Reduction	After Reduction	Determined by the Wada–Snell Method (%)	Calcd from Protein-Bound Radioactivity (%)	Calcd from Reactivation (%)
PLP	0.98	0.94	4	6	7
PLP-ME[b]	1.03	0.90	13	11	13
PL	0.97	0.76	22	24	21
3′-*O*-Me-PLP	1.02	0.77	25	34	21
PLP-N-O[c]	1.05	0.60	43	39	42

[a] Each entry represents the average of at least two experiments. [b] ME = monomethyl ester. [c] PLP-phosphorylase present in the preparation was determined by activity measurements. It was 25% of the total (PLP-N-O + PLP) phosphorylase *b*. The amount of PLP formed was deducted. Therefore only the data on the reduction of PLP-N-O phosphorylase are given. All experiments with PLP-N-O were carried out in the dark and under nitrogen (see Pfeuffer *et al.*, 1972).

rate at which $NaBH_4$ is decomposed in water is greater than the rate of reduction of pyridoxal-P in a hydrophobic environment. At acidic pH or in the presence of other deforming agents pyridoxal-P is turned outward to a more hydrophilic environment and is now readily reduced by $NaBH_4$. The same applies to the resolution of pyridoxal-P from apophosphorylase. As shown by Shaltiel *et al.* (1966), it is necessary to first reversibly de-form the protein with imidazole citrate. Pyridoxal-P is then easily detached from the de-formed protein with a nucleophilic carbonyl reagent such as L-cysteine. L-Cysteine reacts stereospecifically with the C_4–azomethine bond and forms the corresponding thiazolidine derivative. D-Cysteine is ineffective (*cf.* Shaltiel *et al.*, 1969a). The reactivity of pyridoxal-P analogs bound to phosphorylase *b* toward nucleophilic reagents such as $NaBH_4$ and L-cysteine was therefore studied at neutral pH and in the absence of deforming buffers and compared to the reactivity of enzyme-bound pyridoxal-P. The data are summarized in Tables I and II.

The data obtained with three independent assays agree satisfactorily (Table I). Only in the case of 3′-*O*-methylpyridoxal-P was the extent of reduction significantly larger, when calculated on the basis of incorporation of radioactivity. This could have been due to a primary isotope effect. The reactivity of enzyme bound 3′-*O*-methylpyridoxal-P toward L-cysteine was however also greater than expected (Table II). The greater reactivity of enzyme bound 3′-*O*-methylpyridoxal-P might arise because the C_4–azomethine bond which links the 3′-*O*-methylpyridoxal-P to the ε-aminolysyl group in the protein is not stabilized by hydrogen bonds as are pyridine carbaldehydes with a free 3-OH group. Modification of the 5′-phosphate of pyridoxal-P, such as in the pyridoxal-P monomethyl ester, increased the reactivity with $NaBH_4$ or L-cysteine to a lesser extent than did modification at the 3-OH and at the N_1 position of pyridoxal-P.

The most reactive of all the analogs were pyridoxal-P *N*-oxide and 3′-*O*-methylpyridoxal-P *N*-oxide. Pyridoxal-P *N*-oxide phosphorylase *b* could be completely reduced, although 6 hr were required. Since pyridoxal-P bound to phosphorylase was only slightly reduced by $NaBH_4$ under these conditions, the pyridoxal-P formed from pyridoxal-P *N*-oxide should be quantitatively resolved from the reduced pyridoxal-P *N*-oxide phosphorylase on precipitation with trichloroacetic acid. This was the case. The amount of pyridoxal-P recovered from reduced pyridoxal-P *N*-oxide phosphorylase and determined as phenylhydrazone (Wada and Snell, 1961) was that expected from the activity of the pyridoxal-P *N*-oxide phosphorylase (Pfeuffer *et al.*, 1972). Moreover, the activity of pyridoxal-P or pyridoxal-P *N*-oxide phosphorylases did not change on reaction with $NaBH_4$, since the specific activity of pyridoxal-P enzyme was 62 and of pyridoxal-P *N*-oxide enzyme was 14 μmoles of $P_i \times min^{-1} \times mg^{-1}$ before and after reaction. The data obtained with the 3′-*O*-methylpyridoxal-P *N*-oxide phosphorylase *b* are not included here because this analog became partially detached from the protein in the course of reduction. In 3 hr, 3′-*O*-methylpyridoxal-P *N*-oxide phosphorylase *b* was about 60–70% reduced, but about one-half of the reduced cofactor was unbound. Neither of the pyridoxal-P *N*-oxides seem to fit properly into the hydrophobic binding region in phosphorylase.

