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Evidence for the Binding of Pyridoxal 5′-Phosphate in a Hydrophobic Region of Glycogen Phosphorylase *b* Dimer*

Don Carl Jones† and Robert W. Cowgill

ABSTRACT: Although pyridoxal 5′-phosphate (PLP) is known to be required for the catalytic activity of glycogen phosphorylase, the role of this cofactor in the catalytic process is uncertain. Pyridoxal 5′-phosphate does appear to stabilize the dimeric form of phosphorylase *b*, and several kinds of evidence suggested that this cofactor is buried in a hydrophobic region in the dimer, and perhaps lies between the two monomers. The purpose of this study was to examine the fluorescent properties of phosphorylase *b* and its reduced form for additional evidence that this cofactor is indeed buried in the dimer. An estimate of the rigidity of binding of pyridoxal 5′-phosphate to the apoenzyme also was investigated by polarization of fluorescence. One suggestion of the inaccessibility of pyridoxal 5′-phosphate on the dimer came from the effect of hydroxylamine on the fluorescence of the cofactor. Hydroxylamine reacted directly with bound pyridoxal 5′-phosphate to form a unique fluorescent species only in the presence of "deforming agents," which are known to dissociate phosphorylase *b* to monomers. Another suggestion that pyridoxal 5′-phosphate is buried in the hydrophobic interior of the dimer came from studies on phosphorylase *b* which had been reduced with sodium borohydride. The low fluorescence efficiency of this reduced enzyme was similar to that of pyridoxamine 5′-phosphate in nonpolar solvents. When the reduced phosphorylase *b* dimer was placed in a solution which was known to dissociate dimers to monomers, the fluorescence intensity of reduced phosphorylase *b* increased to that characteristic of pyridoxamine 5′-phosphate free in aqueous solution. Two kinds of observations indicate that pyridoxal 5′-phosphate is firmly bound to phosphorylase *b*. The polarization of fluorescence was near the maximum value for pyridoxal 5′-phosphate and reduced pyridoxal 5′-phosphate bound to both the dimer and monomer of phosphorylase *b*, which suggested that more than one binding interaction exists between the cofactor and the apoenzyme. Also, in connection with the hydroxylamine studies, it was observed that there was energy transfer from tryptophan residues to the pyridoxal 5′-phosphate–hydroxylamine complex. This demonstrated that the complex was still bound to the protein and suggested a binding site in addition to that of the 4′-aldehyde group of pyridoxal 5′-phosphate and the enzyme. A polarization of fluorescence study of reduced phosphorylase *b* in imidazole–citrate suggested that the PLP binding site on the monomer did not change upon dimer dissociation. This implies PLP may be bound to the surface of the dissociated monomer and not buried in a hydrophobic pocket within each monomer. In summary, these fluorescence studies strengthen the hypothesis that pyridoxal 5′-phosphate is firmly bound in a hydrophobic environment between the monomers of the phosphorylase *b* dimer.

The function of PLP[1] on phosphorylase *b* has remained elusive for many years. A catalytic role for the 4′-aldehyde group of PLP has been eliminated (Fischer *et al.*, 1958; Hedrick and Fischer, 1965; Strausbauch *et al.*, 1967), and there is only indirect evidence that the 5′-phosphate (Fischer and Krebs, 1966; Shaltiel *et al.*, 1969) or the ring nitrogen (Bresler and Firsov, 1968) of PLP may be involved in the catalytic process. There is clear evidence that PLP stabilizes the dimeric form of phosphorylase *b*, and perhaps that is its role. PLP could not be removed from the dimer of phosphorylase *b* by carbonyl reagents unless a "deforming buffer" was present to dissociate the dimers to monomers (Shaltiel *et al.*, 1966). Once the PLP was removed, the apoenzyme monomers did not reaggregate to enzymatically active dimers, but to a random assortment of aggregates. Furthermore, PLP or PLP analogs which restored enzymatic activity to apophosphorylase also restored phosphorylase to its dimeric form (Shaltiel *et al.*, 1969).

How PLP stabilizes the dimeric structure, however, remains unknown. The location of PLP may have an important bear-

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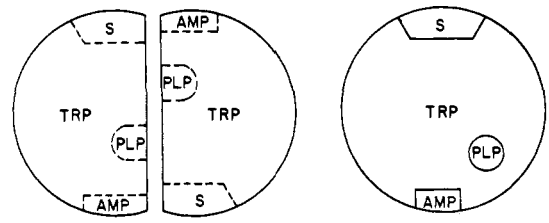
[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; AMP, adenosine 5′-monophosphate; *A* : *E*, activation : emission wavelength maxima, in nanometers.

ing on this question. The observation that PLP on the dimer was inaccessible to carbonyl reagents (Fischer and Krebs, 1966) suggested that PLP was "buried" between the monomeric subunits as illustrated in Figure 1. This was supported by the observation that exchange from [^{32}P]PLP in solution and PLP bound to phosphorylase *b* occurred only when the enzyme was in the monomeric form (Fischer and Krebs, 1966). In addition, Shaltiel and Cortijo (1970) have recently found evidence from fluorescence studies that PLP is in a hydrophobic environment in native phosphorylase *b*.

