Reconstitution of Apophosphorylase with Pyridoxal 5′-Phosphate Analogs*

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ABSTRACT: The purpose of this study was to probe the catalytic role of pyridoxal 5′-phosphate in muscle glycogen phosphorylase (EC 2.4.1.1). Pure apophosphorylases *b* and *a* were reacted with analogs of pyridoxal 5′-phosphate modified in every position around the pyridine ring and the enzymatic activity and state of aggregation of the system were investigated. Slight reactivations observed with high concentrations of pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate could be ascribed to trace contamination by pyridoxal 5′-phosphate. Restoration of enzymatic activity was observed with analogs modified in positions 2, 3, 4, and 6. Removal of the methyl group in position 2 (2-norpyridoxal 5′-phosphate) gave 65% reactivation while the bulkier 2-ethyl derivative did not reactivate the apoenzyme. 3-*O*-Methylpyridoxal 5′-phosphate activated apophosphorylases *b* and *a* 25 and 40%, respectively. None of the analogs modified in position 4 restored enzymatic activity, indicating that a 4-formyl group is required for the binding of the cofactor. Once bound, however, the formyl group can be modified, *e.g.*, by sodium borohydride reduction that covalently fixes the cofactor to the protein with retention of activity. The 6-methyl derivative was also active. Substituion of the pyridine nitrogen and all replacements of the 5′-phosphate group led to inactive products. It is concluded that these two groups, in addition to the 4-formyl group, are required for the binding of the cofactor or enzymatic activity or both. In all instances, restoration of activity was accompanied by restoration of the state of aggregation of the enzyme. However, reaggregation in itself did not confer catalytic activity on the protein since several analogs that restored the quaternary structure of the enzyme yielded inactive products.

The function of PLP[1] in glycogen phosphorylase has never been completely understood since its discovery in the enzyme over ten years ago (Baranowski *et al.*, 1957; Cori and Illingworth, 1957; Kent *et al.*, 1958). In principle, one could visualize three types of functions for this cofactor. First, it could be intimately involved in catalysis participating directly in the bond-breaking or bond-forming reaction. In this instance, the protein would serve to enhance an already existing catalytic function of the cofactor and determine the substrate specificity of the reaction. However, in model systems in which glucose-1-P and P_i were present as substrates (Hedrick and Fischer, 1965) PLP alone displayed none of the catalytic functions of phosphorylase. Moreover, phosphorylase displayed none of the activities typical of other B_6 enzymes. Secondly, PLP could serve as a simple though specific building block required to maintain the structure of the enzyme in an active conformation. Lastly, it could serve to regulate the activity of the enzyme if its level could be physiologically altered.

Removal of PLP from phosphorylase was shown to result in a loss of enzymatic activity that could be partially restored by incubation of the resolved protein with PLP (Cori and Illingworth, 1957; Illingworth *et al.*, 1958). Subsequently, by the introduction of deforming agents and mild conditions during resolution (Shaltiel *et al.*, 1966) apophosphorylase *b* preparations were obtained that regained full catalytic activity upon restoration of PLP; the reconstituted enzyme was indistinguishable from native phosphorylase (Hedrick *et al.*, 1966). Such preparations were ideally suited for the kind of study contemplated here, namely, an attempt to establish which features of the PLP molecule are required to maintain the catalytic function and structural integrity of phosphorylase.

This manuscript reports a comprehensive study of the interaction of both apophosphorylases *b* and *a* with analogs of PLP modified in every single position around the pyridine nucleus. Since phosphorylase *a* does not undergo resolution under mild conditions devised for the removal of PLP from phosphorylase *b*, a new procedure and some of the properties of the resulting apoenzyme *a* are described. This is the sixth publication on the role of PLP in phosphorylase; for the previous publication, see Shaltiel *et al.* (1969).

Materials and Methods

Rabbit muscle glycogen phosphorylase *b* was prepared by the method of Fischer *et al.* (1958) with the modifications described by Krebs *et al.* (1964). Phosphorylase *a* was prepared from phosphorylase *b* using phosphorylase *b* kinase (Krebs *et al.*, 1964). Stock solutions of phosphorylase *b* for resolution were freed from AMP by passage through a small charcoal–cellulose column (Fischer and Krebs, 1958). Apophosphorylase *b* was prepared, characterized, and reconstituted by methods described earlier in this series (Shaltiel *et al.*, 1966,

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[1] Abbreviations used are: PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PLP, PNP, PMP, the corresponding 5′-phosphate derivatives.

1967; Hedrick *et al.*, 1966). Molecular weights of 370,000, 185,000, and 92,500 were used for phosphorylase *a* and *b* and the enzyme monomers as recently determined (Seery *et al.*, 1967; De Vincenzi and Hedrick 1967; Ullmann *et al.*, 1968); reduced phosphorylase *b* was prepared according to Kent *et al.* (1958; see also Strausbauch *et al.*, 1967).

Absorption spectra were taken with a Beckman DK-1 recording spectrophotometer. Sedimentation coefficients were determined in a Spinco Model E analytical ultracentrifuge using either double-sector or wedge window cells. The temperature of the rotor during the run was maintained within ±0.2° of the indicated value. Movement of boundaries was calculated from direct microcomparator measurements of the schlieren diagrams; corrections for viscosity and density of the various buffers were applied in calculating the sedimentation coefficients.