Similar experiments as those reported here for phosphorylase *b* are presently carried out with phosphorylase *a*. In addition, the effect of AMP on the reactivity of pyridoxal-P analogs bound to phosphorylase *b* is being studied.

TABLE II: Reactivity of Pyridoxal-P and Pyridoxal-P Analogs Bound to Phosphorylase *b* toward L-Cysteine.[a]

Cofactor for Reconstitution	Moles of Coenzyme Bound/Mole of Monomer		Coenzyme Lost (%)
	A[c]	B[d]	
PLP	1.02	0.95	7
PLP-ME	0.97	0.83	14
PL	0.96	0.71	26
3′-*O*-Me-PLP	1.05	0.54	49
PLP-N-O[b]	0.95	0.60	37

[a] Each entry represents the average of at least two experiments. [b] See footnote to the experiment with PLP-N-O phosphorylase *b* in Table I. [c] After dialysis *vs.* 2-mercaptoethanol buffer. [d] After dialysis *vs.* L-Cys buffer.

TABLE III: pK^* Values of the Phosphate Groups of Pyridoxal-P in Dioxane–Water Mixtures.

Dioxane (%)	PLP		H_3PO_4		Benzoic Acid	Morpholine
	pK^*_1	pK^*_2	pK^*_1	pK^*_2		
0	<2.5[a]	6.20[a]	2.0	6.71	4.20[b]	8.35[c]
					3.90 ± 0.05	8.30 ± 0.05
10		6.40		7.00	4.10	8.30 ± 0.05
20		6.75		7.30	4.55	8.30 ± 0.05
30		7.15		7.62	5.25	8.30 ± 0.05
40	4.75	7.45	3.75	8.10	5.65	8.30 ± 0.05
50	5.05	7.90	4.25	8.50	6.10	8.30 ± 0.05
60	5.40	8.45	4.80	8.85	6.85	8.30 ± 0.05
70	~6.0	~8.95	~5.30	~9.35	7.70	8.30 ± 0.05
80	~6.5	~9.30			8.05	8.30 ± 0.05

[a] Compare Williams and Neilands (1954). [b] Compare Rauen and Kuhn (1964). [c] Compare Simon (1964).

It seemed worthwhile to study the influence of a hydrophobic environment similar to that of the pyridoxal-P binding site in phosphorylase on the apparent pK's of pyridoxal and especially of the 5'-phosphate group of pyridoxal-P.

The Apparent pK's of Pyridoxal-P, and Pyridoxal in Dioxane–Water Mixtures. Metzler and Snell (1955) and Williams and Neilands (1954) have reported pK_1 and pK_2 values of pyridoxal and pyridoxal-P in aqueous solution, which are nearly identical: pK_1 is 4.20 and 4.14 and pK_2 is 8.66 and 8.69, respectively. It must be pointed out that the apparent pK's measured in dioxane–water mixtures only provide relative estimates of acidity, since pK values are only exactly defined for dilute aqueous solutions. We therefore refer to the apparent acidity constants in dixoane–water as pK^*.

There are two types of acid–base equilibria (*cf.* Simon, 1964)

$$AH + \text{solvent-H} \rightleftharpoons A^- + \text{solvent-H}_2^+ \quad (1)$$

$$AH^+ + \text{solvent-H} \rightleftharpoons A + \text{solvent-H}_2^+ \quad (2)$$