The concept that the fluorescence characteristics of PLP could be informative about its binding to an enzyme and the detection of enzyme conformational changes has beem employed fruitfully by Churchich in his studies of alanine aminotransferase (Churchich, 1969) and lysozyme (Churchich and Irwin, 1970). The use of fluorescence for the study of phosphorylase was apparently initiated at about the same time in our laboratory and that of Shaltiel (Shaltiel and Fischer, 1967). The purpose of the work reported in this paper was to examine the fluorescent properties of PLP and its reduced form when bound to phosphorylase *b*. Also included were measurements of polarization of fluorescence as a guide to the firmness of binding of PLP to apoenzyme in order to establish whether one or more points of attachment exist. These results in part confirm the reports of Shaltiel and Cortijo (Cortijo and Shaltiel, 1970; Shaltiel and Cortijo, 1970) and in part extend their observations.



GLYCOGEN PHOSPHORYLASE b

FIGURE 1: Postulated relationships between the binding sites on phosphorylase *b*. Left, the dimeric form of phosphorylase *b* suggesting the location of PLP binding sites in the association region of phosphorylase *b* monomers. The juxtaposition of S (substrate) and AMP sites of opposite monomers indicates the requirement of both AMP and the dimeric form for catalytic activity. Right, postulated frontal view of the association region of phosphorylase *b* monomer.

Methods

Phosphorylase *b* (EC 2.4.1.1) was extracted from rabbit skeletal muscle and crystallized according to the procedure of Fischer and Krebs (1962), except that the cysteine buffer was replaced by 0.05 M β-mercaptoethanol–0.05 M β-glycerophosphate. After the third crystallization, the phosphorylase was stored at 4° in an atmosphere containing toluene to prevent bacterial growth. The enzyme was assayed by the method of Hedrick and Fischer (1965). These preparations had specific activities between 70 and 80 units per mg of protein. The enzyme which had been crystallized three times migrated as a single band at pH 9.5 on disc electrophoresis and remained as one band at the point of application at pH 4.3. In a pilot experiment AMP was removed from the phosphorylase *b* stock solution by passage through a charcoal-cellulose column, with complete removal of AMP indicated by an $A_{260}:A_{280}$ ratio of 0.48 (Fischer and Krebs, 1958). Hydroxylamine in imidazole–citrate (as in Figure 5) was reacted with the AMP-free preparation and a preparation in which the AMP concentration had been diluted to about 5×10^{-5} M. No difference in the reaction rate or $A:E$ values were noted. The AMP concentrations were reduced by dilution in all experiments reported in this paper. The approximate AMP concentration in the native phosphorylase *b* experiments (Figures 4 and 5) was 5×10^{-5} M; and in the reduced phosphorylase *b* experiments (Figures 8–10) 1×10^{-5} M.

Phosphorylase *b* was reduced with sodium borohydride according to the method of Strausbauch *et al.* (1967). The reduced phosphorylase *b* was 97% reduced, according to the phenylhydrazine assay (Strausbauch *et al.*, 1967). The specific activity was 71 units per mg of protein before reduction and 21 units per mg of protein after reduction.

Protein was determined either by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the reference standard; or by direct spectrophotometric measurement with the absorbancy index for phosphorylase *b* of $A^{1\%}_{278} = 11.9$ (Appleman *et al.*, 1963). Disc electrophoresis on polyacrylamide gel was done by the general method of Davis (1965). The pH 4.3 electrophoresis was prepared according to the directions of Reisfeld *et al.* (1962), with the gel composition changed to 7.5% acrylamide.

Fluorescence was measured by an Aminco-Bowman spectrophotofluorometer with a 1P28 photocell. The activation and emission spectra were corrected (Chen, 1967) and are accurate within 5 nm. Pyridoxine was used as the reference standard for the PLP and PMP measurements in order to correct for lamp intensity fluctuations. For polarization of fluorescence measurements two Glan-Thompson polarizing prisms (American Instrument Catalogue No. 4-8172) were mounted on a constant-temperature cell holder. The temperature of the cell holder was maintained by a Forma Jr. refrigerated bath. The polarization (P) was calculated from the equation: $P = (I_{vv} - I_{vh}G)/(I_{vv} + I_{vh}G)$, where I_{vv} and I_{vh} are the fluorescence intensities and G is the grating factor, where $G = I_{hv}/I_{hh}$ (Chen and Bowman, 1965).

Materials

PLP, PMP, β-glycerophosphate (Grade I), AMP (Type II and III), and β-D-glucose 1-phosphate (Grade I) were purchased from Sigma Chemical Co. Pyridoxine (U. S. P.) was obtained from Calbiochem and glycogen from Nutritional Biochemicals. Eastman Organic Chemicals supplied the imidazole, which was recrystallized twice from benzene in this laboratory. Urea (Ultra Pure) was supplied by Mann Research Laboratories. All other chemicals used were A. C. S. Reagent grade or the equivalent.