Source of PLP Analogs. PL, PN, and PM and their corresponding 5′-phosphates were obtained from Sigma; 4-deoxypyridoxal 5′-phosphate and pyridoxic acid from Calbiochem. Pyridoxal 5′-sulfate (Kuroda, 1963) was obtained from Wakamoto Pharamaceutical Co., Tokyo, Japan. α^5-Pyridoxalacetic acid (in which the 5-hydroxymethyl group of PLP has been replaced by a propionic acid side chain) was a generous gift from Dr. T. T. Tchen. Synthesis of this derivative was also reported by Iwata and Metzler (1967) under the generic name of 3-(4-formyl-3-hydroxy-2-methyl-5-pyridoxyl)propionic acid or α^5-carboxymethyl-5-deoxypyridoxal. ω-Methylpyridoxal 5′-phosphate and 2-norpyridoxal 5′-phosphate (Mühlradt *et al.*, 1967) were kindly supplied by Dr. E. E. Snell. 3-Hydroxypyridine-4-aldehyde (Heinert and Martell, 1959) was provided by Dr. T. C. Bruice. *N*-Methylpyridoxal (Heyl *et al.*, 1951) and 3-*O*-methylpyridoxal (Heyl and Harris, 1951) were synthesized in this laboratory. Pyridoxic acid 5′-phosphate was prepared by oxidation of PLP with $KMnO_4$ (A. Pocker and E. H. Fischer, unpublished results); this compound had identical properties with those described in the literature (Morrison and Long, 1958). 6-Methylpyridoxal 5′-phosphate was kindly provided by Dr. Karpeisky.

The synthesis of *N*-methylpyridoxal 5′-phosphate and 3-*O*-methylpyridoxal 5′-phosphate are described in the previous publication (Pocker and Fischer, 1969). All other chemicals were the best available from commercial sources.

Determination of PLP. The PLP content in phosphorylase was determined spectrophotometrically after release of the prosthetic group from the enzyme by precipitation of the protein with perchloric acid, added to a final concentration of 0.3 N (Baranowski *et al.*, 1957). A molar extinction coefficient of 6250 at 295 nm was used in 0.3 N $HClO_4$ (Shaltiel *et al.*, 1966). PLP was also determined by the phenylhydrazone procedure of Wada and Snell (1961), or by the use of apophosphorylase *b* according to Shaltiel *et al.*(1967).

Results

Preparation and Properties of Apophosphorylase a. Resolution of phosphorylase *a* is obtained in the following system: 2 mg/ml of protein is diluted in 0.4 M imidazole, 0.1 M sodium maleate, and 0.1 M hydroxylamine adjusted to pH 5.5 with citric acid and incubated at 22°. After 35 min, the reaction mixture is cooled to 0° and precipitated by addition of one volume of saturated ammonium sulfate in 0.05 M sodium glycerophosphate (pH 7.0). The precipitate is centrifuged and the pellet dissolved in 0.02 M Tris buffer containing 0.3 M NaCl and 0.05 M β-mercaptoethanol (pH 7.0); this solution is then filtered through a Sephadex G-25 column equilibrated in the same buffer to remove excess reagents and ammonium sulfate. Because of the marked instability of apophosphorylase *a*, the material obtained was used as soon as possible for reconstitution studies. For this reason and the fact that relatively drastic conditions had to be used for resolution of this form of the enzyme, reactivations of the order of 50% only were generally observed.

Resolution follows first-order kinetics with a half-life of approximately 7 min; this rate is decreased by tenfold if maleate is omitted from the incubation reaction mixture and totally abolished if the pH is increased to 6.0 and the temperature lowered to below 5°. At pH 5.0 or at 37°, resolution is very rapid but accompanied by some irreversible denaturation of the enzyme.

Apophosphorylase *a* crystallizes when held at 0°; crystallization is not increased by addition of 10^{-2} M Mg and 10^{-3} M AMP. The crystals form half-sheaves similar to those obtained for apophosphorylase *b*.

In general, solutions of apophosphorylase *a* were markedly less stable than those of apophosphorylase *b* and their stability was not increased by addition of 10^{-2} M Mg^{2+} and 10^{-3} M AMP, 5×10^{-4} M EDTA, or 1% glycogen. As observed for apophosphorylase *b*, apophosphorylase *a* behaves as a typical associating–dissociating system in which aggregation and disaggregation occur much more readily than with the holoenzyme; this will be discussed in a later section. Dissociation of apophosphorylase *a* increases with temperature (Figure 1) and with the acidity of the medium (31% monomer at pH 7.5, 47% at pH 7.2, and 61% at pH 6.5, 20°). As observed with apophosphorylase *b*, monomerization was prevented by 10^{-3} M AMP and to a lesser extent by 10^{-1} M glucose-1-P. Contrary to the resolution of phosphorylase *b*, glucose-6-P (2.5×10^{-3} M) was without effect.

