The Purification and Mechanism of Action of Yeast Phosphoglucomutase*

Ernest E. McCoy and Victor A. Najjar

From the Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee

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Phosphoglucomutase is present in tissue extracts of various organisms and catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate (1). The mechanism of this conversion was worked out for the crystalline rabbit muscle enzyme (2, 3) and consists of two reversible steps. In the forward reaction it involves the transfer of phosphate from phosphoenzyme to glucose 1-phosphate to yield glucose 1,6-diphosphate. In the second step, the glucosyl phosphate is transferred to the resulting dephosphoenzyme to produce glucose 6-phosphate and phosphoenzyme. The phosphoenzyme is then available for a repetition of the cycle.

Glucose 1-phosphate + phosphoenzyme
Step 1
Glucose 1,6-diphosphate + dephosphoenzyme
Step 2
Glucose 6-phosphate + phosphoenzyme

It was possible to identify each component of the rabbit muscle enzyme system and derive the equilibrium constant of each step (3, 4). At pH 7.5 and 30° the constants for the first and second reactions were 4.58 and 3.76, respectively. The ΔΦ0 of hydrolysis of the enzyme-phosphate bond was found to be about −3900 calories. Acid hydrolysis (5) as well as proteolysis of the phosphoenzyme (6) yielded phosphoserine or peptides containing phosphoserine.

It was possible to purify phosphoglucomutase from bakers' yeast and obtain it free from interfering enzymes, thus making it amenable for similar exploration. The properties and mechanism of action were found to be similar to those of the muscle enzyme. The following findings, reported below in detail, support this conclusion. For full activity the enzyme requires a metal-binding agent, Mg++, and glucose 1,6-diphosphate.

EXPERIMENTAL

Materials and Methods

The source of the enzyme was bakers' yeast (Fleischmann). Glucose 1-phosphate (Nutritional Biochemicals) was used for all enzyme assays in the purification procedures. It contained glucose 1,6-diphosphate in sufficient quantity to saturate the enzyme and thereby obtain full activity under the conditions of the assay. For the study of the enzyme mechanism, glucose 1-phosphate, glucose 6-phosphate, and glucose 1,6-diphosphate, obtained from Schwarz Laboratories, were used. The glucose 1,6-diphosphate preparation contained no glucose 1-phosphate and 0.12 mole of glucose 6-phosphate per mole of the diphosphate. The monophosphate samples were free of any measurable glucose 1,6-diphosphate.

Glucose 1-phosphate was assayed by its hydrolyzable phosphate liberated by 1 N H2SO4 in 3 minutes at 100° or by enzymatic conversion to acid-stable glucose 6-phosphate. The latter was measured with the combined glucose 6-phosphate and 6-phosphogluconic dehydrogenase system as described below. Glucose 1,6-diphosphate was assayed by its coenzymatic activity (3, 8) or as glucose 6-phosphate after acid hydrolysis in 0.1 N HCl for 10 minutes at 100°.

Enzyme activity measurements were made as previously described for the muscle enzyme and based on the disappearance of acid hydrolyzable phosphate under standard optimal conditions of glucose 1-phosphate 4 × 10⁻⁴ M, glucose 1,6-diphosphate 1.5 × 10⁻⁴ M, Mg++ 1.2 × 10⁻² M, histidine 4 × 10⁻² M, pH 7.5, and 30°. The unit of activity is expressed as milligrams of acid stable phosphorus produced in 5 minutes (1). Phosphorus was measured as the reduced molybdate with 0.05% semidine hydrochloride (Eastman) (9). Because of the occasional presence of inhibitory substances in distilled water, deionized distilled water was used throughout this study.