The purity of PLP, PMP, and pyridoxine was verified by paper electrophoresis according to Siliprandi *et al.* (1954), and all three compounds migrated as single bands. PLP and PMP were further identified by fluorometric titrations which were unique for each compound (Bridges *et al.*, 1966). Other reagents used in fluorescence studies were checked for the absence of fluorescence impurities in the spectral region of interest in this study.

Results

Fluorescent Properties of Phosphorylase b. The fluorescence of PLP bound to phosphorylase *b* ("bound PLP") is compared

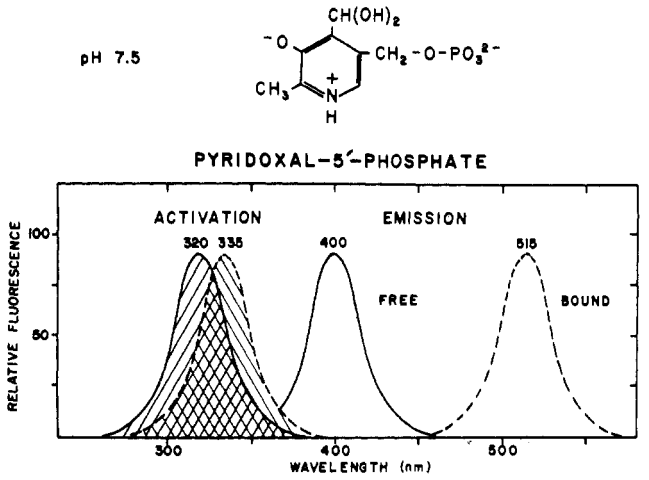


FIGURE 2: Comparison of the $A:E$ spectra of free PLP and PLP bound to phosphorylase *b*. PLP was dissolved in 0.05 M β-glycerophosphate; phosphorylase *b* in 0.05 M β-glycerophosphate + 0.05 M β-mercaptoethanol, pH 7.5 at 23°. At top is the ionic form of free PLP at pH 7.5. Free PLP is indicated by solid lines; bound PLP is indicated by dotted lines.

to the fluorescence of PLP free in solution ("free PLP") in Figure 2. Note that free PLP at neutral pH, with a hydrated aldehyde group and ionic charges as indicated, has activation and emission maxima that are different from the maxima for PLP bound to phosphorylase *b*. In particular, the emission spectrum of bound PLP is shifted to much longer wavelengths.

In addition to fluorescence from the bound PLP, phosphorylase *b* exhibited fluorescence by the Trp residues. Note in Figure 3 (top) that the emission maximum of these Trp residues coincided with the activation maximum of the bound PLP at 335 nm. This suggested that resonance energy transfer (Förster, 1959) from Trp to PLP might occur. Evidence

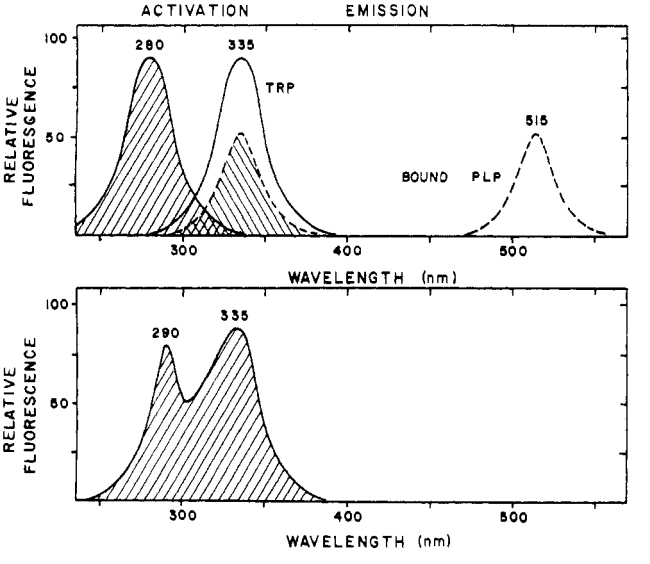


FIGURE 3: The relationship between Trp and PLP fluorescence of phosphorylase *b*. Top: schematic representation of the activation (shaded areas) and emission (unshaded areas) spectra of Trp (—) and PLP (- - - -) of phosphorylase *b* at pH 7.0 and 23°. Bottom: activation spectrum of phosphorylase *b* with emission wavelength at 515 nm.

