INTERACTION OF MUSCLE GLYCOGEN PHOSPHORYLASE WITH PYRIDOXAL 5'-METHYLENEPHOSPHONATE

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Summary: Rabbit muscle glycogen phosphorylase (EC 2.4.1.1) was reconstituted with pyridoxal 5'-methylenephosphonate with *ca.* 25% restoration of enzymatic activity. The modified enzyme has very similar chemical and physical properties to native phosphorylase including UV and fluorescence spectra, quaternary structure, high energy of activation in the reconstitution reaction, optimum pH and susceptibility to phosphorylase kinase in the *b* to *a* conversion. While V_{max} is reduced to *ca.* one-fifth, affinities for the substrate glucose 1-P and the effector AMP are increased. This is the first analog of pyridoxal 5'-P modified in the 5'-position found to restore catalytic activity to apophosphorylase.

The exact role of pyridoxal 5'-P (PLP)‡ in glycogen phosphorylase, whether catalytic or structural, has never been elucidated (1). Recent findings that the PLP-binding site in yeast phosphorylase has been largely conserved (2) lends further support to the view that the co-factor has a direct catalytic function since *Saccharomyces* have separated from the main line of evolution more than a billion years ago.

Reconstitution of rabbit muscle apophosphorylase with analogs of PLP modified in positions 2, 3, 4 and 6 around the pyridine ring (3), resulted in partial or total restoration of activity (4,5). However, a possible involvement of either the pyridine nitrogen or the 5'-phosphate group could not be ruled out. As of now, no PLP derivative modified in the

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‡ Abbreviations used are: Glc-1-P, glucose-1-phosphate; PLP, pyridoxal 5'-phosphate; PLMP, pyridoxal 5'-methylenephosphonate.

5'-position could confer enzymatic activity on apophosphorylase; both pyridoxal and pyridoxal 5'-monomethyl phosphate ester (6) which lack a free 5'-phosphate group, bind to the apoenzyme and restore its allosteric properties, but not its catalytic activity. Pyridoxal 5'-sulfate and 5'-acetic acid (4), which contain acidic groups in the 5'-position cannot reactivate the enzyme either, but then, their pK_a's are well below the physiological range. It seemed therefore of interest to investigate the interaction of phosphorylase with pyridoxal 5'-methylenephosphonate (PLMP) since the phosphonate group is at an atomic distance similar to that of the phosphate and its pK_a is close to neutrality (7.3 - 7.6), although more than one unit above that of the natural cofactor.

Materials and Methods: Rabbit muscle phosphorylase *b* was prepared (7), assayed (8), resolved (9), and reconstituted (4) as previously described. Residual PLP on the apoenzyme was determined according to Wada and Snell (10) and by enzymatic activity measurements. (PLMP)-phosphorylase *a* was prepared from (PLMP) phosphorylase *b* by the action of phosphorylase kinase in the presence of Mg-ATP (11). PLMP was synthesized as described by Hullar (12).

Absorption and difference spectra were determined with a Beckman DK-1 or a Cary 15 spectrophotometer respectively, and fluorescence spectra with a Farrand Model 104244 spectrofluorometer. Buffer consisted mainly of 50 mM Na glycerophosphate, 50 mM mercaptoethanol, pH 7.0, with added 0.4 M NaCl in the case of (PLMP)-phosphorylase *a*. Sedimentation velocity experiments were run in a Spinco Model E analytical ultracentrifuge.

Results: Interaction of pyridoxal 5'-methylenephosphonate with phosphorylase b. Addition of a five-fold excess of PLMP to apophosphorylase *b* resulted in an uptake of stoichiometric amounts of the analog with a 20-27% reactivation (spec. act. 15-20 units/mg) of the enzyme in 10 min

at 37°. As with PLP, inhibition was observed at high concentrations of the analog, undoubtedly as a result of some unspecific binding to the protein (13, 14). Since in one previous instance (reconstitution of apophosphorylase with PLP N-oxide) it was found that restoration of activity was in reality not due to the analog itself, but to PLP generated by deoxygenation of the analog (5, 6), reconstituted enzyme samples were routinely isolated, resolved and characterized as to the chemical nature of the cofactor. Precipitation of (PLMP)-phosphorylase with 0.3 M perchloric acid released material behaving like authentic PLMP on electrophoresis, that is, with a relative migration of 0.51 at pH 3.6 as compared to 1.0 for PLP. Reconstitution was highly temperature-dependent with an energy of activation 27 kcal/mole as compared to 22 kcal/mole for PLP (13).

