Glucose-6-phosphate Dehydrogenase

PURIFICATION AND PARTIAL CHARACTERIZATION

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Glucose-6-phosphate dehydrogenase has been purified 1000-fold from pig liver. This enzyme exists as an active dimer of molecular weight 133,000 and an inactive monomer of molecular weight 67,500. The pH of maximum activity is 8.5 and the ionic strength maximum is 0.1 to 0.5 M. Glucose-6-phosphate dehydrogenase is highly specific for NADP⁺ and glucose 6-phosphate. Apparent Kₘ values of 5.6 μM and 5.4 μM were obtained for glucose 6-phosphate and NADP⁺. This enzyme is located almost entirely within the soluble portion of the cellular cytoplasm.

The pentose phosphate shunt occurs widely in living cells where one of its main functions is to provide the NADPH necessary for the synthesis of fatty acids and other specific reductions. The biosynthesis of fatty acids is related to the oxidative part of the shunt where the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase produce the necessary NADPH. The above facts along with the strategic position of glucose-6-phosphate dehydrogenase (d-glucose 6-phosphate:NADP oxidoreductase EC 1.1.1.49) at a metabolic branch point, suggest that the glucose-6-phosphate dehydrogenase reaction is an important site of metabolic control. This view is supported by several investigators who have shown variations in enzyme activity as a function of hormone and nutritional levels (1, 2), enzyme quaternary structure, and various metabolites including ATP, ADP(3), spermidine, and palmitoyl-CoA(4).

It is our purpose to study glucose 6-phosphate dehydrogenase from pig liver and to determine the effect that various metabolites have on its kinetic properties. Glucose-6-phosphate dehydrogenase has been previously isolated from microorganisms, erythrocytes, and various mammalian tissues (5-17). In this work, we report the isolation, purification, and partial characterization of this enzyme from pig liver. The purification scheme includes homogenization, acid deamination, Triton X-100 treatment, Sephadex gel filtration, and ion exchange chromatography with DEAE-cellulose. Characterization studies include molecular weight determination, pH, and ionic strength effects, a subcellular fractionation, and preliminary kinetics.

MATERIALS AND METHODS

Chemicals—Glucose 6-phosphate, glucose oxidase, NADPH, NAD⁺, bovine serum albumin, catalase, and lactate dehydrogenase were purchased from Sigma Chemical Co. Blue dextran, yellow dextran, and Sephadex were purchased from Pharmacia Fine Chemicals. DEAE-cellulose was obtained from Bio-Rad Laboratories. Other reagent grade chemicals were purchased either from the California Biochemical Co or Sigma Chemical Co.

Experimental Procedures—Glucose-6-phosphate dehydrogenase was routinely assayed spectrophotometrically. The usual assay mixture contained 2.6 ml of 0.05 M NaOH/glycine buffer, pH 8.5, 0.1 ml of 30 mM MgCl₂, 0.1 ml of 1.5 mM NADP⁺, 0.1 ml of 30 mM glucose 6-phosphate, and 0.1 ml of enzyme. The reaction was followed at 340 nm using a Gilford DU spectrophotometer equipped with a Texas Instruments recorder and connected to a constant temperature bath set at 30 ± 0.1°C. The formation of NADPH produced a linear increase in absorbance readings for periods of 3 min or longer after the addition of glucose 6-phosphate to a 1-cm cuvette. All activities were recorded as change in absorbance per min and later converted to concentrations of NADPH/min at 30°C. Protein concentration was determined either by the method of Warburg and Christian (18) or by the microburet method of Goo (19).

Localization of Enzymes—For the determination of the subcellular localization of the enzyme, freshly slaughtered pig liver was homogenized and fractionated according to a modified method of Schneider and Hogeboom(20). The liver was first cut into small pieces and 2 g were suspended in 14 ml of 0.25 M sucrose containing 0.02 M sodium phosphate buffer, pH 7.4. The cells were homogenized in a Potter-Elvehjem type glass homogenizer. The operation was repeated four times such that 8 g of liver were homogenized in a total volume of approximately 60 ml. The homogenate was centrifuged at 1,000 x g for 20 min and the precipitate containing nuclei, blood, and cellular debris was discarded. The supernatant fraction was then centrifuged for 10 min at 9,000 x g. The resulting pellet was washed twice in the same medium and centrifuged twice at the same speed. The washed pellet was designated as the mitochondrial fraction. The 9,000 x g supernatant fraction was further centrifuged at 20,000 x g for 20 min to remove any unwanted mitochondria still remaining. The supernatant portion of this treatment was centrifuged at 100,000 x g for 40 min. The pellet collected at this stage was washed once in the same medium and was presumed to contain the microsomal fraction. The liquid remaining after 100,000 x g centrifugation was designated as the soluble supernatant fraction. The soluble supernatant fraction was treated with ammonium sulfate at 55% saturation and the precipitate redissolved in 8 ml of 0.02 M sodium phosphate buffer, pH 7.4. The washed mitochondrial and microsomal fractions were finally disrupted by freezing and thawing four times in 6 ml of 0.02 M sodium phosphate buffer containing 0.5% sodium cholate. The disrupted mitochondrial...
fraction was finally centrifuged at 40,000 \times g for 20 min and the precipitate discarded. The microsomal fraction was centrifuged at 100,000 \times g for 20 min, and the supernatant portion retained. Both glucose-6-phosphate dehydrogenase and glucose dehydrogenase were assayed in the clear supernatant portions of the various fractions. Glucose dehydrogenase was assayed with glucose (55.3 mM) and NAD (50 \mu M) as substrates in 0.05 mM glycine buffer (1 mM EDTA) at pH 10, 30°.