Interaction of Apophosphorylase b with PLP, PMP, and PNP. Several factors were taken into consideration in this study. Interaction of apophosphorylase with PLP or its analogs may result in the restoration of two of its original properties, namely, enzymatic activity or quaternary structure or both. Therefore, reconstituted samples were routinely examined with respect to these two characteristics. PLP analogs were tested at concentrations varying from 1- to 1000-fold molar excess, since some of these may have a low affinity for the apoenzyme. However, there are inherent difficulties in doing so. Some analogs might be contaminated by traces of PLP that would suffice to reactivate the apoenzyme. Also, PLP (and perhaps some of its analogs) is known to inhibit the enzyme when added in large excess, probably because of interaction with unspecific side chains on the molecule. These difficulties are illustrated in Figure 2; it can be seen that PLP at concentrations above 10^{-3} M (100-fold molar excess or more) strongly inhibits phosphorylase (Figure 2A) and that both PNP (Figure 2B) and PMP (Figure 2C) seem to reactivate phosphorylase when added in a 100:1 or 1000:1 molar ratio. That this reactivation was not genuinely due to the derivatives themselves but to trace contamination by PLP (less than 1 part/10,000) was demonstrated in several ways. (a) If one reduces the stock PMP or PNP solution with $NaBH_4$ (Kent *et al.*, 1958) prior to the reconstitution experiment (a treatment that would convert contaminating PLP to PNP) no significant ac-

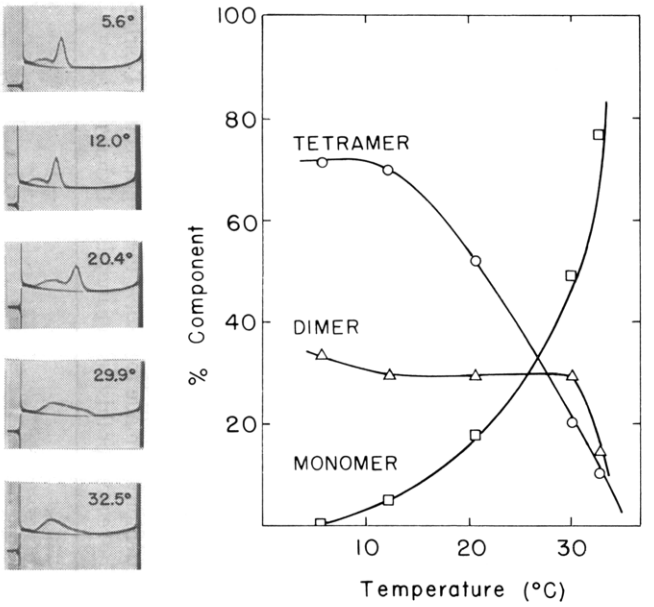


FIGURE 1: Effect of temperature on the sedimentation properties of apophosphorylase *a*. Apophosphorylase *a* (7.25 mg/ml) was dissolved in 0.05 M sodium glycerophosphate–0.05 M mercaptoacetic acid (pH 7.0). On the left, the ultracentrifuge patterns after 24 min at 52,000 rpm. On the right, a rough estimate of the relative proportion of heavy (13–14.5 S), medium (7.5–8.2 S), and light (5.2–6 S) components obtained with the aid of a DuPont 310 curve resolver is plotted as a function of temperature. These components are attributed to tetramer, dimer, and monomer, respectively. At the two highest temperatures, equilibrium is too fast to allow for reliable resolution of the components (Gilbert, 1959).

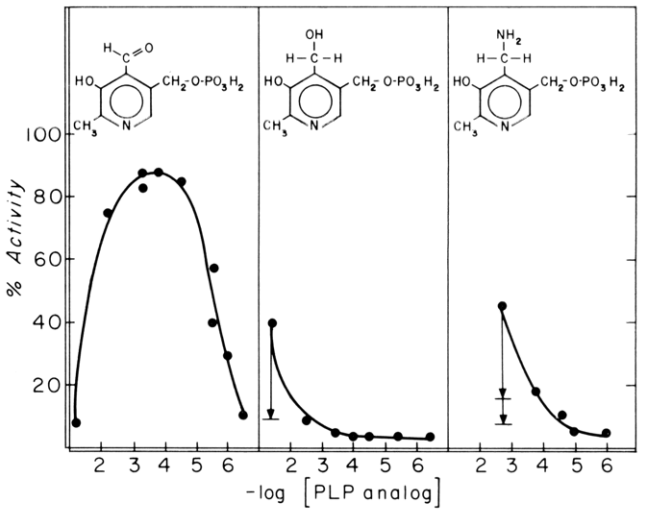
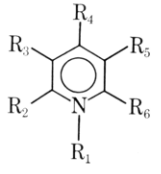


FIGURE 2: Effect of PLP (A), PNP (B), and PMP (C) concentration on the reactivation of apophosphorylase *b*. Reconstitution mixtures contained apophosphorylase *b* (1.2×10^{-5} M), 0.025 M sodium glycerophosphate and 0.025 M β-mercaptoethanol (pH 7.0). Reconstitution was allowed to proceed for 20 min at 30°. Arrows indicate the drop in activity when stock solutions of PNP or PMP were reduced with an excess of $NaBH_4$ before reconstitution.

tivation occurs (see arrows in Figure 2B, C). (b) Apophosphorylase *b* was reconstituted with a 3350 molar excess of PNP and a 420 molar excess of PMP and then subjected to gel filtration on Sephadex G-25. Analysis of the protein fraction for bound cofactor following precipitation with 0.3 N $HClO_4$ showed the presence of PLP only (rather than PNP or PMP), and in amounts commensurate with the observed activity. (c) Freshly prepared solutions of PMP or PNP could be "purified" by addition of apophosphorylase *b:* the apoenzyme specifically trapped the contaminating PLP. After passage through a column of Sephadex G-25 to remove the protein fraction, the remaining solutions of either PNP or PMP no longer reactivated a new batch of apophosphorylase *b*.

The above experiments emphasize the kinds of precaution that have to be taken in interpreting reconsititution data. In the instance of phosphorylase, trace contamination by 1 part of PLP per 10,000 or 100,000 parts of a given analog would result in noticeable reactivation of the apoenzyme; clearly, these would escape detection by the usual chromatographic or physicochemical methods.