These equilibria are mainly affected by the dielectric constant and the protolytic character of the solvent. Equilibrium 1 is shifted to the left in solvents with small dielectric constants (the dielectric constant for 80% dioxane–water is 11.86, *cf.* Critchfield *et al.*, 1953), whereas equilibrium 2 is little affected. This is demonstrated by the behavior of benzoic acid and morpholine. The pK^*'s of benzoic acid which is a representative example of equilibrium 1 and of morpholine which follows equilibrium 2 in 80% methyl Cellosolve–water were determined by Simon (1964). They are similar to the corresponding pK^* values in 80% dioxane–water, indicating that these compounds, which served as controls, behaved under our experimental conditions as expected (compare Table III with Simon, 1964). In pyridoxal-P the protonation of the 3-OH and of the phosphate group follow equilibrium 1 and that of the ring nitrogen follows equilibrium 2. The scheme and the equations of Metzler and Snell (1955) who have determined the equilibrium constant (K_z) for several 3-hydroxypyridines were used to calculate the apparent dissociation constants for the phenolic and the ring nitrogen group of pyridoxal

$$K_1 = K_A + K_B$$

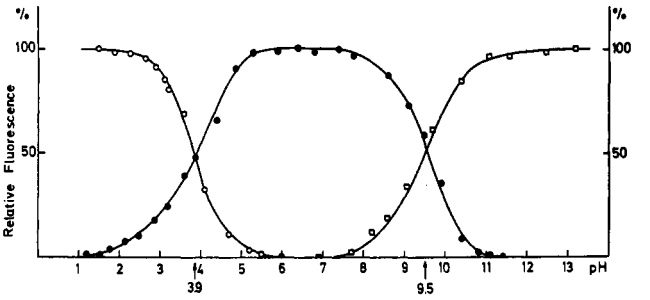
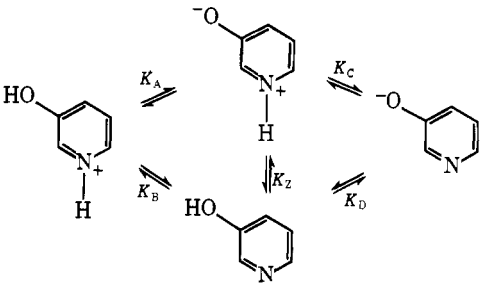


FIGURE 3: The pH-dependent changes in fluorescence emission of pyridoxal in 80% dioxane–water. The changes are given in per cent of arbitrary fluorescence intensity units. (○) refers to excitation at 290 nm and emission at 390 nm; (●) is excitation at 280 nm and emission at 320 nm, and (□) is excitation at 302 nm and emission at 360 nm. The concentration of pyridoxal was 1×10^{-5} M, and the temperature 25°.

$$\frac{1}{K_2} = \frac{1}{K_C} + \frac{1}{K_D}$$

$$K_z = \frac{K_A}{K_B} = \frac{K_D}{K_C}$$



(Metzler and Snell, 1955). Since pyridoxal exists in water to 92% in the dipolar form (K_z = 11.5), the equilibria represented by K_A and K_C are relevant. In 80% dioxane–water, however, pyridoxal exists to 90% in the uncharged form (K_z = 0.1) and K_B and K_D are important. In water pK_1 is 4.20 and pK_2 is 8.66 and pK_A is 4.24 and pK_C is 8.62. In 80% dioxane–water pK_1* is 3.90, pK_2* is 9.50, pK_B is 3.94, and pK_D is 9.46 (see Figure 3). From a comparison of the ab-