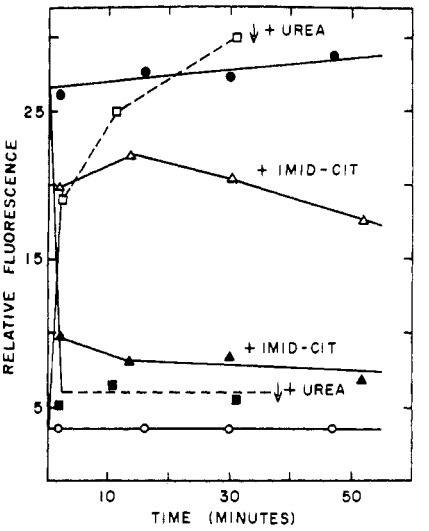


FIGURE 4: The effect of enzyme deforming agents on bound PLP fluorescence. The fluorescence intensity was monitored with time for phosphorylase *b* (6.3 mg/ml) in 0.05 M β-mercaptoethanol–0.05 M β-glycerophosphate (335:515, ●; 420:515, ○); plus 4 M urea (335:515, ■; 420:505, □), or 2 M imidazole–0.4 M citrate (335:515, ▲; 420:505, △). The pH was 7.0 and the temperature was 6°. The arrows indicate the start of protein precipitation in urea.

that energy transfer did actually occur is found in the excitation spectrum of phosphorylase *b* (Figure 3, bottom). The observation of a peak in the excitation spectrum at 290 nm indicated that energy absorbed by Trp at 290 nm was transmitted to PLP and emitted as 515-nm PLP fluorescence. (PLP free in solution rather than bound to protein was not activated by light at 290 nm.) This has also been observed recently by Cortijo *et al.* (1971).

Effect of Enzyme Deforming Agents on PLP Fluorescence. The presence of urea or imidazole–citrate caused a shift of the wavelength for excitation of bound PLP from 335 to 420 nm (Figure 4). In β-glycerophosphate–mercaptoethanol buffer no changes in the 335-nm activation with time were observed. However, in 4 M urea or 2 M imidazole–0.4 M citrate, there was a conversion to the 420-nm form in the first 10 min after adding the phosphorylase *b* to the solution. After 30 min the phosphorylase *b* in urea began to precipitate, but the phosphorylase *b* in imidazole–citrate solution remained clear for more than 50 min. Lower concentrations of either urea or imidazole–citrate produced partial conversion, and required longer time periods. Lowering the pH to 4.0 in β-glycerophosphate–mercaptoethanol buffer also caused this same fluorescence change.

Inactivation of Phosphorylase b by Hydroxylamine as Monitored by Change of PLP Fluorescence and Loss of Activity. Bound PLP is required for phosphorylase *b* to be catalytically active (Illingworth *et al.*, 1958). Comparison of the rates of inactivation of phosphorylase *b* and the reactivity of PLP with aldehyde reagents as monitored by fluorescence indicated that the two processes occurred simultaneously. A deforming buffer was known to be required for both inactivation and PLP removal (Shaltiel *et al.*, 1966). The absence of reactivity of the PLP bound to phosphorylase *b* in the absence of a deforming agent (imidazole–citrate) was observed with hydroxylamine as the carbonyl reagent. Figure 5 demonstrates that phosphorylase *b* was rapidly inactivated in the presence of hydroxylamine and imidazole–citrate. At the same time a new fluorescent species appeared with an $A:E$ = 360:450

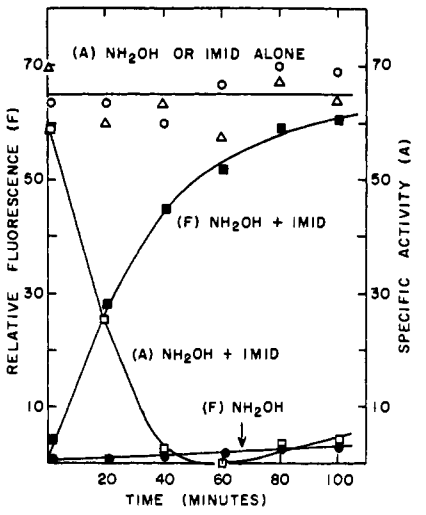


FIGURE 5: The effect of hydroxylamine and imidazole–citrate on the enzymatic activity and PLP fluorescence of phosphorylase *b*. At zero time phosphorylase *b* at 23° was added to 0.5 M imidazole (imid) previously adjusted to pH 7.0 with citric acid (△); 0.1 M hydroxylamine, pH 7.0 (●, ○); or 0.5 M imidazole–citrate + 0.1 M hydroxylamine (pH 7.0) (■, □), all at 23°. At the time intervals indicated, the fluorescence intensities (*F*) of each solution (closed symbols) were recorded at *A*:*E* = 360:450 nm. Aliquots of each solution were diluted 1:100 with assay buffer (see Methods) to prevent further reaction and stored at 23° until they could be assayed for enzymatic activity (open symbols) two hours later. All solutions contained 6.2 mM β-mercaptoethanol and 6.2 mM β-glycerophosphate (pH 7.0) and a final phosphorylase concentration of 2.1 mg/ml.