PLMP appears to be buried within the protein since it does not readily exchange with the natural cofactor. Incubation of apophosphorylase *b* with varying PLP/PLMP ratios indicated, however, that the apoenzyme has a greater affinity for the natural cofactor, as illustrated by the Job Plot in Figure 1: the observed specific activity was higher than

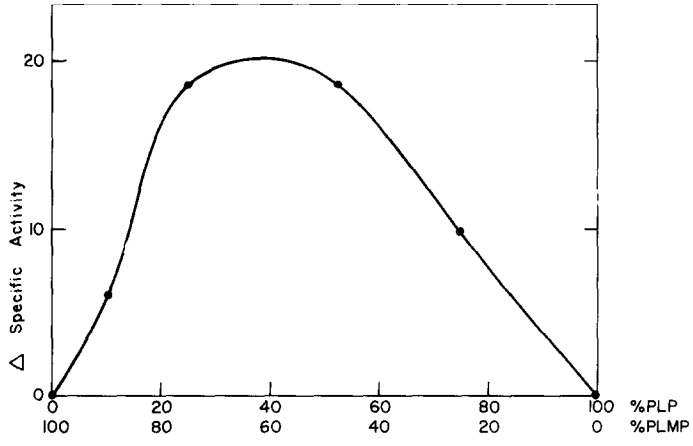


FIGURE 1: Job plot for the reconstitution of apophosphorylase *b* (2 mg/ml) with varying molar ratios of PLP and PLMP (0.1 mM total concentration) for 10 min at pH 7, 30°. The curve represents the difference between the observed and theoretical specific activities assuming that the apoenzyme has equal affinities for both cofactors.

expected had the apoenzyme equal affinities for the two derivatives.

The conversion of (PLMP)-phosphorylase b to a is catalyzed by purified rabbit phosphorylase kinase Mg^{2+} and ATP (15). The final material, (PLMP)-phosphorylase *a*, has a specific activity of 12 and 17.5 units/mg when assayed without and with 1 mM AMP, respectively, as compared to 55 and 85 units/mg for the native enzyme. Both (PLMP)-phosphorylase *b* and *a* were crystallized in sheaves following dialysis (Fig. 2) against NaCl-free buffer but only the latter crystals were stable at room temperature.

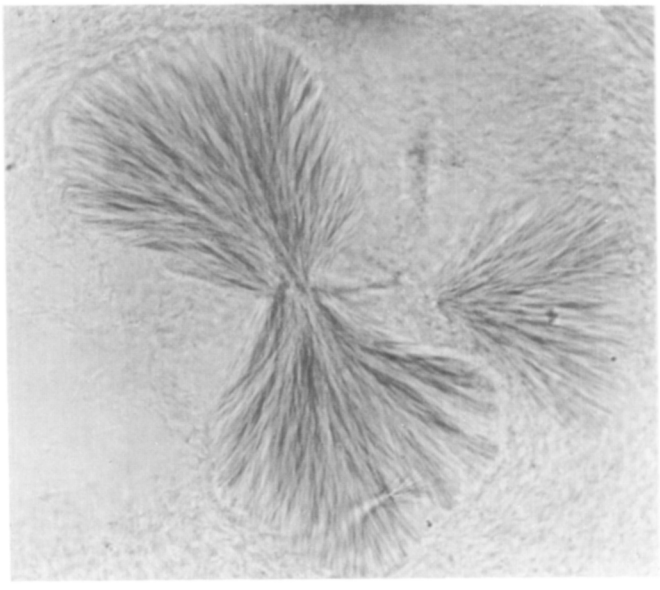
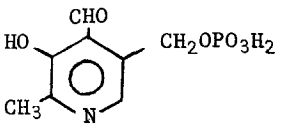
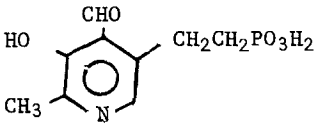


FIGURE 2: Crystals of (PLMP)-phosphorylase *a* magnified 180-fold.