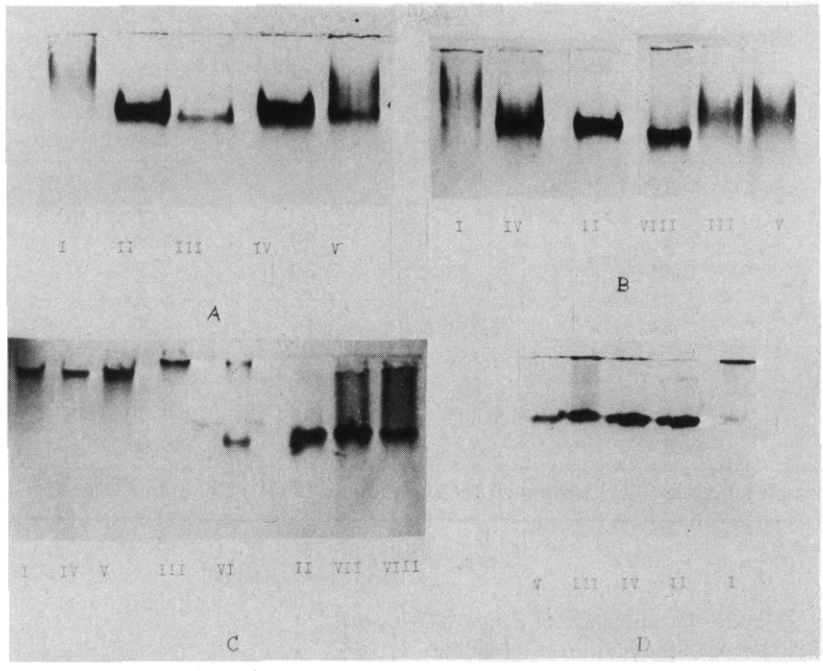


FIGURE 4: Polyacrylamide gel electrophoresis of holophosphorylase *b*, apophosphorylase *b*, and analog-carrying phosphorylases. I, apophosphorylase *b;* II, holophosphorylase *b;* III, pyridoxal; IV, pyridoxal-P monomethyl ester; V, 3′-*O*-methylpyridoxal-P *N*-oxide; VI, pyridoxal-P *N*-oxide; VII, 5′-deoxypyridoxal methylenephosphonate; VIII, 3′-*O*-methylpyridoxal-P phosphorylase *b*. The temperatures are A, 4°; B, 20°; C, 35°; D, 35° in the presence of 1×10^{-3} M AMP.

sorption spectra of holophosphorylase *b* in aqueous buffer and a Schiff base derivative of pyridoxal-P in dioxane–water, it would appear that pyridoxal-P is bound in phosphorylase in an environment as hydrophobic as that provided by a >95% dioxane–water mixture (see Shaltiel and Cortijo, 1970). Hence near neutrality pyridoxal-P in phosphorylase should exist mainly in the neutral form.

Less equivocal are the assignments of the phosphate pK*'s of pyridoxal-P. The pK_2 value of the 5′-phosphate group of pyridoxal-P is 6.2 in water and increased to pK_2* of *ca.* 9.3 in 80% dioxane–water. pK_1 increased from <2.5 in water to pK_1* of about 6.5 in dioxane (see Table III). For comparison the corresponding pK* values for orthophosphate are also given. They change in a similar manner.

State of Aggregation of Phosphorylase Proteins Carrying Pyridoxal-P or Analogs of Pyridoxal-P. At concentrations of 5 mg/ml in glycerophosphate buffer (pH 6.8, T/2 = 0.14) and at temperatures ranging from 20 to 35°, all phosphorylases carrying analogs sedimented like dimer holophosphorylase *b* as a symmetrical peak. The $s_{20,w}$ values ranged from 8.1 to 8.5 S (*cf.* Pfeuffer *et al.*, 1972). Thus, these derivatives of phosphorylase *b* could not be distinguished on the basis of their sedimentation behavior in the ultracentrifuge. Only apophosphorylase *b* sedimented as monomers at 35°. Chignell *et al.* (1968) and Hedrick *et al.* (1969b) have pointed out differences regarding the aggregation state of muscle phosphorylases *b* and *a* and their corresponding apoenzymes when these were studied under identical conditions by gel electrophoresis and by sedimentation velocity in the analytical ultracentrifuge.

Previous work had shown the aggregation state of phosphorylase *b* to be strongly influenced by temperature (*cf.* Graves *et al.*, 1965; Kastenschmidt *et al.*, 1968b). The experiments were therefore carried out at temperatures ranging from 4 to 35°.