Purification Procedure—Air dried fresh yeast, 400 g, was autolysed at 38° for 4 hours in 1000 ml of Na2HPO4, 7 × 10⁻² M, containing 1 ml of toluene. It was then centrifuged at 13,000 × g for 10 minutes. The precipitate was extracted with 500 ml of the phosphate solution and centrifuged. The combined supernatants, about 900 ml, had a pH of about 5.5 and a specific activity of about 0.08 unit per mg of protein. Solid ammonium sulfate, 5.3 g per 100 ml, was added giving 0.1 saturation at 4°. Aliquots of 250 ml of this were then heated at 60° in a 90° water bath with constant stirring. The preparation was maintained at this temperature for 5 minutes and was then rapidly cooled in an ice bath. Further manipulations were performed at 4°. The resulting precipitate was separated by centrifugation. The
supernatant, which had a specific activity of about 0.25 unit, was raised to 0.6 saturation with solid ammonium sulfate, 30.1 g per 100 ml, and the precipitate discarded. The supernatant was then similarly raised to 0.7 saturation, by further addition of 6.2 g of ammonium sulfate per 100 ml. The precipitate obtained was dissolved in about 25 ml of 0.15 M acetate buffer, pH 5.0. The specific activity at this point was 0.8 unit. At this stage the enzyme could be stored for 3 months without loss of activity. The preparation was then dialyzed for 16 hours against three successive changes, 1 liter each, of 0.05 M acetate buffer, pH 5.0. Over half of the protein precipitated from the solution without loss of enzyme, thus raising the specific activity to about 2.0 units. To aliquots of 10 ml of the supernatant solution, containing 60 to 100 mg of protein, 2 mg of calcium phosphate gel (Sigma) were added per mg of protein. This was allowed to stand for 10 minutes with occasional stirring. Over half the protein, containing little or no enzyme, was adsorbed on the gel. The combined supernatant solution was then dialyzed overnight against 500 ml of 0.1 M sodium citrate buffer, pH 4.8. The adsorption procedure was then repeated in detail, except that alumina gel Cy was used. By contrast, 90% of the protein and about half of the protein were adsorbed on the centrifuged gel. The enzyme was then eluted twice, each with a half volume of citrate buffer, 0.05 M, pH 4.8, containing 15 g of ammonium sulfate per 100 ml. The combined eluates contained over half of the adsorbed enzyme and had a specific activity of about 9 units. A sample protocol is given in Table I.

**Properties of Enzyme**—Like the muscle enzyme, yeast phosphoglucomutase requires for full activity, glucose 1,6-diphosphate, Mg++, and a metal-binding agent (10). In the absence of Mg++, it is about 15% as active. Fig. 1 shows the activation at various magnesium concentrations, the maximum being attained at 1 to 3 × 10⁻³ M. Zn++, Mn++, and Co++ also activate the enzyme but to a lesser degree than Mg++. Ca++ and Al+++ are inhibitory. Fig. 1 also shows Zn++ activation of the enzyme for comparison.

Fig. 2 depicts the range of activity as affected by pH. Maximal activity is attained at pH 7.5 in 0.04 M histidine. Complete suppression is observed at pH 5.0 or below, whereas at pH 8 and above there is a precipitous decline in activity. This decline is not due to destruction of the enzyme during activity measurements since it is stable for hours at this pH. Maximal stability is, however, obtained at pH 5 in 0.15 M acetate buffer or in ammonium sulfate.

The enzyme prepared according to the above procedure was free of glucose 1-phosphate transphosphorylase. The latter catalyzes the formation of glucose 1,6-diphosphate and glucose from glucose 1-phosphate (11). There was also complete absence of glucose 1-phosphate kinase which forms glucose 1,6-diphosphate and ADP from glucose 1-phosphate and ATP (12). Both of these interfering enzymes were eliminated at the third step in purification. The preparation, however, contained very active glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. This property was quite useful for measuring glucose 6-phosphate when catalytic amounts, 5 to 10 µg of protein per ml of reaction mixture, were used. Two moles of reduced triphosphopyridine nucleotide were formed per mole of glucose 6-phosphate added. However, trace amounts of TPNH oxidase activity could be detected when the concentration of enzyme was 1 mg per ml or greater.