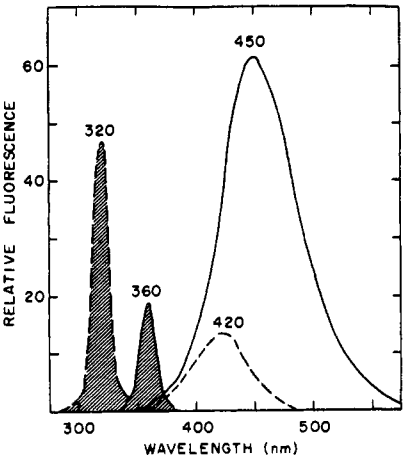


FIGURE 6: Change in *A*:*E* of free PLP in the presence of hydroxylamine. The fluorescence of 2×10^{-5} M PLP in 0.05 M β-glycerophosphate (pH 6.8) (- - - -); and after the addition of 0.1 M hydroxylamine (—). Shaded areas represent scattered activating light; unshaded areas represent emission light. Spectra were recorded 10 min after mixing at 23°.

nm. In the presence of either hydroxylamine or imidazole–citrate alone no change in activity of fluorescence was observed. This new fluorescent species appeared to be a complex of PLP and NH_2OH, because free PLP after mixing with NH_2OH showed excitation and emission maxima (Figure 6) identical with those found for phosphorylase *b* in NH_2OH and imidazole–citrate. After the reaction at least some of the PLP–NH_2OH complex remained bound to phosphorylase *b*, because energy transfer from tryptophan to the complex was found. That is, excitation spectra of the PLP–hydroxylamine complex (emission at 450 nm) demonstrated, in addition to the 355-nm excitation maximum of the PLP–hydroxylamine complex, a second excitation maximum at 295 nm that must arise from absorption by Trp residues on the protein. This energy transfer must occur intramolecularly, for the average distance between molecules is too far for intermolecular transfers in these dilute solutions.

Fluorescence Characteristics of Reduced Phosphorylase b. The Schiff base form of PLP bound to phosphorylase *b* can be reduced with sodium borohydride to form an acid-stable, covalent bond with an ε-amino group of a lysyl residue (Fischer *et al.*, 1958). This reduced PLP ("bound PMP") has a covalent structure different from free PMP only by the linkage of the PMP to a lysyl carbon atom of the protein. This structural similarity to PMP is evident when one compares the fluorescence properties of free PMP and reduced phosphorylase *b*. The observed *A*:*E* was 330:395 nm at pH 6 for both.

At pH 7, however, the fluorescence intensity of bound PMP was less than one-fifth that of free PMP in the same solution. One explanation for this difference might be that the bound PMP was located in an environment within the protein which quenched the fluorescence. This possibility was tested with the model system shown in Figure 7. When the solvent environment of free PMP became more nonpolar with increasing concentrations of ethanol or dioxane, the PMP fluorescence was increasingly quenched. It is probably significant that the fluorescence intensity of the bound PMP on reduced phosphorylase *b* in β-glycerophosphate–mercaptoethanol buffer at pH 7 was as quenched as that of free PMP in 95% EtOH (compare Figure 7 with the fluorescence changes of bound PMP in Figure 8).

This quenching of bound PMP fluorescence could be abolished by procedures known to dissociate and/or denature

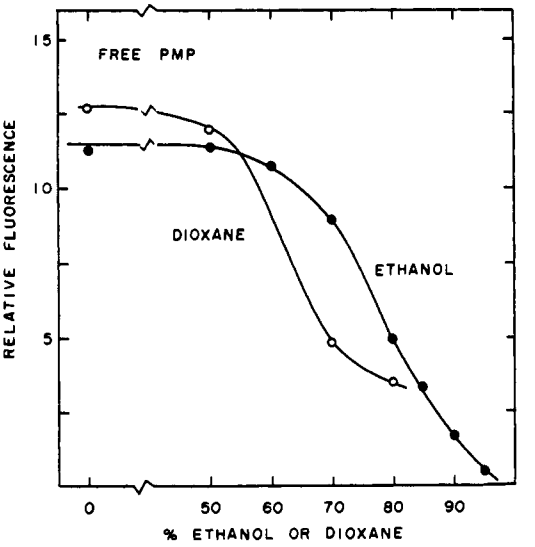


FIGURE 7: Quenching of free PMP fluorescence by ethanol and dioxane. PMP in β-glycerophosphate (pH 7.0) was mixed with ethanol (●) or dioxnae (○) and water to the percentage (by volume) indicated on the graph. Final concentrations were: PMP, 1.25×10^{-6} M; β-glycerophosphate, 2.5×10^{-4} M. Temperature = 23°. The *A*:*E* for PMP in 50% ethanol was 330:395, which gradually shifted to 340:400 in 95% ethanol. The *A*:*E* for PMP remained constant at 330:400 from 50 to 80% dioxane. The *A*:*E* was 325:390 in the absence of any organic solvent. All fluorescence intensities were measured at maximum nm values.