Interaction of Apophosphorylase with Analogs of Pyridoxal 5′-Phosphate. Schematically, the general formula for pyridoxal 5′-phosphate analogs can be represented as follows



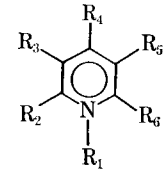
For PLP, R_1 is a proton at acid pH ($pK_a > 4.6$ when the phenolic group is protonated);[2] $R_2 = CH_3$; $R_3 = OH$ ($pK_a = 3.6$ when the pyridine nitrogen is protonated);[2] $R_4 = CHO$ (predominantly in the hydrated form); $R_5 = CH_2OPO_3H_2$; and $R_6 = H$. Reconstitution of apophosphorylase *b*, and in some instances apophosphorylase *a*, was investigated with the analogs listed in Table I. Reactivation occurred only with PLP (I), 2-norPLP (VI), 3-*O*-MePLP (VIII), and the pyridoxyl 5′-phosphate residue covalently bound to the protein by $NaBH_4$ reduction (X). As already pointed out by Illingworth *et al.* (1958) all the nonphosphorylated derivatives were totally inactive, even when the 5′-phosphate group was replaced by other negatively charged derivatives such as a sulfate (XIX) or a carboxyl (XX) group.

The only derivative modified in the one position that was tested, namely, *N*-MePLP (III), did not reactivate either apophosphorylase *b* or *a*. This is an important point in view of the proposal by Bresler *et al.* (1966) that PLP could directly participate in catalysis by binding either P_i or glucose-1-P, two of the substrates of the enzyme, through formation of an ion pair with the pyridinium nitrogen. However, sedimentation experiments indicated that this derivative was not even taken up by the protein since it did not restore the quaternary structure of the enzyme.

The methyl group in position 2 is not essential for enzymatic activity since 2-norPLP (VI) in which the methyl group is replaced by a hydrogen atom will reactivate apophosphorylase *b* by 65%. Surprisingly, this methyl group could not be replaced by the bulkier ethyl side chain: ω-MePLP (V), previously reported to reactivate apophosphorylase *b* to the extent of *ca.* 50% (Illingworth *et al.*, 1958), was inactive in our hands.

[2] Private communication from Dr. D. Metzler. Anderson and Martell (1964) have assigned different pK_a values which could not be confirmed by Morozov *et al.* (1967).

TABLE 1: Reconstitution of Apophosphorylase *b* with Analogs of PLP.



No.	Compound Added[a] to Apophosphorylase *b* or *a*	Position Modified	Ring-Substituent Modified						% Reactivation with		Aggregation[b] State of Apophosphorylase *b*
			R_1	R_2	R_3	R_4	R_5	R_6	Apophosphorylase *b*	Apophosphorylase *b*	
	Control apophosphorylase *b*:								0	0	6.7
	Control apophosphorylase *a*:								0	0	
I	Pyridoxal 5′-phosphate	None							95–100	50–60	8.3
II	Pyridoxal	5					CH_2OH		0	0	8.2
III	*N*-Methylpyridoxal 5′-phosphate	1	CH_3						0	0	7.5
IV	*N*-Methylpyridoxal	1,5	CH_3				CH_2OH		0	0	7.3
V	ω-Methylpyridoxal 5′-phosphate	2		CH_3CH_2					0[c]	0	
VI	2-Norpyridoxal 5′-phosphate	2		H					65	42	8.3
VII	3-Hydroxypyridine-4-aldehyde	2,5		H			H		0		8.4
VIII	3-*O*-Methylpyridoxal 5′-phosphate	3			OCH_3				25	40	8.5
IX	3-*O*-Methylpyridoxal	3,5			OCH_3		CH_2OH		0		7.1
X	Pyridoxyl 5′-phosphate reduced onto phosphorylase	4				CH_2-protein			60		8.1
XI	4-Deoxypyridoxine 5′-phosphate	4				CH_3			0	0	6.7
XII	Pyridoxamine 5′-phosphate	4				CH_2NH_2			0	0	6.9
XIII	Pyridoxamine	4,5				CH_2NH_2	CH_2OH		0	0	6.6
XIV	Isopyridoxal 5′-phosphate	4,5				CH_2OH	CHO		0	0	
XV	Pyridoxine 5′-phosphate	4				CH_2OH			0	0	6.5
XVI	Pyridoxine	4,5				CH_2OH	CH_2OH		0	0	6.7
XVII	Pyridoxic acid 5′-phosphate	4				COOH			0	0	7.7
XVIII	Pyridoxic acid	4, 5				COOH	CH_2OH		0	0	7.3
XIX	Pyridoxal 5′-sulfate	5					CH_2OSO_2H		0		8.2
XX	α^5-Pyridoxalacetic acid	5					CH_2CH_2COOH		0		8.2
XXI	6-Methylpyridoxal 5′-phosphate	6						CH_3	8	4	

[a] Reconstitution was performed in each case in the presence of 2×10^{-3} M of the analog listed except for PLP^+ which was added at a concentration of 1×10^{-4} M. [b] Ultracentrifuge experiments were carried out at 30°, in 0.05 M sodium glycerophosphate–0.05 M β-mercaptoethanol (pH 7.0) in the presence of 2×10^{-3} M of the appropriate analog. Sedimentation constants are given for the composite peak as a rough indication of the behavior of the system; no attempt was made to resolve the diagrams into their individual components. [c] Illingworth *et al.* (1958) reported up to 50% reactivation of apophosphorylase *b* with this analog. The reason for this discrepancy is not known.

