Paradoxical Inhibition of Phosphorylase by Pyridoxal Phosphate

II. CHARACTERIZATION OF THE PYRIDOXAL PHOSPHATE-PHOSPHORYLASE DERIVATIVE*

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SUMMARY

Reaction of phosphorylase b with pyridoxal-P followed by reduction with sodium borohydride results in a derivative which shows a homogeneous band upon polyacrylamide gel electrophoresis. This modified enzyme has a dimeric form with an average S20w of 8.5. Unlike the native phosphorylase b, the aggregation state of this derivative is not affected by the presence of AMP. The evidence suggests that pyridoxal-P reacts with one specific site per monomer, and further reactions show only minimal effects on the properties of the enzyme. Imidazole citrate, which causes dissociation of phosphorylase b, has the same effect on the pyridoxal-P-phosphorylase b derivative. Absorption spectra of the reduced pyridoxal-P-phosphorylase b show an increased absorbance at 325 nm. Fluorescence spectra show two peaks upon excitation at 325 nm, one peak at 535 nm found in the native enzyme, and a peak at 400 nm characteristic of pyridoxamine derivatives. Crystals of the reduced pyridoxal-P-iodoacetamide-phosphorylase a have been prepared in the presence and absence of AMP. The reduced pyridoxal-P-phosphorylase b derivative shows different kinetic and allosteric properties from the native enzyme, most notably in that the Kₐ values for substrate and activator have been greatly increased with no change in Vₘₐₓ. In addition, the nature of the homotropic interactions between substrate sites has been altered, whereas that between activator sites has been abolished.

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Glycogen phosphorylase has been studied extensively concerning its kinetic and physical properties and the changes induced by various reagents. Of particular interest in this paper are the changes in regulatory properties induced by reaction of one amino group with pyridoxal-P. After removal of the prosthetic group, pyridoxal 5'-phosphate, from phosphorylase the enzyme activity is lost (3–5). Incorporation of one mole of pyridoxal-P into apophosphorylase restores enzymic activity and quaternary structure (6). Incorporation of one more pyridoxal-P group is accompanied by changes in regulatory activity, aggregation-disaggregation properties, and spectral characteristics. The purpose of this investigation is to explore the relationship between such reagent-induced effects and the function of the enzyme by means of specific amino acid modification. Under conditions used by other authors, modification by KCNO resulted in an inactive enzyme with 20 altered amino groups (7), whereas modification by glutaraldehyde (8) involved 7 to 11 amino groups, and modification by dinitrophenylation involved 4 to 5 lysyl residues (9). In our studies, under specified experimental conditions, modification of only one lysyl group is achieved. Controlled modification of phosphorylase b by pyridoxal P decreased the sensitivity to ATP inhibition, changed the allosteric properties, and increased the stability of the dimeric form in solution.

MATERIALS AND METHODS

Rabbit muscle phosphorylases b and a were prepared and assayed by the methods given in the preceding paper (1). Methods for the preparation of reduced pyridoxal-P-phosphorylase derivatives and iodoacetamide-phosphorylase derivatives and the sources of chemicals were also given in the preceding paper (1). Ultracentrifugal sedimentation analyses were performed with an analytical ultracentrifuge (Beckman Spinco, model E) operated at a speed of 60,000 rpm and a temperature of 20°C.

RESULTS

Aggregation State of NaBH₄-reduced Phosphorylase b Derivative—The reduced pyridoxal-P-phosphorylase b derivative was subjected to disc gel electrophoresis according to the method of Chignell et al. (10) and Davis (11), using a pH of 7.8 for the small pore gel. Preparation of the gel column was by the method of Davis et al. (12). Only a single band of protein was seen in the gels. This contrasts with the results obtained with phosphorylase modified by isocyanate (7) or glutaraldehyde (8): multiple bands were seen which indicate the appearance of heterogeneous fractions as the result of different extents of modification and different aggregation states (10). Thus heterogeneity of both charge and size would appear to be ruled out for the reduced pyridoxal-P derivative of phosphorylase b.

This derivative moved faster toward the anode than did the native enzyme, a result to be expected from the replacement of one positive charge by two negative charges.