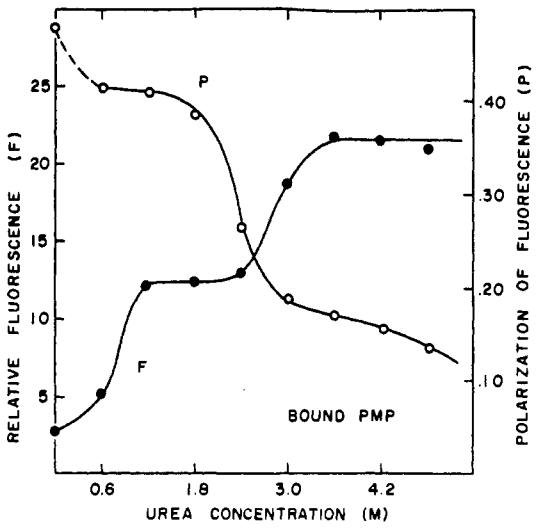


FIGURE 8: The influence of urea on the fluorescence intensity and fluorescence polarization of PMP bound to phosphorylase *b*. Reduced phosphorylase *b* (0.5 mg/ml) in solutions of urea, 0.05 M β-mercaptothanol, and 0.05 M β-glycerophosphate (pH 7.0) at 7°. Fluorescence intensities, *F* (●), were measured 30 min after mixing; and polarization of fluorescence, *P* (○), 60 min after mixing. The fluorescence intensities did not change between 30 and 60 min after mixing. *A*:*E* = 330:395 nm for both *F* and *P* measurements. The fluorescence intensities were corrected for the effect of urea on free PMP (PMP relative fluorescence increased 1.12-fold per 1.0 M increase in urea concentration).

native or reduced phosphorylase *b* (Strausbauch *et al.*, 1967; Hedrick *et al.*, 1969a). Figure 8 demonstrates the increase in bound PMP fluorescence with increasing urea concentrations at pH 7. There was a plateau region between 1.2 and 2.0 M urea where the fluorescence intensity is about one-half that of free PMP; and a second plateau at 3.6 M urea with a fluorescence intensity about the same as free PMP at pH 7. Increasing concentrations of urea also increased the fluorescence of free PMP slightly; this is probably attributable to the commonly observed solvent effect of urea on fluorescence (Cowgill, 1964). Reduced phosphorylase *b* was also exposed to an imidazole–citrate deforming buffer at pH 7. As in the urea experiment at pH 7, the bound PMP fluorescence increased as the imidazole–citrate concentration increased (Figure 9). At 1.0 M imidazole the fluorescence intensity was within 10% of that of bound PMP in 3.6 M urea (Figure 8). No intermediate plateau was evident in the presence of this deforming agent. At pH 6 the initial fluorescence of the reduced phosphorylase *b* even in the absence of imidazole–citrate was as intense as that of free PMP, and there was no change with increasing imidazole–citrate concentrations. To characterize this effect of pH on bound PMP fluorescence, a fluorometric titration of reduced phosphorylase *b* was conducted from pH 7 to 5 (Figure 10). The fluorescence of the bound PMP again increased to an intensity quite similar to that in 4 M urea. A fluorometric titration of free PMP did not show dramatic changes in fluorescence yield in this pH range of 5–7.

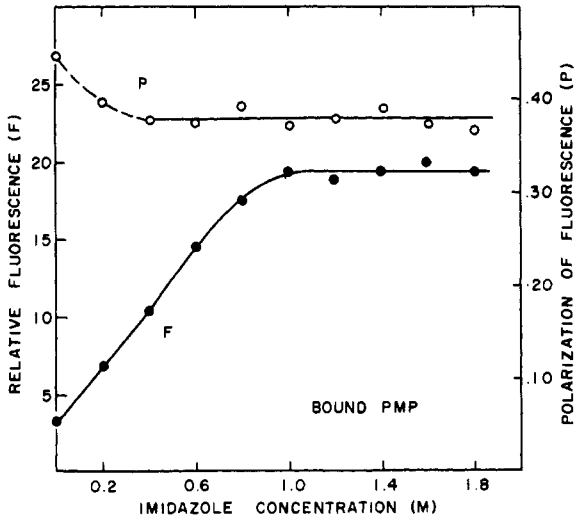


FIGURE 9: The effect of imidazole–citrate on the fluorescence intensity and fluorescence polarization of PMP bound to phosphorylase *b*. The fluorescence intensity, *F* (●), and fluorescence polarization, *P* (○), of reduced phosphorylase *b* (0.5 mg/ml) in imidazole–citrate and 0.05 M β-mercaptoethanol–0.05 M β-glycerophosphate (pH 7.0) at 6°, were measured 30 min after adding the enzyme. The citrate concentration was one-fourth that of imidazole at all concentrations of imidazole used. The *A*:*E* was 330:395 nm. The relative fluorescence was corrected for the effect of imidazole on free PMP. (PMP relative fluorescence increased 1.62-fold per 1.0 M increase in imidazole concentration.) The relative fluorescence scale is the same as in Figure 8.

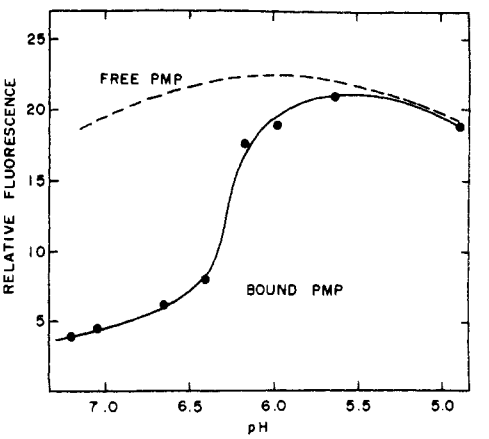


FIGURE 10: The effect of pH on the fluorescence intensity of PMP bound to phosphorylase *b*. Reduced phosphorylase *b* (0.5 mg/ml) in 0.005 M β-mercaptoethanol and 0.05 M β-glycerophosphate, pH 7.2 at 7°, was titrated to pH 4.9 with 1 N HCl. The *A*:*E* was 330:395 nm. The relative fluorescence scale is the same as in Figures 8 and 9. The titration of free PMP is indicated by the dotted line.