The comparative properties of (PLP) and (PLMP)-phosphorylase b are summarized in Table I. (PLMP)-phosphorylase *b* has an optimum pH essentially identical to that of the native enzyme (Fig. 3) though *ca.* 0.2 pH units higher. On the other hand, it displays an almost ten-fold greater affinity for the substrate Glc-1-P and the effector AMP. Contrary to the native enzyme, binding of AMP to (PLMP)-phosphory-

PROPERTIES OF RABBIT MUSCLE PHOSPHORYLASE *b* RECONSTITUTED WITH:

COFACTOR	PYRIDOXAL 5'-PHOSPHATE	PYRIDOXAL 5'-METHYLENE-PHOSPHONATE
Formula		
pK_a	2.12, 4.14, 6.20, 8.69†	2.87, 4.35, 7.35, 8.75‡
State of Aggregation ($S_{20,w}$)	9.22	9.20
for phosphorylase *a*:	13.23	12.86
Specific Activity	80	20
pH Optimum	6.8	7.0
K_m for Glc-1-P	20 mM	3.1 mM
V_{max}	125	23.8
K_m for AMP	.15 mM	.015 mM

† Perrin, D., *Dissociation Constants of Organic Bases in Aqueous Solution* (1965).

‡ Hullar, T. L., (12).

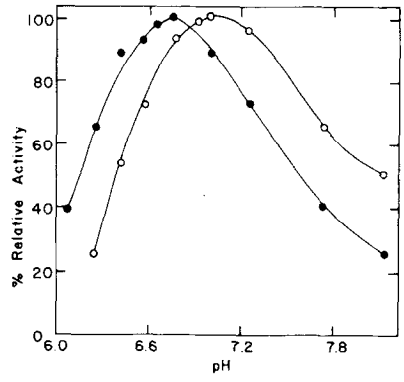


FIGURE 3: pH profile of native *b* (●) and (PLMP)-phosphorylase *b* (o).

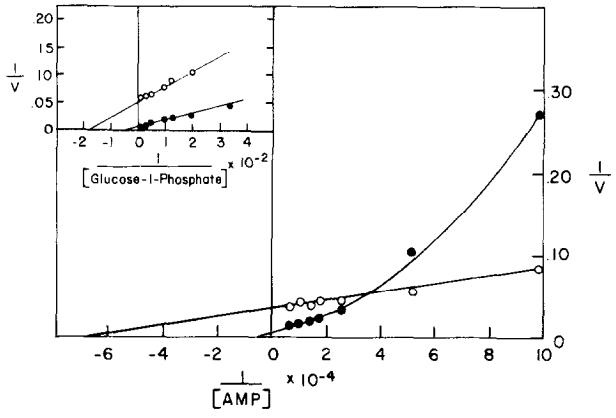


FIGURE 4: Lineweaver-Burk plots of native (●) and (PLMP)-phosphorylase *b* (o), as a function of AMP or Glc-1-P concentration (insert).

lase *b* obeys Michaelian kinetics (Fig. 4). Sedimentation properties are also extremely similar to those of the native enzyme: the analog unequivocally restores the quaternary structure of phosphorylase *b* (dimer), but is perhaps slightly less effective in promoting the subunit association characteristic of phosphorylase *a* (tetramer, Fig. 5).

Absorption and fluorescence spectra of (PLMP)-phosphorylase a and b. Both forms of the enzyme displayed maximum absorption at 332 nm, with absorbance indices of 6090 and 5220 for the *b* and *a* species, respectively.