At 4°, all inactive and active phosphorylase analogs migrated as dimers (Figure 4A) with identical R_m values. Only apophosphorylase *b* was present in the form of higher aggregates (trimers, tetramers, etc.). As the temperature was increased, some of the inactive phosphorylases began to migrate differently. For example, 3′-*O*-methylpyridoxal-P *N*-oxide phosphorylase *b*, started to aggregate at 12° (not shown) and pyridoxal phosphorylase *b* around 20° (Figure 4B). A notable exception was the inactive pyridoxal-P monomethyl ester phosphorylase *b*. This enzyme migrated as dimer over a wider range of temperatures from 4 to 30°. Thus, it behaved at these temperatures exactly like all the active phosphorylases including pyridoxal-P phosphorylase *b*. Only at still higher temperatures, *i.e.*, 35°, did the pyridoxal-5′-P monomethyl ester phosphorylase *b* finally aggregate (Figure 4*C*). Apparently it is less stable at higher temperatures than the active phosphorylase. At 35° the aggregation state of the pyridoxal-P monomethyl ester phosphorylase *b* resembled that of apophosphorylase. In order to assure that this was not due to a separation of cofactor and protein, the pyridoxal-P monomethyl ester was secured by $NaBH_4$ reduction. The reduced phosphorylase behaved exactly like the unreduced enzyme. The allosteric effector of muscle phosphorylase *b*, AMP, profoundly affected the aggregation state of the phosphorylase in the gel (Hedrick *et al.*, 1969b). At 35°, AMP apparently induced a conformational change which caused the inactive phosphorylases to migrate as dimers like the active phosphorylases in the absence of AMP (Figure 4D). Even apophosphorylase *b*, which still binds AMP (*cf.* Kastenschmidt *et al.*, 1968a) formed dimers in the presence of 5′-AMP although the greater portion of the protein was still aggregated.

Pyridoxal-P *N*-oxide phosphorylase *b*, with 20–25% the activity of pyridoxal-P phosphorylase *b*, behaved exactly as was to be expected on the basis of the previous findings (Pfeuffer *et al.*, 1972). Pyridoxal-P *N*-oxide phosphorylase could be separated by gel electrophoresis into two forms.

TABLE IV: Inhibition and Aggregation of Phosphorylase *b* Treated with Dimethyl Suberimidate.[a]

Dimethyl Suberimidate (M)	Sp Act.[b] (%)	Monomers (%)	Cross-Linked Dimers (%)	Cross-Linked Trimers (%)	Cross-Linked Tetramers and Higher Aggregates (%)
8×10^{-6}	56.5	98	2		
4×10^{-5}	53.2	94.7	3.7	1.5	
2×10^{-4}	48.5	84.4	12.6	3.1	
1×10^{-3}	27.3	55.2	10.4	21.2	13.2
5×10^{-3}	4.6	18.3	17.5	14.8	49.4

[a] The experimental conditions are described in "Methods" and in the legend to Figure 6. [b] The specific activity of the untreated enzyme was 52 μmoles of $P_i \times min^{-1} \times mg^{-1}$. This value corresponds to 100%.

One had the same R_m value as pyridoxal-P phosphorylase with the pyridoxal-P formed from pyridoxal-P *N*-oxide. The other more aggregated form did not migrate and probably represents that part of the enzyme which is inactive and contains pyridoxal-P *N*-oxide.

Formation of Cross-Links between the Subunits of Pyridoxal-P and Pyridoxal-P Analog Phosphorylases. Recently Davies and Stark (1970) showed that the bivalent reagent dimethyl suberimidate (like glutardialdehyde (Wang and Tu, 1969)) formed with oligomeric proteins interprotomeric cross-links between exposed ϵ-aminolysyl groups. Moreover, they found that oligomeric proteins with identical subunits preferentially form such cross-links. Apparently, formation of cross-links is greatly facilitated between proximal ϵ-aminolysyl groups in homologous sequences. Thus, the amount of cross-linked subunits is a measure of structural homology in oligomeric proteins.

Treatment with dimethyl suberimidate almost completely inactivated holophosphorylase *b*. The half-time of inactivation was about 15 min at 25°. At high dimethyl suberimidate concentrations (1×10^{-3} M), inactivation is initially a pseudo-first-order reaction (Figure 5). Neither 0.5% glycogen, 1 mM glucose-1-P, nor 1 mM AMP (singly or in combination) protected against inactivation. Preincubation of the enzyme at 30° for 30 min with the above compounds also had no effect. The extent of cross-linking and inactivation are not related (see Table IV and Figure 6). For example, reaction of phosphorylase *b* with equimolar concentrations of dimethyl suberimidate caused 43% inactivation but formed only 2% cross-linked oligomers. This is in agreement with reports by Phillipp and Graves (1967) and Fasold *et al.* (1969) that one exposed ϵ-aminolysyl group per monomer *b* reacts preferentially with FDNB at pH 6.3. The reactivation with FDNB, like that with dimethyl suberimidate, also resulted in almost complete inactivation.