Polarization of Fluorescence of Native and Reduced Phosphorylase b. The fluorescence polarization (*P*) of bound PMP in urea (Figure 8) was found to be above 0.40 in urea concentrations up to 1.2 M. Between 1.2 and 3.0 M urea the polarization decreased to below 0.20. These polarization values for bound PMP can be compared to values for bound PLP and free PLP and PMP in Table I. Note that only free PLP and PMP in viscous glycerol had the high values of polarization comparable to those of PLP and PMP bound to the apoenzyme. It is significant that the polarization of fluorescence of bound PMP even in imidazole–citrate (Figure 9) remained high.

Discussion

The fluorescence data in this paper substantiate the conclusions of Shaltiel and Cortijo (1970) that PLP of phosphorylase *b* and its PMP derivative in reduced phosphorylase are buried in the hydrophobic interior of the dimeric form of the enzyme.

Specifically, the following observations are in accord with this proposal and further imply that the PLP may be located between the monomeric units of phosphorylase *b* as illustrated in Figure 1.

(1) The low fluorescence intensity of reduced phosphorylase *b* at pH 7 (Figure 10) could be simulated by unbound PMP in a nonpolar solvent (Figure 7). Shaltiel and Cortijo (1970) have also reported that there was a loss of fluorescence when PMP was transferred from water to a nonaqueous solvent. As reduced phosphorylase *b* is enzymatically active and dimeric at pH 7 (Strausbauch *et al.*, 1967), these observations suggest that PMP is located in a relatively nonpolar environment in the reduced phosphorylase *b* dimer.

(2) The dissociation of reduced phosphorylase *b* dimer to monomers caused a dramatic increase in the fluorescence intensity of the bound PMP. Thus, when reduced phosphorylase *b* was exposed to solvent conditions (Figures 8 and 9) which are known to cause formation of monomers in native phosphorylase *b* (Hedrick *et al.*, 1969a,b), the bound PMP fluorescence increased to an intensity very close to that of free PMP. This implied that the bound PMP, while in a relatively nonpolar environment in the dimer, had become fully exposed to the aqueous solvent in the reduced phosphorylase *b* monomer. This further suggests that the PMP was bound to the monomer in the region where two monomers associate to form the phosphorylase *b* dimer.

The fluorescence intensity of reduced phosphorylase *b* was increased also by lowering the pH. Note in the fluorometric titration of reduced phosphorylase *b* (Figure 10) from pH 7 to 5 that the fluorescence intensity of the bound PMP increased until equivalent to that of free PMP. This pH effect was first noticed in 1963, when fluorescence was not detected from reduced phosphorylase *b* at pH 6.8, but fluorescence similar to that of pyridoxyllysine was found at pH 4.7 (Fischer *et al.*, 1963). The pH effect on reduced phosphorylase *b* fluorescence has also been described by Cortijo and Shaltiel (1970), who found a midpoint for the fluorescence increase of pH 6.1–6.2. These authors suggested that bound PMP is sensitive to changes in environment; and more recently that a "conformational change" exposed the bound PMP to a more polar environment (Shaltiel and Cortijo, 1970). We agree that the environmental change in this case is probably that from a nonpolar to a polar solvent environment. Furthermore, we suggest that the conformational change with pH is probably a dimer to monomer transition of phosphorylase *b*.

The increase in fluorescence of reduced phosphorylase *b* in urea was a two-stage process (Figure 8). The fluorescence intensity of bound PMP in 4.2 M urea is quite similar to the fluorescence intensity of bound PMP in 1.0 M imidazole–citrate (Figure 9) or free PMP at the same pH (Figure 10). This suggests that the bound PMP on reduced phosphorylase *b* is fully exposed to the aqueous solvent in 4.2 M urea. Then what is the form of reduced phosphorylase *b* in 1.8 M urea? As the fluorescence intensity of the plateau at 1.8 M urea was approximately one-half the fluorescence intensity at 4.2 M urea, perhaps only one of the two PMP molecules bound per dimer was exposed to the aqueous solvent at 1.8 M urea. That is, it is possible that the two monomers have opened as if hinged on one side (see Figure 1) to an incomplete dissociated state in 1.8 M urea. This is a relatively stable state, whatever its identity, as the fluorescence intensities did not change for at least 30 min after the measurements were taken. This raises the intriguing possibility that there may be nonidentical dissociation sites on the monomers.

