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Conformational Transitions in Glycogen Phosphorylase Reported by Covalently Bound Pyridoxamine Derivatives†

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ABSTRACT: $NaBH_4$ reduced rabbit skeletal muscle phosphorylases *b* and *a* (α-1,4-glucan:orthophosphate glycosyltransferase (EC 2.4.1.1.)) dissociate on acidification completely to monomers. The conformational change leading to the disruption of the interprotomeric bonds is reported by the absorbance and fluorescence of the bound pyridoxamine-5′-P and analogs modified at the 5′ position. The structural alteration was shown to involve changes in dimer conformations followed by monomerization. A comparison of the responses to pH of several reduced phosphorylase derivatives carrying the pyridoxamine—the 5′-deoxypyridoxamine methylenephosphonate—and the pyridoxamine 5′-monomethyl ester analogs indicated that the ionization of the 5′ group is not related to the structural change. Neutralization (or 5′-AMP addition) completely reversed the pH perturbation of reduced phosphorylases resulting in reassociation of monomers to oligomers and in reactivation, the rate of which was enhanced by substrates. 5′-AMP and substrates also protected against inactivation by acidification. But, in the absence of 5′-AMP substrates alone were ineffective. The absorbance and fluorescence intensity of reduced phosphorylase *b* at a given pH (6.25) was concentration dependent whereas the quantum yield was independent of concentration. This together with the change of the fluorescence intensity of glutardialdehyde cross-linked reduced dimer *b* with pH change indicated that the spectral properties including the fluorescence polarization of bound pyridoxamine-5′-P are the same in the monomer and in at least one of the dimeric forms. This makes it unlikely that the chromophore is buried between the two subunits and can only be exposed on dissociation. The spectral properties of the cofactor in oligomeric reduced phosphorylases *b* and *a* at neutral pH can be explained without assuming that the chromophore is completely immersed in a hydrophobic crevasse. The structure of the binding site must only enable the 3′-OH group of the cofactor to be hydrogen bonded. There is no convincing reason why other protonatable groups, especially the 5′-P moiety, could not react in a more hydrophilic environment.

Pyridoxal-5′-P is essential for the activity of all known α-glucan phosphorylases, but its function is not known (Fischer *et al.*, 1970). But whatever its role one has to bear in mind that the pyridoxamine-5′-P derivative which is covalently linked to phosphorylase as secondary amine is still active (Fischer *et al.*, 1958). This should make $NaBH_4$-reduced phosphorylase a suitable object for the study of pyridoxal-5′-P function.

Cortijo and Shaltiel (1970) reported that the quantum yield of the fluorescence of bound pyridoxamine-5′-P in reduced phosphorylase *b* increased about 10-fold when the pH was lowered from 7.0 to 5.8. The pH effect was interpreted as to result from a conformational change of the enzyme. This stimulated us to study the nature of the conformational change which reduced phosphorylase undergoes on lowering pH and which is reversed by 5′-AMP (see Johnson *et al.*, 1970).

On the basis of previous experiments on the role of 5′-AMP in phosphorylase activation (Helmreich and Cori, 1964a,b; Helmreich *et al.*, 1967; Kastenschmidt *et al.*, 1968a,b), it seemed likely that in the case of reduced phosphorylase the allosteric nucleotide would also affect conformational transitions between active and inactive forms. Recently we could show that the phosphorylase monomer bound to Sepharose is nearly inactive (Feldmann *et al.*, 1972). This led us to a study of monomer–oligomer equilibria in reduced phosphorylase which verified the suggestion of Jones and Cowgill (1971) that reduced phosphorylase *b* and as is shown here *a* as well, dissociate on acidification to monomers and reassociate slowly on addition of 5′-AMP (or neutralization). Substrates (mainly glycogen) enhanced reassociation and reactivation but

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only in the presence of the nucleotide resulting in the formation of an active dimer which also had the highest affinity for glucose-1-P.

The conformational changes accompanying association–dissociation of reduced phosphorylases *b* and *a* were reported by the absorbance and fluorescence of the bound pyridoxamine-5′-P chromophore. This made it possible to study the pH response of reduced phosphorylase derivatives carrying analogs of pyridoxamine-5′-P modified at the 5′ position in order to decide what role the 5′-P group of the cofactor might play in the assembly of dimers. Significant differences between pyridoxamine-5′-P and analog containing phosphorylases became apparent on pH perturbation, but structural differences could not be correlated with the varying degree of activity of these phosphorylase derivatives. This suggested to us that the 5′-P group of pyridoxal-5′-P may not be necessary "to stabilize the active dimer conformation"—a role commonly ascribed to the cofactor—but may be needed for catalysis. Pyridoxal-5′-P analogs which lack the phosphate group or a phosphoryl oxygen protonatable at near-neutral pH are inactive with phosphorylase (Shaltiel *et al.*, 1969; Pfeuffer *et al.*, 1972a,b).

Materials and Methods

Enzymes. Phosphorylase *b* was prepared from frozen rabbit skeletal muscle by the procedure of Fischer and Krebs (1958) as modified by Krebs *et al.* (1964) and recrystallized at least three times. All calculations and molar concentrations are based on a molecular weight of 100,000 g per phosphorylase monomer (Cohen *et al.*, 1971). Phosphorylase *b* was freed of 5′-AMP by passage over activated charcoal. The $A_{260}:A_{280}$ ratios at pH 7.0 of native and reduced AMP-free phosphorylases were 0.53 and 0.48, respectively, reflecting spectral differences of the bound cofactor in the two enzymes. Apophosphorylase *b* was prepared essentially by the procedure of Shaltiel *et al.* (1966). Reconstitution was carried out with pyridoxal-5′-P and analogs in 50 mM β-glycerol-P–50 mM β-mercaptoethanol buffer (pH 7) for 45–60 min at 30°, except with pyridoxal and with 3′-*O*-methylpyridoxal-5′-P where reconstitution was for up to 120 min at 25°. Reconstitution of apophosphorylase *b* with inactive or partially active analogs was considered complete when addition of pyridoxal-5′-P did not result in further activation. Reconstituted or native phosphorylase *b* preparations were reduced with $NaBH_4$ in principle following the method of Graves *et al.* (1965) with the exception that the pH was 6.5 and 0.1 M imidazole chloride was present prior to cooling to −5°. Reduced phosphorylase *b* was recrystallized by addition of $^1/_{100}$th the volume of a 0.1 M 5′-AMP–1 M magnesium acetate solution (pH 7.0) and stored in the cold under toluene vapor. For conversion to phosphorylase *a* the $(NH_4)_2SO_4$ precipitate of reduced phosphorylase *b* was dissolved in 50 mM β-glycerol-P–50 mM β-mercaptoethanol buffer (pH 7) and exhaustively dialyzed against the same buffer. Following dialysis, the pH was raised to 8.5 with 2 M Tris and the phosphorylase solution was incubated with phosphorylase *b* kinase, 10^{-3} M ATP, and 10^{-2} M Mg^{2+} at 30° for 60 min. Phosphorylase *b* kinase was prepared and activated according to Danforth and Helmreich (1964). Reduced phosphorylase *a* was recrystallized at least twice prior to use. The preparations retained full activity for at least 3–5 days when kept at 4° under toluene vapor. After 15 days reduced phosphorylase *a* had lost about 50% of its original activity whereas reduced phosphorylase *b* was still fully active.