**MECHANISM OF ACTION OF THE ENZYME**

All experiments reported below were done with dialyzed fractions at 0.7 ammonium sulfate saturation (Step 3). At this

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**Table I**  
Sample protocol for purification of phosphoglucomutase from bakers’ yeast

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total activity</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>mg</td>
<td>units/mg</td>
</tr>
<tr>
<td>1. Autolysate</td>
<td>3,250</td>
<td>39,000</td>
<td>0.08</td>
</tr>
<tr>
<td>2. Heat 60°</td>
<td>2,160</td>
<td>8,880</td>
<td>0.24</td>
</tr>
<tr>
<td>3. Ammonium sulfate 0.7 fraction dialyzed</td>
<td>1,720</td>
<td>810</td>
<td>2.10</td>
</tr>
<tr>
<td>4. Calcium phosphate and alumina gel Cy</td>
<td>800</td>
<td>87</td>
<td>9.20</td>
</tr>
</tbody>
</table>

**Fig. 1.** The effect of Mg++ and Zn++ on the activity of the enzyme under otherwise standard optimal conditions (see text). Glucose 6-phosphate (G6-P) was taken as the amount of acid stable phosphorus.

**Fig. 2.** The effect of pH on the activity of the enzyme under otherwise standard optimal conditions (see text). Glucose 6-phosphate (G6-P) was taken as the amount of acid stable phosphorus.
stage, the enzyme was entirely in the phospho form as judged by the lack of formation of glucose 6-phosphate when it was treated with diphosphate.

Formation of Glucose 1,6-Diphosphate and Dephosphoenzyme through Interaction of Hexose Phosphate and Phosphoenzyme

Formation of Glucose 1,6-Diphosphate—Substrate quantities of the enzyme were incubated for 30 to 60 seconds at 30° with either glucose 1-phosphate or glucose 6-phosphate under standard conditions of Mg++ 1.2 X 10^{-2} m, histidine 4 X 10^{-3} m, pH 7.5. After incubation, the reaction tubes were placed in a boiling water bath for 5 minutes. After centrifugation, the supernatant solution was diluted with water or evaporated under reduced pressure to obtain the desired concentration of glucose diphosphate for the appropriate assay. The amount of enzyme varied from about 1 to 20 units and the glucose phosphate from about 0.01 to 0.4 μmole per ml of reaction mixture. In all cases, the amount of glucose 1,6-diphosphate formed could readily be measured by its coenzymatic activity. With this method as little as 5 X 10^{-4} μmole could be measured with an error of ±15%. Samples from over 25 preparations of the enzyme were tested and the formation of glucose diphosphate readily demonstrated. The coenzymatic activity of the diphosphate was completely destroyed by heating in 0.1 M HCl for 10 minutes at 100°. This treatment hydrolyzes the glucosyl phosphate bond yielding stoichiometric amounts of glucose 6-phosphate. It was possible to identify and measure the latter with the combined dehydrogenase system. Tables II and III show representative data illustrating the synthesis of glucose diphosphate with glucose 1-phosphate for the forward reaction of Step I and glucose 6-phosphate for the reverse reaction of Step 2.

Formation of Dephosphoenzyme—Dephosphoenzyme is formed with the transfer of phosphate from the enzyme to the glucose phosphate. At equilibrium both phospho- and dephosphoenzyme are present in the reaction mixture. It was, however, possible to convert all the enzyme into the diphospho form by repeated treatment with glucose 1- or glucose 6-phosphate, with dialysis after each treatment. The dephosphoenzyme so prepared was characterized by complete loss of enzymatic activity in the absence of glucose diphosphate and by its failure to form the diphosphate on reaction with glucose 1-phosphate as shown in Table III. It was not possible to measure the loss of phosphate from the enzyme insnsuch as the enzyme preparation contained relatively large amounts of organic phosphates as contaminants. The dephosphoenzyme was also characterized by its ability to react with glucose diphosphate to produce glucose 6-phosphate and phosphoenzyme. This is shown in Table IV. The enzyme can now act catalytically in the absence of added diphosphate. It is also capable of forming the coenzyme, by reaction with either glucose 1- or 6-phosphate (Table III).