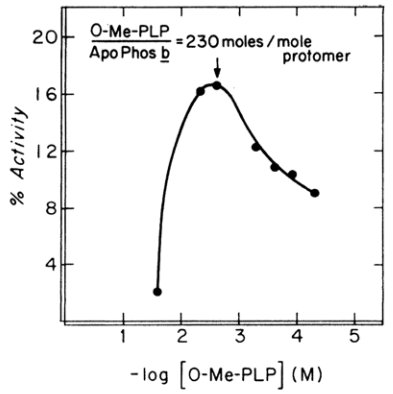


FIGURE 3: Dependence of apophosphorylase *b* reconstitution as a function of *O*-MePLP concentration. Reconstitution mixtures contained apophosphorylase *b* (1 mg/ml or 1.1 × 10^{-5} M), 0.025 M sodium glycerophosphate–0.025 M β-mercaptoethanol, and the indicated concentrations of 3-*O*-MePLP. Reactivation was allowed to proceed for 12 min at 37° (pH 7.0).

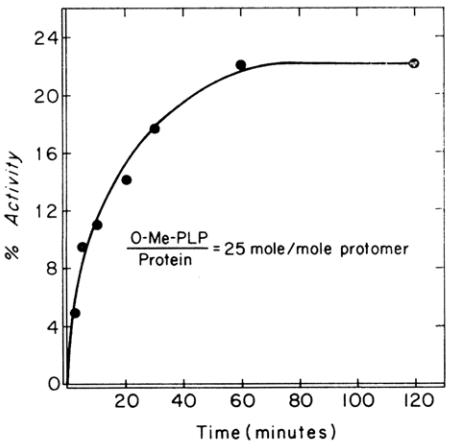


FIGURE 4: Rate of reconstitution of apophosphorylase *b* with 3-*O*-MePLP. Solution contained apophosphorylase *b* (1 × 10^{-5} M), *O*-MePLP (2.5 × 10^{-4} M), 0.025 M sodium glycerophosphate, and 0.025 M β-mercaptoethanol (pH 7.0). Reactivation was allowed to proceed at 37° for the period indicated.

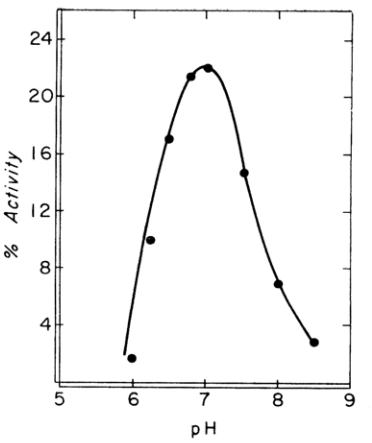


FIGURE 5: pH optimum of activity of 3-*O*-MePLP phosphorylase *b*. The enzyme was assayed as previously described (Hedrick and Fischer, 1965) except that the pH of the dilution buffer and the substrate solution was adjusted to the indicated values with either HCl or NaOH.

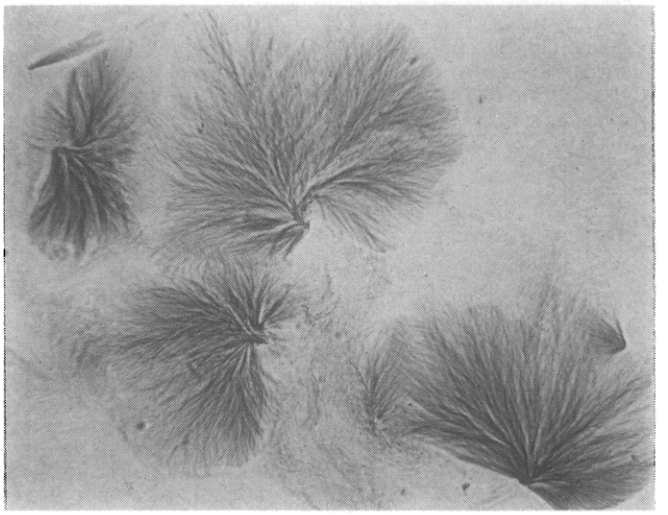


FIGURE 6: Crystals of 3-*O*-MePLP phosphorylase *b*. The enzyme (20.7 mg/ml) was crystallized at 0° from 0.05 M sodium glycerophosphate, 0.05 M mercaptoethanol, 10^{-3} M AMP, and 10^{-2} M magnesium acetate (pH 7.0). Specific activity of the crystals was 13 units/mg or 16% of that of the native enzyme.

It was of interest to determine whether a free phenolic group on PLP was essential for the activity of the enzyme. Being a nucleophile, the phenoxide ion would be a good candidate for catalysis if the cofactor were assigned such a role in phosphorylase; therefore, interaction with this analog was studied in some detail.

3-*O*-MePLP (VIII) was found to reactivate both apophosphorylases *b* and *a*, but reactivation required higher concentrations of this analog and longer periods of incubation as compared with reconstitution with PLP. For instance, a 230-fold molar excess of 3-*O*-MePLP was needed for maximum reactivation, whereas, under the same conditions (12-min incubation at 37°, pH 7.0), complete reconsititution occurred with a 3–5-fold molar excess[3] of PLP (Figure 3). Stoichiometric amounts of PLP would suffice under optimum conditions. At a 25-fold molar excess of 3-*O*-MePLP, maximum reactivation required 60-min incubation (Figure 4) (Hedrick *et al.*, 1966).