Phosphorylase *b* (11.2 mg/ml) was reduced with $NaBH_4$ and cross-linked at near 0° for 10 min with 0.05% glutardialdehyde in 40 mM β-glycerol-P–1 mM EDTA buffer at pH 7.5 and reduced again with $NaBH_4$ as Wang and Tu (1969) have described for native phosphorylase *b*. Further purification and analysis was by chromatography at pH 5.5 on a Bio-Gel A 0.5 column and by sodium dodecyl sulfate gel electrophoresis. On the basis of sodium dodecyl sulfate gel electrophoresis the fraction containing 60% of the cross-linked dimers and small amounts of higher aggregates was used for further study. In the analytical centrifuge this fraction traveled however at pH 5.5 as a single boundary with a $s_{20,w}$ value of 8.6 which is that for dimer *b*. Heat treatment did not result in further enrichment of cross-linked material. The cross-linked reduced phosphorylase *b* had a specific activity of 12–14 μmol/min per mg under the test conditions described below.

Activity Measurements. Initial velocities were measured in the direction of glycogen synthesis at 30°. P_i was analyzed according to Fiske and Subbarow (1925). With reduced phosphorylase *b*, the reaction was started by addition of about 10 μg of enzyme/ml of 10 mM β-glycerol-P–20 mM β-mercaptoethanol buffer (pH 6.8), containing 1% glycogen (corresponding to 5×10^{-3} M terminal nonreducing glucose residues), 100 mM glucose-1-P, and 1 mM 5′-AMP. Reduced phosphorylase *a* was preincubated in the above buffer with 1% glycogen at the indicated pH (from 6 to 7) with or without 1 mM 5′-AMP for 30 min at 30°. The reaction was started with glucose-1-P. Initial velocities were calculated from the linear portion of the rate curves. Native and reduced phosphorylase *b* preparations usually had under assay conditions specific activities of 85–90 and 50–55 μmol of $P_i \times mg^{-1} \times min^{-1}$, respectively. The specific activities of freshly prepared native and reduced phosphorylases *a* were the same and ranged without and with 1 mM 5′-AMP from 57 to 60 and from 63 to 68 μmol of $P_i \times mg^{-1} \times min^{-1}$, respectively. Reduced and nonreduced 3′-*O*-methylpyridoxal-5′-P phosphorylases *b* (with 1 mM 5′-AMP) had specific activities of 15 μmol of $P_i \times mg^{-1} \times min^{-1}$ and up to 45 μmol of $P_i \times mg^{-1} \times min^{-1}$, respectively, mainly dependent on enzyme concentrations. Reduced and nonreduced 5′-deoxypyridoxal methylenephosphonate containing phosphorylases *b* had specific activities of 18–22 μmol of $P_i \times mg^{-1} \times min^{-1}$ and of 24–28 μmol of $P_i \times mg^{-1} \times min^{-1}$, respectively (Vidgoff, 1971). Pyridoxamine and pyridoxamine-5′-P monomethyl ester phosphorylases were completely inactive.

Analytical Procedure. 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). In some cases the method of Lowry *et al.* (1951) was used with a calibration curve of phosphorylase *b* against bovine serum albumin as standard. Analysis of reconstituted phosphorylase proteins for acid dissociable pyridoxal-5′-P or analogs were carried out before and after reduction with $NaBH_4$. They involved precipitation of the protein with 0.3 M $HClO_4$ release of the nonreduced cofactors and their determination by the method of Wada and Snell (1961).

Absorbance and Fluorescence Measurements. Absorbance measurements were carried out with a Zeiss PMQ III spectrophotometer. Temperature was kept constant by using jacketted thermostated cuvets from Hellma, Müllheim, F.R.G. The samples were centrifuged at 30,000*g* for 20 min at 15° and kept dust free. Scattering of the protein solution was determined at long wavelengths starting at 600 nm. The data were graphed as log *A vs.* log $\tilde{\nu}$ in accordance with the equation: $A = k\tilde{\nu}^n$; where *k* is a constant characteristic for polymers, $\tilde{\nu}$ (1/λ) is the wave number and *n* is the exponent which is 4 for

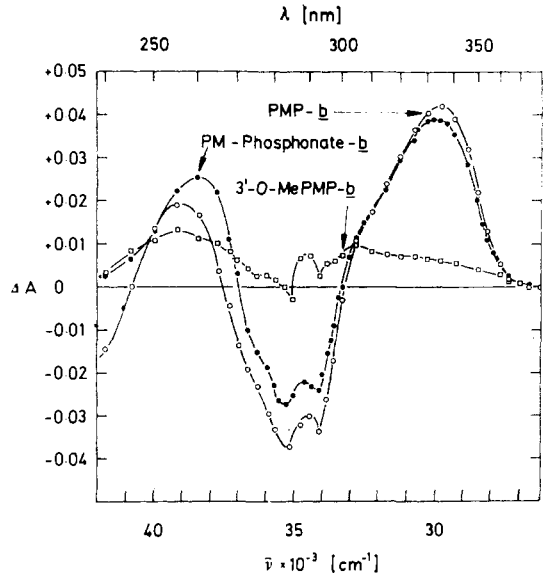


FIGURE 1: pH difference spectra of reduced phosphorylase *b*. Enzyme concentration 3 mg/ml in each case. Measurements were made in cuvets of 0.2-cm light path in 50 mM β-glycerol-P buffer adjusted with HCl to the pH indicated; temperature 20°. The 380-nm absorbancy was taken as zero base line. The spectra were not corrected for differences in scattering due to various aggregation states (*cf.* Leach and Scheraga, 1960): (○) PMP *b*, pyridoxamine-5′-P phosphorylase *b* at pH 5.26–7.6; (●) PM-phosphonate *b*, 5′-deoxypyridoxamine methylenephosphonate phosphorylase *b* at pH 5.65–7.14; (□) 3′-*O*-MePMP *b*, 3′-*O*-methyl pyridoxamine-5′-P phosphorylase *b* at pH 5.52–7.44.