(3) The fluorescence of PLP bound to native phosphorylase *b* dimer was mimicked by model studies of PLP in nonpolar solvents (Shaltiel and Cortijo, 1970). Their studies have shown that the absorption and fluorescence of a Schiff base between free PLP and *n*-hexylamine in chloroform ($A:E$ = 335:535 nm) was identical to that of PLP bound to native phosphorylase *b*. When this model compound was placed in water the $A:E$ shifted to 420:535 nm. This is similar to the shift from $A:E$ = 335:515 to $A:E$ = 420:505 nm that we observed when native phosphorylase *b* was placed in urea or imidazole–citrate (Figure 4), except for an unaccountable difference in the emission maximum reported by the two laboratories. We concur with Shaltiel and Cortijo that, like bound PMP, bound PLP on native phosphorylase *b* dimer appears to be in an environment which is less polar than water. We suggest further that the dissociation of the dimer to monomers in urea or imidazole–citrate allowed the bound PLP, like the bound PMP, to become fully exposed to the aqueous environment. In this connection it is interesting that Johnson *et al.* (1970) have suggested that a tautomeric imine of PLP would have the 330-nm absorption which is observed for native phosphorylase *b*, and that this form would be favored in nonpolar solvents.

(4) Carbonyl reagents reacted only with PLP bound to the monomer form of phosphorylase *b*. Shaltiel *et al.* (1966), using a variety of carbonyl reagents including cysteine and hydroxylamine, could demonstrate no PLP removal from or enzymatic inactivation of phosphorylase *b* in the absence of a "deforming buffer" which caused dimer dissociation to monomers. We have confirmed these results by demonstrating a reaction of bound PLP and hydroxylamine which occurred only in the presence of the "deforming buffer" imidazole–citrate. This reaction was monitored by the appearance of the new fluorescent species of the PLP–hydroxylamine complex (Figure 5). Thus PLP appears inaccessible to hydroxylamine in the dimer. This provides further evidence that the cofactor is buried when phosphorylase *b* is in the dimeric form.

We have used polarization of fluorescence (P) to determine the firmness with which PLP and PMP are attached to the phosphorylase *b* dimer and monomer. A chromophore firmly attached to a protein will rotate in solution at the same rate as the protein, and if the rate of rotation is slow relative to the fluorescence lifetime of the chromophore, then the emitted

TABLE I: Polarization of Fluorescence of Free PLP and PMP and Bound PLP in Viscous and Nonviscous Solvents.

Compound[a]	pH	$A:E$ (nm)	Polarization of Fluorescence: Nonviscous Soln[b]	Polarization of Fluorescence: Viscous Soln[c]
Free PLP	6.0	325:410	0.08	0.39
Free PMP	7.0	330:400	0.04	0.41
Bound PLP	6.0	335:515	0.38	
		420:505	0.40	

[a] Concentrations and temperatures were 2×10^{-5} M and 9° for free PLP; 5×10^{-6} M and 10° for free PMP; 13 mg/ml and 12° for bound PLP (phosphorylase *b*). [b] The nonviscous solution was 0.05 M β-glycerophosphate for free PLP and free PMP; and 0.025 M β-mercaptoethanol–0.025 M β-glycerophosphate–0.06 M imidazole–citrate for bound PLP. [c] The viscous solution was 98% glycerol.

light will be highly polarized. Under conditions where the chromophore does not rotate, the measured polarization is defined as the limiting polarization (P_0) value (Weber, 1952). Thus an observed polarization (P) value of a bound chromophore which is comparable to P_0 indicates firm attachment to the protein. P_0 for free PLP and PMP was 0.4 (Table I) when the chromophores were in a viscous solution of 98% glycerol to restrict their rotation. The value of P (0.38) for bound PMP at all concentrations of imidazole–citrate used (Figure 9) was quite close to P_0, and indicated that PMP was firmly attached to the phosphorylase *b* monomer as well as the dimer. (Churchich (1970) has found a rigid attachment of PMP to aspartate aminotransferase reduced with $NaBH_4$ by this same technique, and observed a polarization of fluorescence value of 0.36.) Polarization was also high for bound PMP in 1.2 M urea (Figure 8), but between 1.8 and 3.0 M urea P decreased sharply to values approaching that of free PMP in aqueous solution (Table I). This suggested that the higher concentrations of urea caused a loss in one or more binding interactions, or perhaps a loss of steric hindrance, between PMP and the protein which allowed the bound PMP to rotate more freely. Dimer dissociation by imidazole–citrate, however, did not disrupt these interactions, as P remained high. Thus, the PLP binding site on the monomer did not seem to change upon dissociation of the dimer. This implies PLP may be bound to the surface of the dissociated monomer and not buried in a hydrophobic pocket within each monomer. Shaltiel and Cortijo (1970) have suggested that PLP may be in a hydrophobic pocket on the phosphorylase *b* dimer surface, and that this pocket "opens" at acidic pH to expose the PLP to the solvent. We suggest that the hydrophobic pocket may in fact be the region between the two monomers of the phosphorylase *b* dimer, and that PLP is bound on the surface of the dissociated phosphorylase *b* monomer and fully exposed to the solvent.

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