The pH dependence of activity of apophosphorylase *b* reconstituted with 3-*O*-MePLP (Figure 5) was found to be extremely similar to that of phosphorylase *b* with an optimum at pH 7.0 as compared with 6.8 for the native enzyme (Hedrick and Fischer, 1965). Like the latter, 3-*O*-MePLP phosphorylase crystallized in the presence of 10^{-2} M Mg^{2+} and 10^{-3} M AMP (Figure 6); the crystals are very similar to those obtained with both apophosphorylases *b* and *a* (see also Hedrick *et al.*, 1966).

3-*O*-MePLP restores the quaternary structure of the holoenzyme: whereas the apoenzyme exists partly in the tetrameric form at a low temperature (1.8°) and in the monomeric form at 30° or higher temperatures, addition of the analog

[3] The molecular weight of phosphorylase is taken as that of the enzyme monomer, *i.e.*, 92,500.

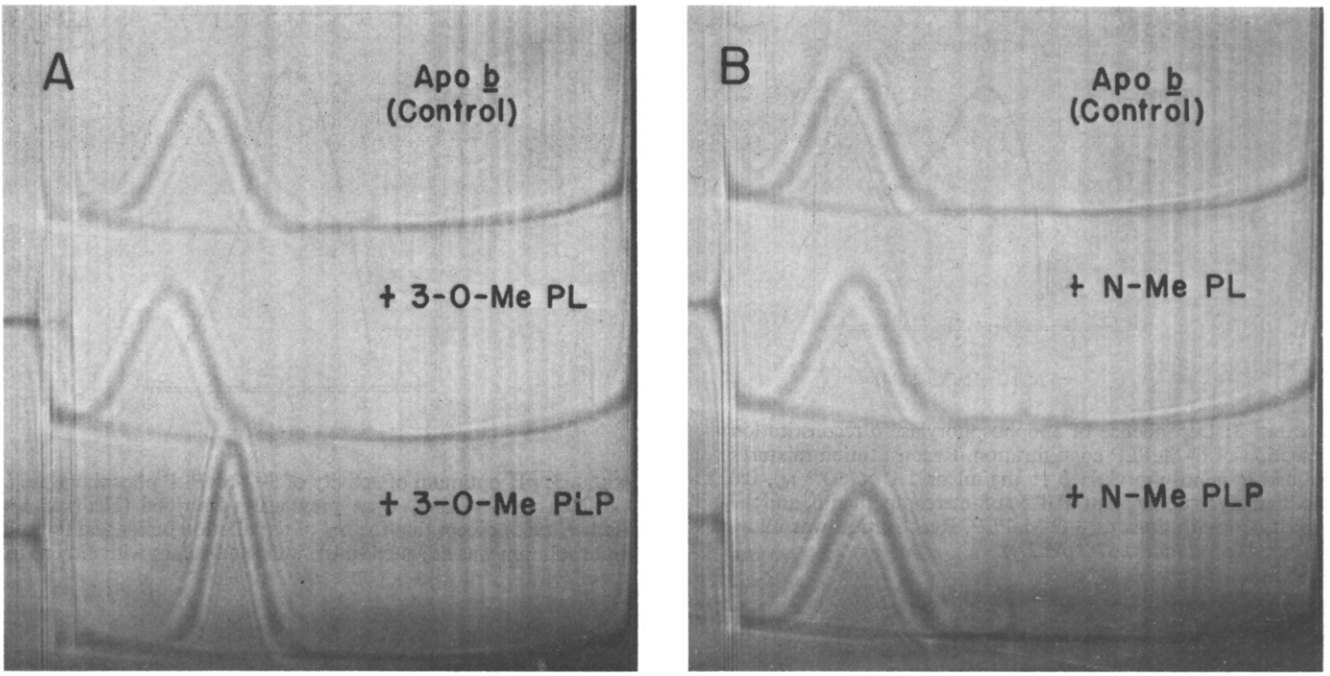


FIGURE 7: Effects of 3-*O*-Me (A) and *N*-Me analogs of PLP on the sedimentation properties of apophosphorylase *b*. The apoenzyme (5.25 mg/ml or 5.7×10^{-5} M) was dissolved in 0.05 M sodium glycerophosphate–0.05 M β-mercaptoethanol (pH 7.0), and incubated with 10^{-3} M of the specified analogs. Ultracentrifuge runs were carried out at 30°. Rotor speed was 52,649 rpm; pictures were taken 24 min after attainment of maximum speed.

will restore the dimeric state characteristic of the native enzyme (see Figure 7).

The aldehyde group in position 4 could not be substituted by either an alcoholic, amino, carboxylic, or methyl function (XV, XII, XVII, and XI, respectively). On the other hand, this aldehyde group cannot be involved in direct catalysis since PLP can be reduced onto the enzyme (X) to yield a material displaying 60% of the activity of the native enzyme.

As already mentioned, all modifications of R_5 led to complete inactivation. A single analog of PLP modified in position 6 was tested, namely, 6-MePLP (XXI) synthesized and kindly provided by Dr. Karpeisky. This compound led to some reactivation of apophosphorylases *b* and *a* indicating that the hydrogen atom in position 6 is not indispensable for enzymatic activity.