particles smaller than $^1/_{20}$th the wavelength of light; the extinction due to scattering was linearly extrapolated to the uv absorbing range and deducted. A justification for that correction is given in Treiber *et al.* (1955) and in Schauenstein and Bayzer (1955). Fluorescence emission spectra were measured with a spectrofluorometer designed and built in our laboratory. The sample in a thermostated cuvet of 1-cm light path was illuminated with a 200-W Hanovia DC xenon mercury compact arc lamp (type 901 B1) supported by a power supply (Metronic 102CC; K. Lüdeke, Göttingen, F.R.G.). The light was passed through a Bausch and Lomb double-grating monochromator (bandwidth = 2.0 nm). The light emitted at a right angle to the exciting light was passed through a Zeiss M20 monochromator (bandwidth = 5 nm/mm). An EMI photomultiplier tube (9558Q) was used as fluorescence detector. A constant proportion of the monochromatic excitation light was reflected to a reference multiplier (RCA 1-P28) by means of an uncoated quartz plate (oriented at 45° to the beam). Because pyridoxal and pyridoxamine compounds are exceedingly light sensitive a camera shutter was installed to produce short light pulses. Thus, it was possible to measure a complete emission spectrum without detectable photodestruction of the chromophore. The detector system was calibrated with a Osram Wi 17 lamp using the data of DeVos (1954). The calibration was checked with standard solutions of quinine sulfate (White and Argauer, 1970). For measurements of quantum yields of fluorescence (q) pyridoxamine-5′-P was used as standard: q pyridoxamine-5′-P = 0.17 in 50 mM phosphate buffer (pH 7.0) at 20° (*cf.* Chen, 1965). The excitation wavelength was the 334-nm mercury line and all measurements were carried out 20 ± 0.1°. Quantum yields were calculated using the expression: $q = q_{st}[F/F_{st}][Q_{st}/Q] \cdot [E_{st}/E]$ for $E \rightarrow 0$; the subscript st refers to the standard (pyridoxamine-5′-P); q is quantum yield, E the optical density, F represents the total area under the corrected fluorescence emission normalized to relative quanta between $\tilde{\nu}_i$ and $\tilde{\nu}_f$ where $\tilde{\nu}_{initial}$ and $\tilde{\nu}_{final}$ are the limits of the fluorescence band. This takes into account that the emission maxima for free and bound pyridoxamine-5′-P differed as did the shapes of the bands. The maxima were at 392 and 383 nm, respectively. Q is the intensity of the lamp at the excitation wavelength; since the latter was always 334 nm Q_{st}/Q was 1. Fluorescence polarization was determined according to Chen and Bowman (1965). Polacoat quartz filters were used.

Sedimentation velocity measurements were carried out with a Spinco Model E analytical ultracentrifuge equipped with a uv scanner and electronic speed control. A double-sector cell with an 1.2-cm aluminum filled Epon centerpiece was used. Sedimentation was at 60,000 rpm using an AND rotor at 20 ± 0.1°. All experiments were carried out in 50 mM β-glycerol-P–50 mM β-mercaptoethanol buffer at the indicated pH. Apparent s values were calculated from the second moment positions of the boundaries (*cf.* Goldberg, 1953). For the partial specific volume a value of 0.746 as determined by Cohen *et al.* (1971) for native phosphorylase was used. The $s_{20,w}$ values were not corrected for enzyme concentration which was always 0.5 mg/ml.

Materials. Pyridoxal and pyridoxal-5′-P were products of E. Merck A. G., Darmstadt, Germany. 5′-Deoxypyridoxal methylenephosphonate was prepared by Dr. O. Saiko from E. Merck A. G., according to the procedure of Hullar (1969). Pyridoxal-5′-P-monomethyl ester was prepared according to Pfeuffer *et al.* (1972a). 3′-*O*-Methylpyridoxal-5′-P was provided by Dr. J. Ehrlich. Pyridoxamine-5′-P and quinine sulfate were purchased from the Fluka AG, Buchs, Switzerland. 5′-AMP and glucose-1-P were products of Boehringer and Sons. Oyster glycogen was purchased from E. Merck A. G. It was further purified and freed of nucleotides as described by Helmreich *et al.* (1967). Imidazole (p.A. grade), L-cysteine, β-glycerol-P, and activated charcoal were from E. Merck A. G.; the latter was further purified by treatment with HCl and EDTA. β-Mercaptoethanol was obtained from Serva, Heidelberg.

Results

Absorbance and Fluorescence of Pyridoxamine-5′-P and Analogs Bound to Phosphorylase. Johnson *et al.* (1970) on the basis of their uv and CD spectra of reduced phosphorylase *b* at neutral and acid pH and of previous data of Kent (1959) and of Matsushima and Martell (1967) on pyridoxamine in methanolic solutions have assigned a hidden band at 290 nm to the tautomer of pyridoxamine-5′-P in which the oxygen in position 3 is protonated and the nitrogen in position 1 of the pyridine ring is unprotonated. The appearance of a band near 330 nm as the pH is lowered was explained by the formation of the zwitterionic form with an unprotonated oxygen in position 3. Moreover the appearance of this band was ascribed to a conformational change (Johnson *et al.*, 1970). We have examined this proposal with 3′-*O*-methylpyridoxamine-5′-P phosphorylase *b;* since the 3′-OH group is blocked in this analog no absorbance band near 330 nm should be generated on acidification. Moreover the cofactor should not contribute to pH difference spectra of the phosphorylase derivative in the range from 7.4 to 5.5, because according to Pocker and Fischer (1969) the pK of the pyridinium ring nitrogen of the analog in aqueous solution is as low as 3.60. The results in Figure 1 are therefore expected. Included also are pH difference spectra for the pyridoxamine-5′-P- and 5′-deoxypyridoxamine methylenephosphonate phosphorylases *b* which are

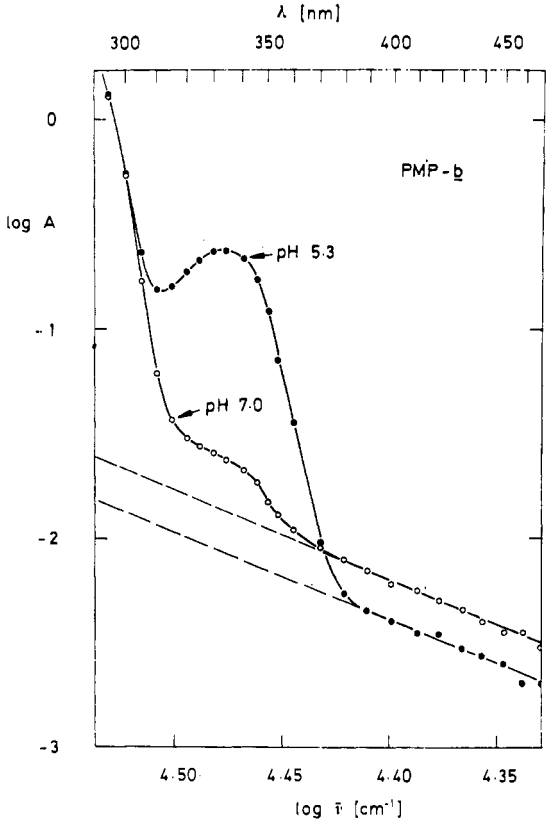


FIGURE 2: Absorption spectra of reduced phosphorylase *b*. Enzyme concentration 3 mg/ml in each case. The buffer was 50 mM β-glycerol-P–50 mM β-mercaptoethanol adjusted to the pH indicated with HCl; temperature 20°: (○) pyridoxamine-5′-P phosphorylase *b* at pH 7.0 and (●) at pH 5.3.