A comparison of cross-linked pyridoxal-P monomethyl ester, pyridoxal, 3′-*O*-methylpyridoxal-P and pyridoxal-P phosphorylase *b* on polyacrylamide gel electrophoresis revealed only minor differences (Figure 7). Both pyridoxal-P

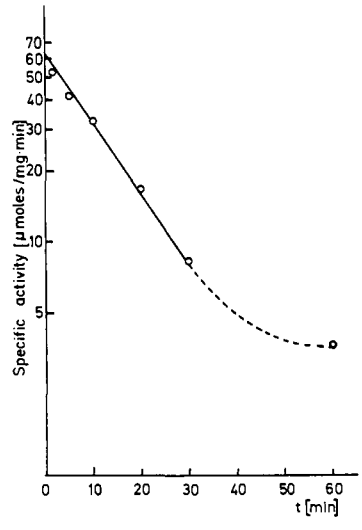


FIGURE 5: Inactivation of phosphorylase *b* by dimethyl suberimidate. The enzyme (1.5×10^{-6} M) was free of AMP. The concentration of dimethyl suberimidate was 1×10^{-3} M in 0.2 M triethanolamine buffer (pH 8.5), containing 0.5% glycogen. The reaction was carried out at 25°. The abscissa gives the time of the reaction. The ordinate is the log of the specific activity. The activity was determined by the routine assay (*cf.* Pfeuffer *et al.*, 1972).

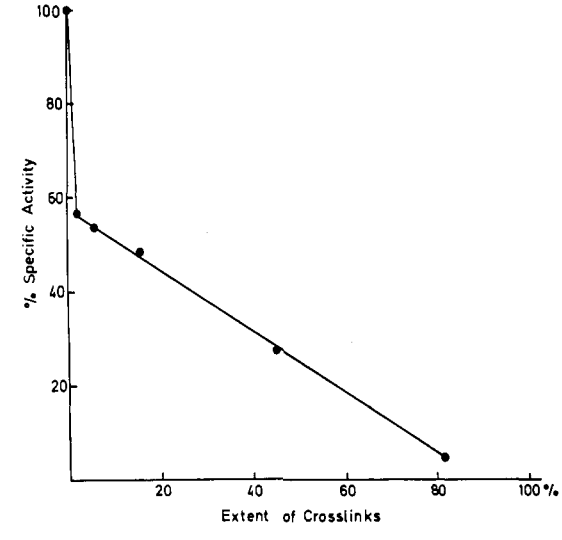


FIGURE 6: Extent of inhibition *vs.* cross-linked oligomers. The phosphorylase *b* concentration was 5×10^{-6} M and that of dimethyl suberimidate ranged from 8×10^{-6} to 5×10^{-3} M. Specific activity of the unreacted enzyme was 52 μmoles of $P_i \times min^{-1} \times mg^{-1}$ (100%). Concentration of the enzyme in the gel was measured densitometrically at 620 nm using a Gilford spectrophotometer, Model 240. The data are taken from Table IV.

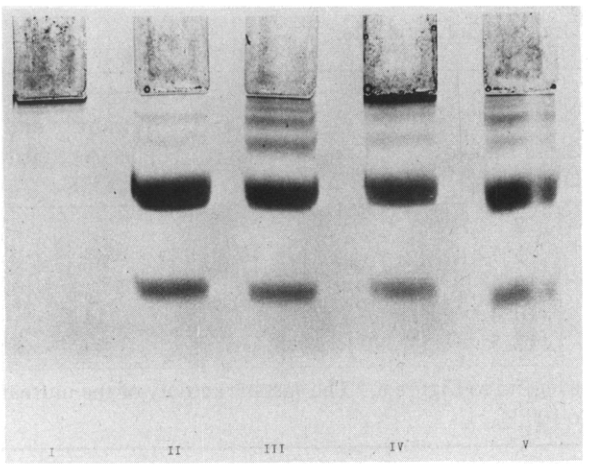


FIGURE 7: Polyacrylamide gel electrophoresis of dimethyl suberimidate cross-linked reconstituted phosphorylase *b* in the presence of sodium dodecyl sulfate: I, apophosphorylase *b*; II, holophosphorylase *b*; III, pyridoxal-P monomethyl ester; IV, pyridoxal; V, 3′-*O*-methylpyridoxal-P phosphorylase *b*.