Fig. 3 illustrates graphically the difference in catalytic activity between phosphoenzyme and partially dephosphorylated enzyme. Dephosphorylation was accomplished as detailed above. Partial dephosphorylation was obtained by two treatments with glucose 1-phosphate. Here activity measurements were carried out under identical conditions with the same mixture. However, the units of activity were assayed as usual with saturation amounts of glucose diphosphate (3, 4). Another characteristic property of the dephosphoenzyme is its relative instability as compared to the phospho form. Fig. 4 shows that the phosphoenzyme retains most of its activity in 0.15 M acetate buffer, pH 5.0, at 4°. Under the same conditions the dephosphoenzyme loses activity steadily.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme</th>
<th>Glucose phosphate* added</th>
<th>Glucose 1,6-diphosphate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>33 (G-1-P)</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>33 (G-5-P)</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>17 (G-1-P)</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>10 (G-5-P)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Glucose 1-phosphate, G-1-P; glucose 6-phosphate, G-6-P.

Formation of Glucose 1,6-Diphosphate through interaction of glucose phosphate with dephosphoenzyme or rephosphorylated enzyme

One milliliter of the original reaction mixture consisted of glucose 1-phosphate or glucose 6-phosphate as indicated; total volume 1 to 3 ml. The reaction was started by adding the enzyme. Glucose 1,6-diphosphate was measured by its coenzymatic activity with glucose diphosphate as standard. Control samples for each experiment were treated exactly as test samples except that the enzyme was heated for 3 minutes at 100° before additions were made (3).

### Table III

<table>
<thead>
<tr>
<th>Units of enzyme</th>
<th>Glucose phosphate* added</th>
<th>Glucose 1,6-diphosphate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dephosphoenzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.0</td>
<td>300 (G-1-P)</td>
<td>0.00</td>
</tr>
<tr>
<td>9.0</td>
<td>300 (G-1-P)</td>
<td>0.00</td>
</tr>
<tr>
<td>2.4</td>
<td>200 (G-1-P)</td>
<td>0.00</td>
</tr>
<tr>
<td>Rephosphorylated enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>400 (G-1-P)</td>
<td>0.27</td>
</tr>
<tr>
<td>1.2</td>
<td>200 (G-1-P)</td>
<td>0.15</td>
</tr>
<tr>
<td>1.3</td>
<td>200 (G-6-P)</td>
<td>0.09</td>
</tr>
<tr>
<td>3.3</td>
<td>250 (G-6-P)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* Glucose 1-phosphate, G-1-P; glucose 6-phosphate, G-6-P.
used, the dephosphoenzyme contained excess amounts of glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. It also contained traces of TPNH oxidase activity. Under these circumstances, it was possible to measure the TPNH formed spectrophotometrically at 340 nm within 20 to 30 seconds after the addition of the diphosphate and subsequently after 15 to 20 minutes upon complete oxidation of the reduced nucleotide by extrapolation to zero time. In this reaction 2 moles of TPNH were formed per mole of glucose 6-phosphate. Table IV shows the amount of glucose 6-phosphate formed when 12 to 16 units of dephosphoenzyme react with 12 to 24 mmoles of glucose diphosphate per ml of reaction mixture. By contrast, glucose 6-phosphate is not detectable when phosphoenzyme is used.

**Formation of Phosphoenzyme**—The rephosphorylated enzyme, formed by reacting glucose diphosphate and dephosphoenzyme, was dialyzed for 3 days against 0.04 M histidine, pH 7.5. It was then characterized by its ability to form the diphosphate when reacted with glucose 1-phosphate or glucose 6-phosphate. This is shown in Table III.