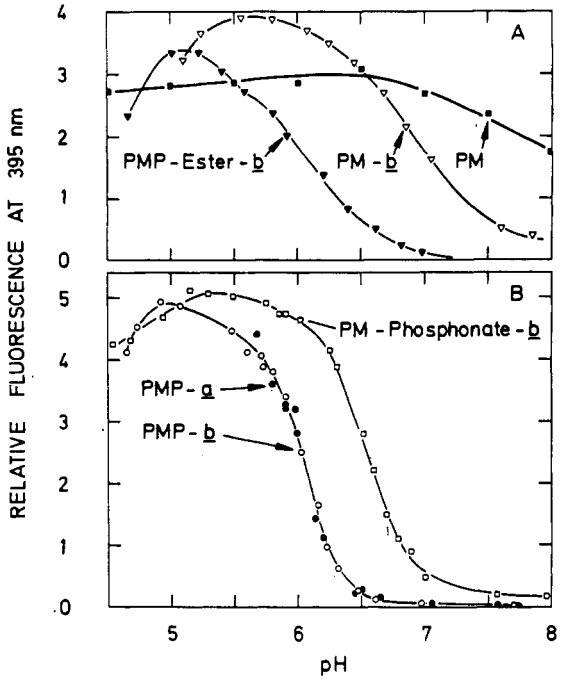


FIGURE 3: pH dependency of relative fluorescence of reduced phosphorylases. Enzyme concentrations 0.5 mg/ml in each case. Buffer and temperature as in Figure 2: (A) (■) PM, pyridoxamine (5×10^{-6} M); (▽) PM *b*, pyridoxamine phosphorylase *b*; (▼) PMP-ester *b*, pyridoxamine-5′-P monomethyl ester phosphorylase *b*. (B) (□) PM-phosphonate *b*, 5′-deoxypyridoxamine methylenephosphonate phosphorylase *b*; (○) PMP *b*, pyridoxamine-5′-P phosphorylase *b*; (●) PMP *a*, pyridoxamine-5′-P phosphorylase *a*.

identical. The spectrum for the pyridoxamine-5′-P phosphorylase agrees with that reported by Johnson *et al.* (1970). The data for the 3′-*O*-methylpyridoxamine-5′-P phosphorylase therefore strongly support the proposition of Johnson *et al.* (1970). Since this phosphorylase derivative is still active the 3′-*O*-methyl analog should be bound like the natural pyridoxamine-5′-P to phosphorylase and hence make this phosphorylase derivative a suitable model to prove the structure of the enzyme-bound cofactor. The structure assigned to pyridoxamine-5′-P bound to phosphorylase explains its fluorescence which was used to follow structural changes in the reduced enzyme. This signal is generated from all pyridoxamine derivatives with an unprotonated 3′-OH group bound to phosphorylase as secondary amines.

The cofactor and the protein each contribute to the absorbance of reduced phosphorylase. In addition the protein as a macromolecule scatters light. In order to evaluate the light-scattering contribution measurements were extended well into the red region of the spectrum where protein and cofactor do not absorb light (see Figure 2). The scattering contribution was deducted as described. It should be noted that at neutral pH (7.0) and 20° the true absorbancy of the bound cofactor at 334 nm is about 25% of the measured absorbancy while at acid pH (5.26) it is more than 95% of the measured absorbancy. Since as shown below reduced phosphorylase *b* is a dimer at pH 7.0 and a monomer at pH 5.26, the scattering contributions indicated by the extrapolated parallel lines should differ by a factor of 2. The observed factor was 1.6.

Absorbance and fluorescence of pyridoxamine-5′-P in phosphorylase *b* and *a* changed little between pH 7.0 and 6.5 but rose steeply between pH 6.5 and 5.8 and approached gradually a maximum around pH 5.0 (see Figure 3B). The pH-dependent fluorescence changes of reduced phosphorylases *b* and *a* were similar, but the curve for the partially active 5′-deoxypyridoxamine-5′-P methylenephosphonate phosphorylase *b* was significantly shifted towards a higher pH range. In Figure 3A the curves for the pyridoxamine-5′-P monomethyl ester and the pyridoxamine phosphorylases *b* are shown. The maxima for these inactive phosphorylase derivatives were lower (compare Figure 3A with 3B). More important the pH at which half-maximal fluorescence of the inactive pyridoxamine-5′-P monomethyl ester derivative of phosphorylase *b* was generated was around 6.0 as in the case of active reduced phosphorylase *b*, but the pH fluorescence relationship for the pyridoxamine enzyme was significantly different. The curve for pyridoxamine phosphorylase *b* was shifted even further towards the more alkaline pH region than the curve for the 5′-deoxypyridoxamine methylenephosphonate derivative in Figure 3B. Between pH 5 and 7, the zwitterionic species of pyridoxamine predominates and only at pH >7 the anionic species with the unprotonated pyridinium N appears (Metzler and Snell, 1955). This is apparent from the pH-dependent fluorescence changes of free pyridoxamine in buffer which are included for comparison in Figure 3A. (Note that the concentrations of free and bound pyridoxamine were equal.) Thus the formation of the anionic species cannot be solely responsible for the shift of the pH dependency of the fluorescence of pyridoxamine bound to phosphorylase toward alkaline pH. Although differences in the maximal fluorescence may in part be due to differences in the chemical structure of the cofactors (*cf.* Chen, 1965), the pH-dependent shifts are mainly determined by the protein conformation. In this respect the role of the 5′-phosphate group of the cofactor concerned us most. The phosphate group does practically not contribute to the uv

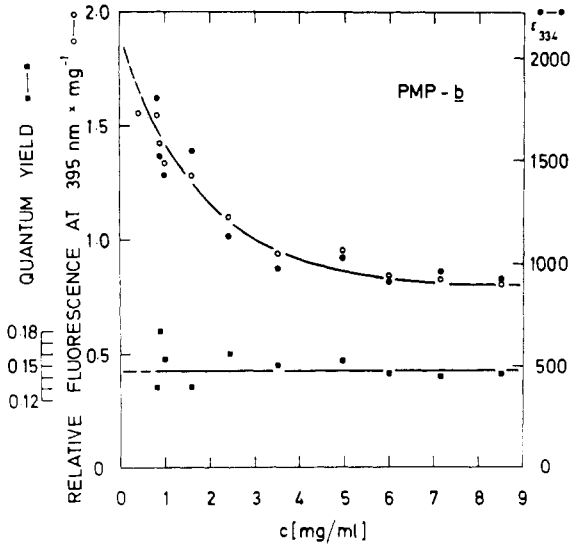


FIGURE 4: Concentration dependence of ϵ, the normalized fluorescence and quantum yield at pH 6.25. The conditions were those of Figure 2. PMP *b* is reduced (pyridoxamine-5′-P) phosphorylase *b*.