monomethyl ester and pyridoxal (inactive analogs) and 3′-*O*-methylpyridoxal-P and pyridoxal-P (active cofactors) seem equally capable of establishing structural homology. The large extent to which stable oligomers are formed with dimethyl suberimidate and muscle phosphorylase indicates a high degree of structural homology. However, there are differences in stability among all of the analog reconstituted phosphorylases, with pyridoxal phosphorylase *b* being the least stable. Incidentally, AMP had an expected effect in that cross-linked phosphorylase *b* formed more tetramers in the presence of 1×10^{-3} M AMP (Hedrick *et al.*, 1969b). The drastic effect of the removal of pyridoxal-P on the interprotomeric bonds is also apparent from Figure 7, since apophosphorylase *b* treated with dimethyl suberimidate formed almost no discrete oligomers.

Finally, it should be mentioned that the restraint imposed by the interprotomeric cross-links prevented the "de-forming" action of imidazole citrate. Pyridoxal-P could not be removed from cross-linked phosphorylase *b* with L-cysteine in imidazole citrate buffer by the procedure of Shaltiel *et al.* (1966). Approximately, 1 mole of pyridoxal-P remained bound per mole of monomer. Thus, monomerization seems to be a prerequisite for resolution.

Discussion

There are two plausible hypotheses concerning the universal requirement of pyridoxal-P for the activity of α-1,4-glucan phosphorylases. First, pyridoxal-P is a structural determinant; and second, pyridoxal-P participates in catalysis.

On removal of pyridoxal-P from rabbit skeletal muscle phosphorylase, a crystallizable apoenzyme is obtained which is highly heterogeneous (Illingworth *et al.*, 1958; Johnson and Graves, 1966; Hedrick *et al.*, 1969a,b). The resolution of pyridoxal-P weakens the rather strong contact forces which hold the monomers together in the dimer. At 35° and pH 7.0, apophosphorylase *b* (3×10^{-5} M) mainly exists as monomers (*cf.* Hedrick *et al.*, 1966). However, the quaternary structure of rabbit muscle phosphorylase can be reassembled by analogs of pyridoxal-P which can bind but do not reactivate apophosphorylase (Kastenschmidt *et al.*, 1968a; Shaltiel *et al.*, 1969b). The results of the comparative study by gel electrophoresis as well as the results obtained with cross-linked phosphorylases emphasize again the role of pyridoxal-P as a structural determinant. Among the inactive phosphorylases reconstituted with analogs of pyridoxal-P which we have studied, the pyridoxal-P monomethyl ester enzyme was on polyacrylamide gel electrophoresis the most similar to active phosphorylase. This analog of pyridoxal-P is apparently capable of correctly realigning the subunits to form dimers, yet the enzyme containing this analog is completely inactive (*cf.* Pfeuffer *et al.*, 1972). Moreover, pyridoxal-P and pyridoxal-P monomethyl ester bind virtually irreversibly to phosphorylase. The latter analog could not be displaced by pyridoxal-P. In native phosphorylase *b*, pyridoxal-P and pyridoxal-P monomethyl ester are bound to hydrophobic regions in the protein. Thus, they are shielded from nucleophilic attack by hydride ions or L-cysteine.

Fluorescence measurements showed that the inactive reduced pyridoxal-P monomethyl ester phosphorylases *b* and *a* are perturbed by acidification exactly like the corresponding active reduced pyridoxal-P phosphorylases (Ehrlich *et al.*, 1971). In both the active and inactive enzymes, the pH-dependent conformational transition was reversed by AMP (Ehrlich *et al.*, 1971; K. Feldmann *et al.*, 1972[2]). Thus, in the case of the reduced phosphorylases at least, the inactive pyridoxamine-P monomethyl ester phosphorylase protein had retained the allosteric control properties of the active pyridoxamine-P phosphorylase. In the following paper Weisshaar and Palm (1972) describe the similarity of active pyridoxal-P and inactive pyridoxal-P monomethyl ester phosphorylase *b*. More recently, we have been able to hybridize phosphorylase monomer *a* bound to CNBr-activated Sepharose with soluble monomers *a* carrying the pyridoxal-P monomethyl ester (K. Feldmann, H. Zeisel, and E. Helmreich, 1972).[2]