Sedimentation Properties of Apophosphorylase b in the Presence of PLP Analogs. One of the most recognizable functions of PLP in phosphorylase is to preserve the quaternary structure of the enzyme. Whereas phosphorylases *b* and *a* are essentially monodispersed in solution (Seery *et al.*, 1967) with structures corresponding to the dimeric ($s_{20,w}$ = 8.2 S) and tetrameric ($s_{20,w}$ = 13.2 S) forms of the enzyme, respectively, removal of PLP results in a polydisperse state typical of freely associating–dissociating systems. Both apophosphorylases can assume predominantly a monomeric state ($s_{20,w}$ = 5.2 S) at 35°, and a tetrameric state or higher states of aggregation at temperatures approaching 0° with mixtures of mono-, di-, and tetramers at intermediate temperatures (Hedrick *et al.*, 1966, and Figure 1). Equilibrium appears to be fast at temperatures above 25° for apophosphorylase *b* and individual components cannot be resolved from the ultracentrifuge patterns (Gilbert, 1959). Because of the technical difficulties of working with apophosphorylase *a*, correlation between reconstitution of enzymatic activity and sedimentation properties was carried out predominantly with apophosphorylase *b*. As seen in Table I, all the PLP analogs that restored enzymatic activity when added to apophosphorylase *b* also restored the state of aggregation of the native enzyme. This is shown in Figure 7 which illustrates typical sedimentation velocity patterns for pure apophosphorylase *b* treated with PLP and two of its analogs. The quaternary structure characteristic of the native enzyme was restored by both PLP and 3-*O*-MePLP but not by *N*-MePLP, an analog that did not reactivate the apoenzyme. However, reaggregation of the protein is clearly not the unique function of the cofactor since several PLP analogs that did not reactivate the apoenzyme nonetheless restored the quaternary structure of the native enzyme. This was the case for pyridoxal (II), pyridoxic acid 5′-phosphate (XVII), pyridoxal 5′-sulfate (XIX), and α^5-pyridoxalacetic acid (R_5 = CH_2CH_2COOH, XX) all of which brought about a reaggregation of the apoenzyme to varying degrees. In fact, the dimeric structure of native phosphorylase *b* was restored by aromatic aldehydes only remotely related to PLP, such as 3-hydroxypyridine-4-aldehyde (VII) or *m*-nitrobenzaldehyde (not listed). The factors involved in the reestablishment of the quaternary structure of the enzymes are not known since many other aldehydes including DL-glyceraldehyde or glycoaldehyde (both added at 1×10^{-3} M) did not restore the state of aggregation of the native enzyme ($s_{20,w}$ = 6.5 and 6.8 S at 30°, respectively).

Discussion

The first question this work attempted to answer concerned the absolute essentiality of the cofactor for phosphorylase catalysis. If it could be demonstrated that the apoenzyme

rigorously freed of PLP possessed some residual activity, then the cofactor could be assigned a subsidiary role such as maintenance of quaternary structure or regulation rather than a direct catalytic function. This proved not to be the case: the apoenzyme lacked enzymatic activity even in assays carried out at high substrate or AMP concentration. Furthermore, no activity was detected at high ionic strength and low temperatures (0–10°) where the apoenzyme reaggregates to dimeric or tetrameric structures. Traces of enzymatic activity found in various apoenzyme preparations were always accounted for by the presence of at least corresponding amounts of residual PLP. Actually, when resolution approached completion, loss of activity often ran ahead of the removal of the cofactor due to some nonspecific irreversible denaturation of the protein.

The apoenzyme has such a high and specific affinity for the cofactor that addition of this protein can serve as a means to remove minute amounts of PLP-contaminating analog solutions. Actually, this procedure was followed routinely whenever a given analog was found to partially reactivate the apoenzyme: the reaction mixture was filtered through a Sephadex G-25 column and apoenzyme was readded to the fractions containing the analog. Lack of reactivation at this point indicated that the reconstitution observed previously was due to contamination by PLP.

The results presented above indicate that substituents in positions 2, 3, and 6 of PLP are not essential for catalysis. The potential aldehyde group in position 4 is necessary for the binding of the cofactor, but not for enzymatic activity since sodium borohydride reduction of the enzyme yields the enzymatically active product (X). Actually, this was the only modification at position 4 that did not abolish catalytic activity. Neither the aldimine nor the secondary amine generated by $NaBH_4$ reduction can by themselves be involved in catalysis. Pyridoxal, which competes with PLP for the cofactor binding site and presumably interacts with the same lysyl residue, is catalytically inactive; no enzymatic activity was generated when the pyridoxal-protein was reduced by sodium borohydride. It is interesting to note that analogs such as pyridoxic acid 5′-phosphate (XVII) that might be expected to bind electrostatically to the lysyl side chain at the PLP site did not reactivate the apoenzyme. The 12% reactivation observed when this analog was added at an 8000-fold molar excess could be entirely attributed to trace contamination by PLP.

Thus, only positions 1 and 5 remain in contention. It has been recently suggested that one of the phosphate ions of PLP ($pK = 6.2$) could be involved in catalysis (Kastenschmidt *et al.*, 1968). There is no evidence for or against this assumption. True, the ascending limb of the pH-dependency curve of the enzyme has a pK around 6.2, but then, purified yeast phosphorylase which also requires PLP for catalysis (L. Neilsen and M. Fosset, unpublished results) has its optimum of activity around pH 5.8 with an inflection point for the ascending limb well below 5. There is, of course, no exchange of the 5′-phosphate group with inorganic phosphate or glucose 1-phosphate, two of the substrates of phosphorylase.