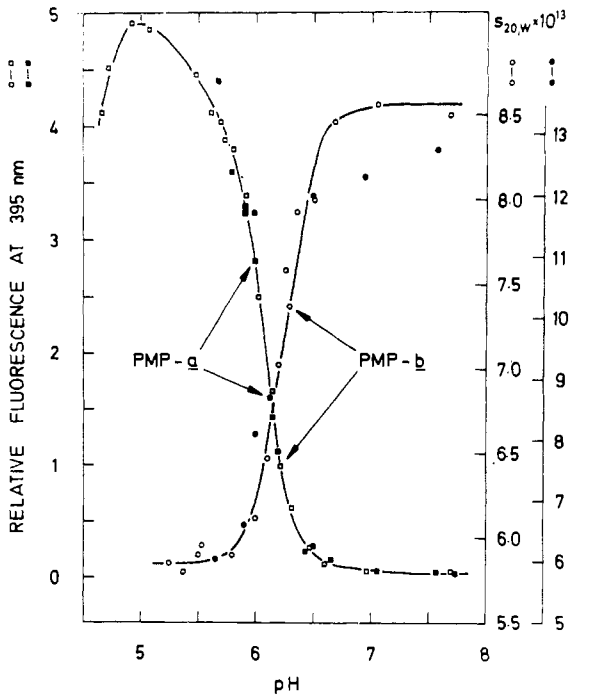


FIGURE 5: pH-dependent monomer–oligomer transitions and fluorescence changes. Enzyme concentrations, buffer and conditions were those of Figure 3: (○) $s_{20,w}$ values and (□) relative fluorescence of reduced (pyridoxamine-5′-P) phosphorylase *b*; (●) $s_{20,w}$ values and (■) relative fluorescence of reduced phosphorylase *a*. Note that the second ordinate scale (●, 5–13 $s_{20,w} \times 10^{13}$) belongs to reduced phosphorylase *a*.

spectroscopic properties of the chromophore. Therefore spectral differences between phosphorylases carrying analogs of pyridoxamine-5′-P modified at the 5′ position should indicate structural differences in the protein. Thus the similar (albeit not identical) pH response of the fluorescence emission for the pyridoxamine-5′-P monomethyl ester and the pyridoxamine-5′-P phosphorylases, would seem to exclude the ionization of the 5′-phosphate group as a factor which determines phosphorylase structure. The phosphate group of the pyridoxamine-5′-P monomethyl ester is not protonatable in this pH range.

For measurements of quantum yield around neutral pH high concentrations, up to 18 mg/ml, had to be used to allow one to measure the very low $A_{334\ nm}$ after correction for scattering. At pH 7.0 and 20° ϵ ($M^{-1} \times cm^{-1}$) was only about 200 but rose to 7200 at pH 5.3. At neutral pH and 20° the ϵ value and the quantum yield (q = 0.07) remained constant in the range of concentrations (5–18 mg/ml) of reduced phosphorylase *b* where these measurements were carried out. On acidification (and dilution to 0.5 mg/ml), q rose to 0.21 which was higher than the value (0.17) for free pyridoxamine-5′-P in phosphate buffer (pH 7.0 and 20°) (Chen, 1965). With the low concentrations (0.5 mg/ml) better suited for ultracentrifugal measurements of the dissociation of reduced phosphorylase to be reported later, the q values at pH above 6.1 could only be estimated but the data pointed to a decrease. The fluorescence sharply increased between pH 6.5 and 6.0 suggesting that in this narrow pH range the fluorescence enhancement exceeded the absorbance increase resulting in an increase in q. Below pH 5.8 where the curve for the relative fluorescence intensities flattened out and quantum yields could be reliably measured they did not further increase but remained constant from pH 5.8 to 5.0. The above findings are in agreement with results of absorption and fluorescence studies with reduced phosphorylase *b* reported by Cortijo and Shaltiel (1972). In order to quantitate the assumption that the quantum yield actually increased in the narrow range between pH 6.5 and 6.0, the dilution experiments at pH 6.25 were carried out (Figure 4). At pH 6.25—the inflection point of the steep part of the curve —the relative intensity of the fluorescence light emitted at 395 nm by the bound pyridoxamine-5′-P and normalized to a concentration of 1 mg/ml of enzyme, decreased with an increase in protein concentration approaching a constant value around 10 mg/ml. The ϵ values changed accordingly and hence the quantum yield remained constant and was independent of concentration at pH 6.25 (Figure 4). The increase in q with lowering pH was as expected as was the numerical value of 0.14 which was inbetween the maximum value of 0.21 at pH 5.8 and the value of 0.07 at pH 7.0.

pH-Dependent Oligomer–Monomer Transitions. DeVincenzi and Hedrick (1970) were first to direct attention on the basis of gel filtration experiments to the greater lability of the quaternary structure of reduced phosphorylase *b*. Subsequently Jones and Cowgill (1971) suggested that the conformational change with pH is probably a dimer–monomer transition. The experiments in Figure 5 prove this assumption to be correct, although as will be explained later other structural changes also play a role. It is remarkable that on lowering the pH reduced oligomeric phosphorylase *a* dissociated as readily as reduced dimer *b*. Reduced like native phosphorylases *a* exist at neutral pH and 20° and at concentrations >1 mg as tetramer while phosphorylase *b* is dimeric. In Figure 5 the change in fluorescence of the bound pyridoxamine-5′-P is shown in relation to the change in the oligomer–monomer population. An interpretation is given later. The instability of the acidified enzyme with time precluded sedimentation equilibrium measurements and sedimentation velocity experiments were therefore carried out. One can calculate a pH-dependent apparent equilibrium constant for the monomer–dimer equilibrium of reduced phosphorylase *b* according to Kawahara *et al.* (1965) which is derived from Ostwald's dilution law: $K_{app} = 4\alpha^2 c_0/(1 - \alpha)M$, where K_{app} is the dissociation constant, α the degree of dissociation, c_0 the protein concentration (in mg/ml), and M the molecular weight of dimer *b* which is 200,000 g (Cohen *et al.*, 1971). One obtains for the inflection

TABLE I: Effect of pH and 5′-AMP on the Relative Fluorescence of Cross-Linked Reduced Dimer b.[a]

pH	Fluorescence at 395 nm	
	Unmodified	Cross-Linked
	Arbitrary Units	
5.0	5.0	5.1
5.5	4.5	4.4
5.5	3.4 (+AMP)	3.5 (+AMP)

[a] The conditions were those of Figure 3. Unmodified refers to (0.5 mg/ml) reduced (pyridoxamine-5′-P) phosphorylase *b* and cross-linked to the same concentration of reduced dimer *b* modified with glutardialdehyde. The preparation is described in Methods. 5′-AMP was 2.0 mM.

point at pH 6.25 a value of $K_{app} = 3.4 \times 10^{-6}$ M. From the pH dependence of K_{app}, one can calculate that at pH 7.0 about 1% of reduced dimer *b* was monomeric. The quaternary structure change of reduced phosphorylase *a* is more complex involving at least one more aggregated (*i.e.*, tetrameric) species.