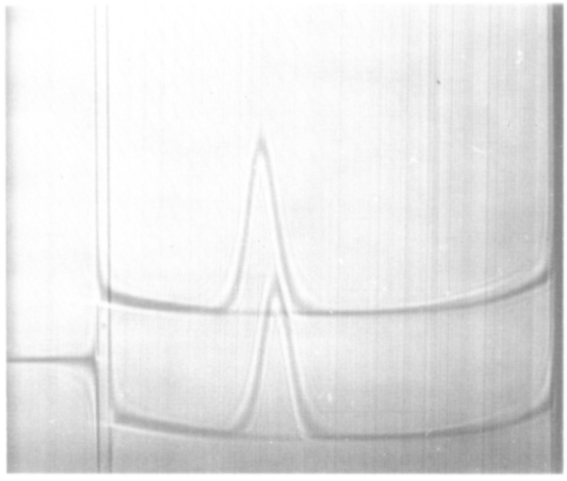


FIGURE 5: Sedimentation velocity analysis of 6.2 mg/ml of (PLMP)-phosphorylase *a* (upper pattern, $S_{20,w}$ = 12.86 S) and 7.17 mg/ml of native phosphorylase *a* (lower pattern, $S_{20,w}$ = 13.23 S). The ultracentrifuge was run at 20°, 48,000 rpm; pictures were taken 32 min after speed was attained.

Attempts to convert the 330 nm into a 420 nm absorbing specie by lowering the pH resulted in precipitation of the protein. Both (PLMP)-phosphorylase *a* and *b* showed maximum fluorescence at 520-530 nm when excited at 335 nm as reported for native phosphorylase (16). Excitation at 280 nm also caused some fluorescence at 520-530 nm as observed with native phosphorylase *b*, in addition to the usual large emission at 330-350 nm resulting from tryptophanyl residues in the protein.

Differential Absorption Spectra of (PLMP)-phosphorylases determined with or without 1 mM AMP and 10 mM Pi were also similar to those displayed by native phosphorylase *b*, with a 360 nm maximum and a 330 nm minimum as reported by Bresler and Firsov (17).

Discussion: While PLMP has been rather ineffective in restoring activity to the apoproteins of several classical PLP enzymes, this is not the case with phosphorylase where most chemical and physical properties remained essentially unchanged (see Table I). Likewise, conversion of

(PLMP)-phosphorylase *b* to *a* proceeded at essentially the same rate as with the native enzyme. The high energy of activation observed during reconstitution with either PLP or PLMP indicates that large changes in conformation must take place.

On the other hand, certain kinetic parameters were altered: maximum velocity was about one-fifth that of the native enzyme despite an increase in affinity for AMP and Glc-1-P. Reduction in V_{max} with an increase in affinities for substrates have been reported for other enzymes reconstituted with PLP analogs. If the 5'-phosphate group functioned merely by interacting with positive residues near the PLP site (*e.g.*, an arginyl side chain two residues removed [18]), it would be difficult to explain why the sulfate or carboxylic derivatives should fail to restore catalytic activity. In enzymatic transamination, the cofactor's 5'-phosphate group and the particular orientation of its oxygen atoms have been postulated to play a role in promoting conformational changes favorable for catalysis (19); clearly, the presence of a C-O-P bond in PLP is not essential for the phosphorolytic cleavage contemplated here. If one assumes that proton donation from the 5'-phosphate group occurs during rupture of the $\alpha(1 \longrightarrow 4)$-glucosidic bond of glycogen (relaxation kinetics have shown a temperature-dependent proton transfer which is first order - or intramolecular - with respect to PLP and second order - or intermolecular - with respect to pyridoxal [20]), then one might expect an upward shift in optimum pH for the PLMP enzyme. While it is difficult to lend significance to the 0.2 pH unit increase in the optimum pH observed here, far more meaningful results might be provided if a study were made concerning the interaction of yeast phosphorylase with this analog since the optimum pH of this enzyme (5.8) is well below the pK of PLMP.

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