If one accepts the evidence presented here and by Ehrlich *et al.* (1971), Weisshaar and Palm, (1972), K. Feldmann *et al.* (1972),[2] and K. Feldmann, H. Zeisel, and E. Helmreich (1972)[2] as proof that pyridoxal-P monomethyl ester fulfills its role as a specific conformational determinant for phosphorylase like pyridoxal-P the most plausible conjecture would be to assume that the pyridoxal-P monomethyl ester phosphorylase *b* is inactive because the 5′-phosphate group ($pK_2 = 6.2$) is esterified and thus not protonatable except at pH <2.5. It is interesting to note in that context that the pK_2 of the phosphate group of 5′-deoxypyridoxal methylenephosphonate is higher than that of pyridoxal-P in water, *i.e.*, 7.3 *vs.* 6.2 (Hullar, 1969). The pH optimum of the active 5′-deoxypyridoxal methylenephosphonate phosphorylase *b* is according to Vidgoff (1971) also higher. It is at pH 7.0, whereas the activities of phosphorylases *b* and *a* are optimum, between pH 6.2 and 6.8 depending mainly on the buffer (*cf.* Helmreich and Cori, 1964b; Vidgoff, 1971; Pfeuffer *et al.*, 1972).

In 80% dioxane–water, the pK_2^* of the 5′-phosphate group of pyridoxal-P was *ca.* 9.3 (see Table III). If the pyridoxal-P site in phosphorylase is indeed as hydrophobic as that of a >95% dioxane–water mixture (see Shaltiel and Cortijo, 1970), there would be no correlation between the pH optima of reconstituted and native phosphorylases and the pK^*_2 of the 5′-phosphate group of pyridoxal-P in dioxane–water. Moreover, in 95% dioxane, the phosphate group pK_2 would be fully protonated over the whole pH range in which phosphorylase is active. Although the phosphate pK_1 increased from 2.5 in water to pK^*_1 of 6.5 in dioxane–water, its participation in proton transfer is unlikely. Otherwise, one should expect the pyridoxal-P monomethyl ester phosphorylase

with the phosphate group (pK_1) fully protonatable to be active. Hence, the phosphate group pK_2 of pyridoxal-P in order to function as proton shuttle must project into a more aqueous environment. This would seem plausible assuming that the phosphate group is less rigidly attached than the pyridinium ring which is held to the protein through its azomethine bond and through additional interactions involving the 3-OH and the N_1 group. This would also be in agreement with the other findings reported above which suggest that the hydroxyl and the pyridinium nitrogen groups are more important in binding pyridoxal-P to its site than is the phosphate group. Moreover, the phosphate group of pyridoxal-P, if it were involved in catalysis, would have to approach the carbonium ion (C_1) of the α-1,4-glycosidic bond and one of the water-soluble substrates, glucose-1-P or P_i, to within a distance of a few Ångstroms. Nothing conclusive can be said with respect to a possible catalytic role of the N_1 group of pyridoxal-P (which is a strong nucleophile) because analogs modified at N_1 (*i.e.*, pyridoxal-P *N*-oxide and 3′-*O*-methylpyridoxal-P *N*-oxide) seem not to fit properly into the hydrophobic pocket of the phosphorylase protein. At the present state, not much would be gained from further discussion of the possible participation of the 5′-phosphate group of pyridoxal-P as a proton shuttle in a general acid–base catalysis accompanied by nucleophilic substitution and simultaneous retention of configuration. The decision whether the phosphate group, in concert with a nucleophilic group of pyridoxal-P or with one in the protein, actually participates in catalysis of glycogen phosphorylases is left to the protein chemist and to the X-ray crystallographer. Maybe the information obtained with these and other analogs of pyridoxal-P (*cf.* Shaltiel *et al.*, 1969b) might guide them on their way.

Acknowledgments

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