Effect of 5′-AMP. Addition of 0.01 M AMP decreased the 330 nm absorbance of reduced phosphorylase *b* at pH 6.0, but not at pH 7.6 (*cf.* Johnson *et al.*, 1970). As was therefore anticipated from Johnson *et al.*'s (1970) work and hence not shown here addition of 5′-AMP to reduced phosphorylase *b* at pH 6.20 and to phosphorylase *a* at pH 6.5 quenched the fluorescence emission at 395 nm generated by excitation of bound pyridoxamine-5′-P at 334 nm. Sedimentation velocity controls indicated that reduced phosphorylases *b* and *a* either adjusted to pH 7.0 or left at pH 6.2 but saturated with 5′-AMP were completely dimeric.[1]

Cofactor Binding Site. Jones and Cowgill (1971) have assumed that the hydrophobic region which harbors the cofactor in phosphorylase is actually the domain in which the monomers make contact. This conclusion seems however premature. In Figure 4 is shown that at pH 6.25 with increasing enzyme concentrations as more and more dimer was formed (up to about 90% as calculated from ultracentrifugal measurements) the normalized fluorescence intensity curve approached a limiting value far above zero. Based on Jones and Cowgill's (1971) suggestion one would have expected much more and nearly complete quenching of the fluorescence of the cofactor as more and finally almost all of the enzyme was associated to dimers. The data in Figure 4 are moreover in agreement with the spectral properties of reduced dimer *b* cross-linked with glutardialdehyde. Though about 60% of the preparation was cross-linked it responded to acidification like the unmodified enzyme which was completely monomeric at this pH (5.5). Moreover, the enhanced fluorescence of the cross-linked enzyme at pH 5.5 was quenched on addition of 5′-AMP like the unmodified control (Table I). The enzymatic activity of the cross-linked enzyme (25% of the unmodified dimer) and its response to the allosteric activator 5′-AMP make it unlikely that other than normal contact surfaces were cross-linked. Thus when monomerization would be the only structural change responsible for the fluorescence increase of the bound cofactor one could not explain that the fluorescence of oligomers whose dissociation was prevented by cross-linking responded to acidification and 5′-AMP exactly like the unmodified enzyme.

It now also becomes more plausible why Jones and Cowgill (1971) and Chignell *et al.* (1972) found a high degree of polarization of the bound pyridoxamine-5′-P which remained unchanged when reduced phosphorylase *b* was exposed at constant pH (7.0) and at 6° to increasing concentrations (0.2–1.8 M) of imidazole citrate or urea, reagents known to dissociate phosphorylase (*cf.* Hedrick *et al.*, 1969). We have obtained similar data with reduced phosphorylase *b* dissociated at low pH. With the exception of extreme pH (2.8) where irreversible unfolding occurs, the fluorescence polarization of the phosphorylase-bound pyridoxamine-5′-P and pyridoxamine-5′-P monomethyl ester remained unchanged indicating comparably low rotational relaxation times at neutral and low pH. The degree of polarization was in both instances 0.35 ± 0.01 between pH 7 and 4.8 in aqueous 50 mM β-glycerol-P buffer at 20° and thus comparable to that of free pyridoxamine-5′-P in 90% glycerol solutions (Chen, 1965). If the cofactor would be buried in between the subunits, it would be difficult to understand that the mobility of pyridoxamine-5′-P which in reduced phosphorylase is only singly bonded to a flexible ε-aminolysyl side chain remained as restricted in the monomer as it was in the dimer.

Structure–Activity Relationship. The fluorescence change of acidified bound pyridoxamine-5′-P on neutralization or on addition of 5′-AMP lets us estimate the rate of the structural transition and can be conveniently used to study the influence of other ligands. Binding of 5′-AMP to phosphorylase was too fast to be measured in a stopped flow fluorescence apparatus and even in fluorescence temperature-jump experiments. (*cf.* Gaugler; work in partial fulfillment of the requirements for a diploma in biology, University of Würzburg, 1973). Similar results were recently reported by Buc *et al.* (1974). The conformational changes associated with dimerization lagged however considerably behind the binding of the effector. The rate of association in the presence of 5′-AMP was pH dependent. The lower the pH the slower the association.[2] The anionic substrates glucose-1-P and P_i had little effect but glycogen increased the rate of association of reduced phosphorylase *a* but only when 5′-AMP was also present (see Figure 6). The effect of glycogen was concentration dependent. At the concentrations used in Figure 6 the relatively large amount of enzyme should have been completely saturated with glycogen and the molar ratio of glycogen$_{(n)}$ to enzyme should have approached the limiting value of 1 to 1 (*cf.* Madsen and Cori, 1958; Palm *et al.*, 1973). The ineffectivity of the high concentrations of glycogen (~1%) in the absence of 5′-AMP makes it unlikely that the only role of 5′-AMP was to increase the affinity of the acidified enzyme for glycogen (*cf.* Helmreich and Cori, 1964a).

The rate acceleration of polymerization by 5′-AMP and glycogen also explains the rapid rate of reactivation of the enzyme. Reduced phosphorylase *b* could not be used for fluorescence measurements in the presence of glycogen and 5′-AMP because it precipitated. However, when brought from pH 6.15 to pH 6.8 by transfer to the substrate mixture and diluted for assay it became fully active within 45 sec.

The final activity was as high as that of the same enzyme preparation not exposed to low pH but in that case the initial

[1] Only at pH <6 was reassociation of monomers to dimers incomplete even after 8 hr, suggesting that some enzyme was irreversibly denatured. Accordingly at pH <6 addition of 5′-AMP did not quench the fluorescence to the expected extent. However 5′-AMP still quenched the fluorescence of reduced dimeric phosphorylase *b* (14 mg/ml) at pH 7.0 indicating conformational changes in the oligomer.

[2] In addition, polymerization was dependent on enzyme concentration and temperature. (The effect of ionic strength has not been studied.)