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Samples of the reduced pyridoxal-P-phosphorylase b derivative containing 1.4 moles of pyridoxal-P per mole of monomer and retaining 39% residual activity (measured with 16 mM glucose-1-P) were subjected to analytical sedimentation velocity studies. A single symmetrical peak with an $S_{obs}$ of 8.8 was obtained. Similar results were obtained with other preparations, indicating that the incorporation of pyridoxal-P did not affect the aggregation state of the protein, which retained its dimeric form and its molecular weight homogeneity.

In other studies we found that 0.4 M imidazole citrate, which causes the dissociation of phosphorylase b into monomers (13), caused the same result with the reduced pyridoxal-P derivative. Other workers have noted that AMP promotes association of phosphorylase b into tetramers, with the appearance of broad diffuse peaks in the ultracentrifuge (14, 15). In contrast, the reduced pyridoxal-P derivative in the presence of 1, 5, and 12 mM AMP exhibited single symmetrical peaks with $S_{obs}$ values of 8.4, 8.4, and 8.2, respectively.

Spectral Studies—The reaction of phosphorylase with pyridoxal-P is accompanied by profound changes in the absorption and fluorescence spectra. The preceding paper discussed the absorption spectra (1). The fluorescence spectra indicate that when the reduced pyridoxal-P-derivative is excited at 325 nm two major emission peaks are seen. One peak at 535 nm is typical of the pyridoxal-P coenzyme in the native enzyme (16, 17) and is not altered by adding extra pyridoxal-P. The other emission, at 400 nm, increases with increasing incorporation of pyridoxal-P and is typical of a pyridoxamine 5-phosphate derivative (18, 19). The fluorescence spectra described here are illustrated for phosphorylase a in Fig. 1; the results for phosphorylase b are very similar.

Preparation of Phosphorylase a from Reduced Pyridoxal-P-Phosphorylase b—It was of interest to determine whether the incorporation of pyridoxal-P into phosphorylase b would affect the sites necessary for the action of phosphorylase b kinase. The kinase was allowed to act on the modified phosphorylase b by the method of Fischer and Krebs (20), and enzymic activities were examined at 0, 15, and 45 min. The results of the latter test are shown in Table I. In addition to the reduced pyri-

![Fig. 1. Fluorescence emission spectra of phosphorylase a and pyridoxal-P derivative. Spectra were obtained in 0.02 M glycerophosphate-0.0015 M EDTA buffer (pH 6.8); protein concentration was 0.15 mg per ml. Pyridoxal-P-phosphorylase a was obtained as described previously, by reacting enzyme with different concentrations of pyridoxal-P for 20 min, reducing with NaBH$_4$, and dialyzing. The excitation was at 325 nm, and the spectrum was followed from 300 to 650 nm. Curves 1 and 2 show a control sample of native phosphorylase a with a maximum peak at 535 (scale expanded 10-fold over that used for peak at 325 nm). Curves 1 to 4 have two peaks, at 395 and 535 nm. The protein in Curves 1 to 4 incorporated 0.19, 0.54, 0.60, and 0.86 moles of pyridoxal-P per mole of monomer, respectively.]

![Fig. 2. Crystals of pyridoxal-P-phosphorylase a. A, grown in 1.2 M (NH$_4$)$_2$SO$_4$ at $10^5$, pH 7.5; B, grown as in A but in the presence of 1 mM AMP.]
The incorporation of pyridoxal-P or the alkylation of the reactive —SH groups did not affect the extent of conversion. However, the two treatments together did result in a lower specific activity of the resultant phosphorylase a. Nevertheless, the ratio of activity without and with 1 mM AMP indicates that no phosphorylase b was left unconverted.

**Crystalization of Reduced Pyridoxal-P-Phosphorylase a Derivatives**—It has been possible to grow large crystals of native phosphorylase a or of derivatives in which the two reactive —SH groups on each monomer have been alkylated or reacted with methyl mercury (21). Phosphorylase a was treated with 1 mM [4°C]iodoacetamide to alkylate the reactive —SH groups. It was isolated and analyzed for catalytic activity (100%) to confirm our results the —SH groups of peptides B₁ and B₂ were alkylated. Phosphorylase a was then reacted with 2 mM pyridoxal-P for 4 min before being reduced with NaBH₄; it was then dialyzed against 0.02 M glyceraldehyde-0.0015 M EDTA (pH 6.8) at room temperature. The protein contained 1.15 moles of pyridoxal-P per mole of monomer. Crystals were grown at 16° by the Zeppezauer technique (22), by using ammonium sulfate concentrations which were increased from 1.0 to 1.2 M over a 20-day period.