On the other hand, all derivatives of PLP modified in position 5 described herein were found to be inactive,[4] which emphasizes the importance of this group. However, most B vitamins are phosphorylated in their coenzymatic form and it is assumed that the phosphate group is involved in the binding or proper positioning of the cofactor, not in catalysis. If this were also the case in phosphorylase, one might expect that introduction of negative charges in position 5 of PLP might serve a similar purpose, assuming electrostatic interactions between this site and positively charged side chains on the protein. Yet, both pyridoxal 5′-sulfate (XIX) and α^5-pyridoxalacetic acid (XX) were inactive.

There remains position 1. The ring nitrogen of PLP has also been implicated in catalysis (Bresler *et al.*, 1966; Bresler and Firsov, 1968), but again, at the present time, there is no solid evidence for or against this hypothesis. On the basis of the present study, no reactivation occurred with *N*-MePLP. However, this analog reacted poorly if at all with the apoenzyme since it did not restore the quaternary structure of the protein (see also Tate and Meister, 1969).

Then, *N*-MePLP has a positive charge on its pyridinium nitrogen at all pH values at which the enzyme is active. It is difficult to know exactly what is the state of ionization of PLP in the native enzyme, all the more that if the cofactor participates directly in catalysis, its charge distribution might fluctuate during the reaction. On the other hand, 3-*O*-MePLP-phosphorylase is active and, in this derivative, the pyridine nitrogen must exist in the uncharged form at or around neutrality. So it is safe to assume that, at least, the neutral form of PLP is active. Recent evidence from this laboratory (J. Vidgoff) indicates that PLP-*N*-oxide kindly provided by Dr. Fukui also reactivated phosphorylase; these results will be published in detail later.

A structural role for PLP in phosphorylase has been amply documented: clearly, it maintains the enzyme in the proper conformation required for enzymatic activity. Whether it participates in the bond-forming or bond-breaking reaction cannot be unequivocally answered at this time. The participation of several substituents of the pyridine nucleus has been definitely excluded; others still remain in contention but proof of their involvement will require different approaches.

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References

Anderson, F. J., and Martell, A. E. (1964), *J. Am. Chem. Soc.* *86*, 715.

Baranowski, T., Illingworth, B., Brown, D. H., and Cori, C. F. (1957), *Biochim. Biophys. Acta* *25*, 16.

[4] Recently, it was found that pyridoxal 5′-methylene phosphonate (R_5 = $CH_2CH_2PO_3H_2$) in which the ester oxygen of PLP is replaced by a methylene bridge reactivated apophosphorylase *b* by *ca.* 20%. This work, carried out in collaboration with J. Vidgoff and T. L. Hullar (StateUniversity of New York at Buffalo), will be published elsewhere.

Bresler, S., and Firsov, L. (1968), *J. Mol. Biol. 35*, 131.
Bresler, S., Firsov, L., and Glazunov, E. (1966), *Nature 211*, 1262.
Cori, C. F., and Illingworth, B. (1957), *Proc. Natl. Acad. Sci. U. S. 43*, 547.
De Vincenzi, D. L., and Hedrick, J. L. (1967), *Biochemistry 6*, 1967.
Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.
Fischer, E. H., Krebs, E. G., and Kent, A. B. (1958), *Biochem. Prepn. 6*, 68.
Gilbert, G. A. (1959), *Proc. Roy. Soc.* (London) *A250*, 377.
Hedrick, J. L., and Fischer, E. H. (1965), *Biochemistry 4*, 1337.
Hedrick, J. L., Shaltiel, S., and Fischer, E. H. (1966), *Biochemistry 5*, 2117.
Heinert, D., and Martell, A. E. (1959), *J. Am. Chem. Soc. 81*, 3933.
Heyl, D., and Harris, S. A. (1951), *J. Am. Chem. Soc. 73*, 3434.
Heyl, D., Luz, E., Harris, S. A., and Folkers, K. (1951), *J. Am. Chem. Soc. 73*, 3430.
Illingworth, B., Jansz, H. S., Brown, D. H., and Cori, C. F. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1180.
Iwata, C., and Metzler, D. E. (1967), *J. Heterocyclic Chem. 4*, 319.
Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E. (1968), *Biochemistry 7*, 3590.
Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 549.
Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964), *Biochemistry 3*, 1022.
Kuroda, T. (1963), *Bitamin (Kyoto) 28*, 21.
Morozov, Yu. V., Bazhulina, N. P., Cherkasina, L. P., and Karpeisky, M. Ya. (1967), *Biophysics 12*, 454.
Morrison, A. L., and Long, R. F. (1958), *J. Chem. Soc.*, 211.
Mühlradt, P. F., Morino, Y., and Snell, E. E. (1967), *J. Med. Chem. 10*, 341.
Pocker, A., and Fischer, E. H. (1969), *Biochemistry 8*, 5181.
Seery, V., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5*, 2108.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1967), *Methods Enzymol. 11*, 675.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1969), *Biochemistry 8*, 2422.
Strausbauch, P. H., Kent, A. B., Hedrick, J. L., and Fischer, E. H. (1967), *Methods Enzymol. 11*, 671.
Tate, S. S., and Meister, A. (1969), *Biochemistry 8*, 1056.
Ullmann, A., Goldberg, M. E., Perrin, D., and Monod, J. (1968), *Biochemistry 7*, 261.
Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.