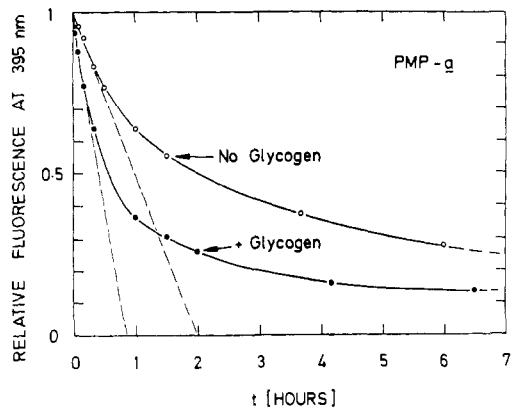


FIGURE 6: Effect of glycogen. Experiments were carried out with reduced (pyridoxamine-5′-P) phosphorylase *a* (0.25 mg/ml). Incubations at 20° in 50 mM β-glycerol-P, 50 mM β-mercaptoethanol buffer pH 6.25 and 2.3 × 10^{-3} M 5′-AMP and with and without 0.9% glycogen corresponding to 4.5 × 10^{-3} M nonreducing end groups.

rate was attained without a lag (see Figure 7). Furthermore, 5′-AMP and substrates also protected reduced phosphorylases against inactivation at low pH. Accordingly, the pH ranges where reduced and native phosphorylases *b* and *a* are optimally active is between 6.2 and 6.4 (*cf.* Helmreich and Cori, 1964b; Pfeuffer *et al.*, 1972a).

Native phosphorylase *b* has an absolute requirement for 5′-AMP for activity whereas phosphorylase *a* saturated with substrates is nearly independent of 5′-AMP for activity (Helmreich and Cori, 1964a,b; Lowry *et al.*, 1964). Since the fluorescence of the bound pyridoxamine-5′-P in reduced phosphorylases *b* and *a* was quenched in a similar manner except that less 5′-AMP was needed in the case of reduced *a*, pH-dependent changes of kinetic parameters were determined with reduced phosphorylase *a*. This made it possible to measure activity with and without 5′-AMP and hence to study the role of the nucleotide. Helmreich and Cori (1964b) have reported an increase in the apparent K_m values of native phosphorylase *a* for glycogen and arsenate on lowering the pH to 6.0. The apparent K_m value of reduced phosphorylase *a* and glucose-1-P at pH 6.1 was more than twice that at pH 6.7, whereas V_{max} was only slightly (about 15%) lower (Figure 8). Incubation of the enzyme at pH 6.1 at 30° with 1% glycogen and 1 mM 5′-AMP restored activity and affinity for glucose-1-P to an even

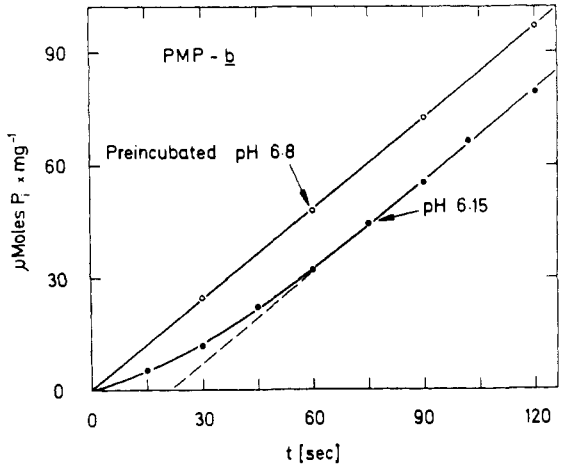


FIGURE 7: Initial velocities of acidified reduced phosphorylase *b*. Enzyme (1.4 mg/ml) was preincubated at pH 6.8 ○—○, and at pH 6.15 ●—●, for one hour and then diluted to a concentration of 22.1 μg/ml for assay at pH 6.8 at 30° as described in Methods.

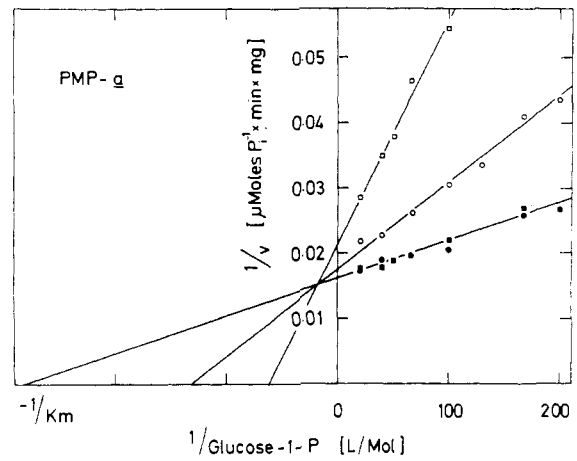


FIGURE 8: Apparent K_m and V_{max} of acidified reduced phosphorylase *a*. Initial velocities were measured as described in Methods. □—□; activity at pH 6.1 and ○—○; activity at pH 6.7 without 5′-AMP; ■—■; activity at pH 6.1 and ●—●; at pH 6.7 in the presence of 1 × 10^{-3} M 5′-AMP.

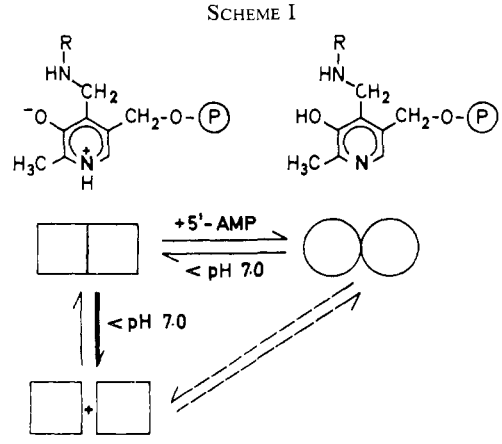
higher level when compared with the same enzyme first neutralized and then preincubated in the absence of 5′-AMP (Figure 8). (Phosphorylase *a* was preincubated with glycogen either with or without 5′-AMP to form an active dimer–glycogen complex (*cf.* Wang *et al.*, 1965; Metzger *et al.*, 1967).) Since either addition of 5′-AMP at low pH or adjustment to neutral pH reactivated reduced phosphorylase *a*, the decrease in V_{max} and the increase in K_m for glucose-1-P at low pH cannot be related to the ionization of glucose-1-P whose pK_2 is 6.2.

Although the structure–activity relationships may be more complex it is quite clear that 5′-AMP not only increased, in concert with glycogen, the rate of reassociation of monomers to oligomers, it also decisively influenced the assembly of active dimers with the highest affinity for substrates. Moreover it stabilized the active dimer conformation. The fact that the structural rearrangement was reported by the pyridoxamine-5′-P cofactor which is essential for activity, invites possibilities for study and speculation on the interdependence of 5′-AMP-induced allosteric transitions and structure and function of the "active" pyridoxal-5′-P site.