Fig. 2A indicates that large single crystals of the modified phosphorylase a could be grown by this technique. The presence of 1 mM AMP in the medium changed the crystal habit drastically, resulting in large needles and rosettes, as shown in Fig. 2B. A similar effect of AMP on the crystal habit of native phosphorylase a has been noted (23).

**Kinetic Analysis**—As mentioned in the preceding paper, the different specific activities of the reduced pyridoxal-P-phosphorylase b derivative at low and high substrate concentrations suggested a changed Michaelis constant rather than an altered $V_{max}$. The reciprocal plot shown in Fig. 3, in which glucose-1-P is the variable substrate, confirms this suggestion. The typical effect of ATP on the native enzyme is shown as well. The pyridoxal-P derivative shows a curved line both in the absence and presence of ATP, whereas a Hill plot of the data indicates that the homotropic cooperativity is not increased by ATP. Fig. 3 shows clearly that the $V_{max}$ of the modified enzyme is the same as the native whereas the $K_m$ has increased. This data is summarized in Table II.

When the concentration of the activator, AMP, is varied, the reciprocal plot shown in Fig. 4 is obtained. Again the $K_m$ for AMP is increased. The decrease in $V_{max}$ shown in Fig. 4 is not due to an intrinsic change in maximal catalytic efficiency but to the fact that the increased $K_m$ for glucose-1-P results in a low activity at the finite substrate concentration used in the experiment. This is documented in Table II, where the data are summarized.

In addition, we may note from Fig. 4 that although ATP can still inhibit the modified enzyme, it does not invoke a positive homotropic cooperativity of the AMP binding sites.

**DISCUSSION**

The reduced pyridoxal-P-phosphorylase b derivative may be of considerable use in studying allosteric properties of phosphorylase because abolition of homotropic cooperativity for AMP can be shown, accompanied by increased $K_m$ values for AMP, glucose-1-P, and Pᵢ, whereas the $V_{max}$ is unaltered. For example, the inhibition by ATP, as shown in Fig. 4, is clearly competitive with AMP. Since, unlike the native enzyme, interpretation is not obscured by curved lines, this suggests that ATP binds to the same site as does AMP.
Michaelis constants for unmodified and modified phosphorylase b derivatives

Phosphorylase b reaction with 2 mM pyridoxal-P was stopped 30 min after addition of NaBH₄. Modified phosphorylase b contained 1.42 moles of pyridoxal-P per monomer of enzyme and 30% residual activity measured in substrate with 1% glycogen, 1 mM AMP, and 16 mM glucose-1-P, and 55% of activity measured with 75 mM glucose-1-P.

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<td>Native (control)</td>
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<td>Reduced-pyridoxal-P-phosphorylase b derivative</td>
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<td>0.82</td>
<td>29</td>
<td>0.98</td>
<td>34</td>
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* Calculated from the Michaelis-Menten equation from the measured Vₘₐₓ, the appropriate Kₘ for glucose-1-P, and the concentration of glucose-1-P used (25.6 mM).

Kₘ for AMP while lowering the Vₘₐₓ (9). The most interesting effect was that reported by Kastenschmidt et al. (24), who showed that when 5,5'-dithiobis(2-nitrobenzoic) acid reacted with two sulfhydryl groups per monomer the Vₘₐₓ was unchanged, whereas the Kₘ for AMP increased and there was a loss of homotropic cooperativity for AMP. Since the two sulfhydryl groups reacted with 5,5'-dithiobis(2-nitrobenzoic) acid were most likely those of the B₁ and B₂ peptides, it is interesting that alkylation of these same sulfhydryl groups did not change the allosteric properties (25). A definite effect of the type of reagent in the same site is therefore seen.

The reduced pyridoxal-P-phosphorylase b derivative is easily prepared, quite stable, and shows different enzymic properties from the derivatives reported above. Further investigation of its physical, chemical, and enzymic properties may prove useful for defining the nature of the subunit interactions of the phosphorylase b dimer.

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REFERENCES

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