In the usual reaction, involving conversion of glucose 1-phosphate to glucose 6-phosphate, the concentration of the enzyme is naturally much lower than that of the substrate. It follows that the concentration of glucose 1,6-diphosphate formed in the reaction mixture will be relatively low. It is therefore advisable to use in the kinetic experiments a limited amount of enzyme in order to prevent interference due to inhibition by the diphosphate.
process is considerably smaller than that of the substrate. In the over-all reaction, therefore, the second step can be shown to be rate limiting. This step can be readily accelerated by the addition of either of the two reactants; glucose 1,6-diphosphate or dephosphoenzyme. It is through this increased forward rate, which would be proportional to the product of the concentration of the reactants, that glucose 1,6-diphosphate exercises its coenzymatic effect.

Table V shows that with the addition of comparable amounts of glucose diphosphate or dephosphoenzyme, the stimulation of the rate of conversion of glucose 1- to glucose 6-phosphate is of the same order of magnitude.

**DISCUSSION**

By analogy with the muscle enzyme, it can be assumed that the yeast enzyme has only one transferable phosphate group per mole. On this basis, it was possible to measure the molar concentration of the enzyme under study and thereby calculate the equilibrium constants of the two steps. This was done by converting all the enzyme to the dephospho form and carrying out the reaction of the second step with excess glucose 1,6-diphosphate. The glucose 6-phosphate formed is removed by the combined dehydrogenase system. All the dephosphoenzyme is thereby phosphorylated. The amount of phosphoenzyme formed would correspond, on a molar basis, to the glucose 6-phosphate oxidized or half the TPNH formed. On this basis, it was estimated from Table IV that each unit of enzyme corresponds to an average of 0.267 mpmole of enzyme.

The values in Table II, representing equilibrium states of the forward reaction of Step 1 and the reverse reaction of Step 2, were used for the estimation of the equilibrium constants in the following manner. The units of enzyme were converted to mmoles. The sum of glucose 1- and 6-phosphate is equivalent to the quantity of monophosphate added less the diphosphate formed. At equilibrium, glucose 1-phosphate and glucose 6-phosphate are present in a ratio of 5.5 to 94.5. From this, actual values of the hexose phosphates were calculated. Further, the amount of dephosphoenzyme present at equilibrium is equal to the amount of glucose diphosphate formed. The phosphoenzyme remaining would then equal the quantity of enzyme added less the dephosphoenzyme formed. The phosphoenzyme remaining would then equal the quantity of enzyme added less the dephosphoenzyme formed. The phosphoenzyme remaining would then equal the quantity of enzyme added less the dephosphoenzyme formed.

Eliminating the aberrant value of 0.84, the average value for the first three constants of \( k_2 = 5.48 \) and \( k_1 = 3.14 \) were obtained; the over-all \( K \) for the whole reaction being 17.2. These values are comfortably close to the equilibrium constants obtained for the muscle enzyme, \( k_2 = 3.76, k_1 = 4.58 \). Thus, the standard free energy change in the second step would be about \(-1000\) calories as compared to \(-800\) calories for the muscle enzyme. The free energy of hydrolysis of the enzyme phosphate bond would be correspondingly close, approximately \( \Delta F^\circ = -3700\) calories as compared to \(-3900\) calories for the muscle mutase. These differences fall within the error of measurements in this system.

**SUMMARY**

A method for purifying phosphoglucomutase (100- to 150-fold) from bakers' yeast is described. The properties and the mechanism of action of the yeast enzyme were found to be similar to those of the corresponding crystalline rabbit muscle enzyme. The catalytic action occurs in two distinct steps during which the enzyme alternates between a phospho and a dephospho form. The latter is relatively less stable.

When glucose 1-phosphate alone is used as substrate with catalytic amounts of phosphoenzyme, the second step can be shown to be rate limiting. The addition of either glucose 1,6 diphosphate or dephosphoenzyme in comparable amounts results in a stimulation of the rate of the reaction to a comparable degree.

On the assumption that the yeast enzyme, like the muscle enzyme, possesses one transferable phosphate per molecule, the molar concentration of the enzyme was determined and the equilibrium constants for the two steps calculated. These were found to be close in value to those obtained for the muscle enzyme.

**REFERENCES**

The Purification and Mechanism of Action of Yeast Phosphoglucomutase
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