Discussion

Scheme I summarizes in the simplest possible way the pH dependent changes of reduced skeletal muscle phosphorylases *b* and *a* and their reversal by 5′-AMP. At the given pH (6.25)

SCHEME I



and at high enzyme concentrations, ≥ 5 mg, fluorescence intensities at 395 nm (corrected for concentration and expressed per milligram of enzyme per milliliter) and molar absorbancies at 334 nm approached constant values (see Figure 4). They are believed to represent the pH-dependent equilibrium between the dimer conformations symbolized by □□ and ○○. With decreasing enzyme concentrations, normalized fluorescence intensities and molar absorbancies rose, indicating that the zwitterionic species of the chromophore increased. Since however both parameters changed proportionately the quantum yield was concentration independent. Absorbance and fluorescence changes and the quantum yield were however concentration and pH dependent, signaling dissociation of the species which absorbs uv light and generates fluorescence (see Figure 4). Dissociation of dimers to monomers was shown to occur in this concentration and pH range by independent measurements in the analytical ultracentrifuge. This led us to assume that the interactions of the chromophore with its environment are very similar if not identical in the monomer and the dimer. This interpretation is compatible with the behavior of the cross-linked reduced dimer *b* (Table I) and with the observation that the high polarization of the chromophore did not decrease on dissociation to monomers. These observations argue against the suggestion that the cofactor is sandwiched between the monomers (*cf.* Jones and Cowgill, 1971). We might add here that even after monomerization was complete (at pH 5.8) fluorescence intensities still increased on further decrease in pH to 5 indicating that still more of the zwitterionic species of the bound cofactor was produced[3] (see Figure 5 and Scheme I). How difficult it is to decide whether the pyridoxal-5′-P binding site in phosphorylase is close to or far from the contact surfaces of the subunits was demonstrated by tritium–hydrogen exchange experiments (Weisshaar and Palm, 1972). These indicated that at least 25% of all potentially exchangeable sites and about 50% of all measurable sites were involved in the interaction of pyridoxal-5′-P with apophosphorylase. The question of the interrelationship between the cofactor binding site and the subunit interfaces in phosphorylase arose again as it was shown that reduced phosphorylase *a* dissociated as readily and completely as reduced phosphorylase *b* at moderately acid pH where native phosphorylases *b* and *a* were completely stable. This was surprising because the introduction of a phosphate group in a seryl residue of the covalent structure in the course of enzymic conversion is known to alter profoundly subunit interactions and to favor tetramer formation in native rabbit muscle phosphorylase. Thus, reduction of the azo–methine bond linking pyridoxal-5′-P to an ϵ-aminolysyl side chain profoundly altered subunit contacts in the phosphorylase oligomer overcoming the quaternary restraint resulting from phosphorylation of the protein. But despite a looser subunit structure reduced phosphorylase *a* was as active, only less stable, as native phosphorylase *a*.

Shaltiel and Cortijo (1970) have suggested on the basis of a comparison of spectral and fluorometric properties of pyridoxamine-5′-P in polar and apolar solvents that the cofactor in native phosphorylase is bound to a hydrophobic region. This could raise a problem if pyridoxal-5′-P should participate in catalysis because the active form of the enzyme would be the one in which the cofactor is embedded in a hydrophobic pocket. In that case it might be more difficult to imagine how protonatable groups of the cofactor, for example the 5′-P group, could participate in phosphorolysis of α-1,4-glucosidic bonds. Actually on the basis of the spectral properties the only group which needs to be hydrogen bonded in phosphorylase is the 3′-hydroxyl group of the cofactor, whereas the pyridinium nitrogen would be unprotonated at physiological pH regardless of whether it is or it is not exposed to solvent. This can be deduced from the ionization scheme of 3′-hydroxypyridine of Metzler and Snell (1955). Incidentally this readily explains why the 3′-*O*-methylpyridoxal-5′-P bound to phosphorylase is still active. However more definitive information on the environment especially of the 5′-P groups of pyridoxal-5′-P bound to phosphorylase requires additional data by other spectroscopic methods. Experiments with ^{31}P nuclear magnetic resonance spectroscopy are in progress.

Although modifications at the 5′ position of the cofactor influenced phosphorylase structure, the structural differences were neither related to the ionization of this group nor to activity. For example, the inactive pyridoxamine and the partially active 5′-deoxypyridoxamine methylenephosphonate phosphorylases already dissociated at near-neutral pH where the active pyridoxamine-5′-P and the corresponding inactive monomethyl ester phosphorylases were stable. Regardless of whether the phosphate group was lacking or protonatable or not (in the pH range studied), these phosphorylase derivatives responded differently to pH changes suggesting that the 5′-P group itself cannot be the group whose ionization affects phosphorylase structure. This directs attention to group(s) in the protein which ionize in this pH range. Incidentally, the moderate pH changes which caused dissociation of the pyridoxamine and of the 5′-deoxypyridoxamine methylenephosphonate phosphorylases would not be expected to have an effect on hydrophobic interactions; electrostatic contacts between subunits are therefore more likely in phosphorylase (*cf.* Kastenschmidt *et al.*, 1968). The behavior of the pyridoxamine-5′-P monomethyl ester phosphorylase deserves a special comment. This analog reconstituted a phosphorylase which was inactive but closely resembled in subunit structure the active pyridoxamine-5′-P phosphorylase (compare Figure 3A and 3B). This analog also appeared in tritium–hydrogen exchange experiments when compared with the natural cofactor, pyridoxal-5′-P equally capable of reconstituting with apophosphorylase *b* the "native" phosphorylase structure (Weisshaar and Palm, 1972). More recently it could be shown that a phosphorylase subunit carrying this analog could form a hybrid dimer with a subunit containing the natural cofactor pyridoxal-5′-P. Although the phosphorylase monomer was nearly inactive, the hybrid enzyme was about half as active as the original dimer. Thus the monomer carrying the inactive analog had properly realigned itself with the potentially active subunit inducing activity in the latter (Feldmann *et al.*, 1972). In this analog compared with pyridoxamine-5′-P only the secondary phosphate group with a pK_2 of 6.2 is blocked and unprotonatable suggesting that this group might have an additional role besides that of a structural ligand. As was pointed out before, it would be an attractive candidate for a proton donor acceptor function in acid–base catalysis (*cf.* Pfeuffer *et al.*, 1972b).

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[3] The fluorescence of free pyridoxamine-5′-P in buffer did not increase between pH 5.8 and 5.0 suggesting a different pH around the bound as compared to the free cofactor.

remove fluorescent impurities. Dr. H. Zeisel ably assisted in the activity assays and Mr. A. Heilos in the preparation of phosphorylase. Professor R. L. Baldwin gave us a helpful hint on the ultracentrifugal measurements.

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