**RESULTS AND DISCUSSION**

**Purification of Enzyme—**IgM liver fresh from the slaughterhouse was cut into 2- cm chunks, and 75-g amounts were homogenized in a Waring Blender for 90 s with 0.2 M pH 7 phosphate buffer. The pH of the resulting solution was lowered to 5.5 with 20% acetic acid, and then stored at 4° for at least 2 hours. At this point, the solution was centrifuged at 6,000 \times g for 30 min at 4° and the pellet discarded. The remaining liquid usually had a specific activity of 0.004 unit/mg of protein. (NH$_4$)$_2$SO$_4$ (21 g/100 ml) was slowly added to the solution with gentle stirring and then allowed to stand for at least 4 hours at 4°. The final suspension was centrifuged at 10,000 \times g for 30 min at 4°, and the pellet retained. The precipitate was treated with Triton X-100 (4 drops of Triton X-100/3 ml of precipitate) in a tissue homogenizer and ground by hand for approximately 9 min. The resulting suspension was increased in volume by 50% using 0.2 M phosphate buffer, pH 7.0 and stored overnight at 4°. The solution was then centrifuged at 10,000 \times g for 30 min at 4° and the precipitate and any suspended material were discarded. The specific activity of the liquid was approximately 0.01 unit/mg of protein.

The solution was then added in 5-ml batches to a column (2.5 \times 45 cm) of Sephadex G-200 previously equilibrated overnight with 0.05 M phosphate buffer, pH 7 containing 10⁻³ M EDTA, 10⁻⁴ M NADP⁺, and 10⁻³ M \beta-mercaptoethanol. Fifteen-minute fractions of approximately 3 ml were collected by means of a Gilson fraction collector.

Fig. 1 is the plot of relative protein and relative activity versus fraction number for glucose-6-phosphate dehydrogenase. The specific activity of the most active fractions is approximately 0.1 unit/mg of protein.

The most active fractions from the Sephadex G-200 column were chromatographed in 7- to 9-ml batches on a DEAE-cellulose column (5 \times 1 cm) previously equilibrated with 0.005 M phosphate buffer, pH 8 containing 10⁻³ M EDTA, 10⁻³ M \beta-mercaptoethanol, and 10⁻⁴ M NADP⁺.

The enzyme was then eluted in a stepwise fashion with 0.005, 0.01, 0.05, 0.1, and 0.2 M phosphate buffers, pH 8. Three-minute fractions of approximately 3 ml were collected. Fig. 2 is the plot of relative protein and activity versus fraction number. The specific of the best fractions 1 approximately 1 to 1.5 units/mg of protein which represents 10- to 20-fold purification from the Sephadex G-200 enzyme.

A summary of typical values obtained during the purification is shown in Table I. As indicated, a purification of about 1000-fold over the first homogenate was obtained. The enzyme loses considerable activity after a week at 4° for each of the first four purification steps listed in Table I. The enzyme loses almost all activity on a Sephadex column unless the column is equilibrated with 10⁻¹ M NADP⁺. Finally, the enzyme is stable and retains the original activity for a period of several weeks at 4° after DEAE-cellulose chromatography. The enzyme showed no reaction with glucose concentration levels in the range of 0.33 to 3.3 M.

The reduced form of NADP⁺ has been used to reassociate the enzyme and reduce the interaction of the subunits of the enzyme (21). However, NADPH has not been used to protect the enzyme during the purification procedure. In this work, it was discovered that the enzyme could be purified with the same yield and degree of purity using NADPH to protect the enzyme from denaturation as shown in Table I.

**Gel Electrophoresis—**Electrophoresis of the highly purified enzyme was carried out according to the method of Ornstein (22) and Davis (23). The high molecular weights and low mobility of glucose-6-phosphate dehydrogenase required the use of a 6% gel. Protein samples were taken directly from the DEAE-cellulose column. Amido black was used as a stain and a single dark protein band was observed for both the NADP⁺- and NADPH-prepared enzymes.

**Molecular Weight Determination—**The molecular weight of glucose-6-phosphate dehydrogenase has been the subject of several studies. The molecular weight of the monomer has been reported as follows: rat mammary gland, 62,000 (24), rat liver, 64,000 (25), bovine adrenal, 64,600 (26), and human erythrocyte, 43,000 (27). The subunits are seen to interact as a function of pH and ionic strength. By sedimentation measurements, it has been found to aggregate as a tetramer (16) and hexamer (24).

In this work, the molecular weight of glucose-6-phosphate dehydrogenase was determined in 5 mM pH 7 phosphate buffer containing 0.1 mM NADP⁺, 1 mM EDTA, and 1 mM \beta-mercaptoethanol using a Sephadex G-200 column. The standards were run on the same column in 5 mM phosphate buffer, pH 7. Blue dextran was used to determine the void volume, V₀, while glucose oxidase (150,000), lactate dehydrogenase (70,000), catalase (240,000), and bovine serum albumin monomer (66,200) were used as standards. The protein profile of the glucose-6-phosphate dehydrogenase elution pattern showed two absorption peaks which corresponded to the dimmer (molecular weight, 133,000) and the monomer (molecular weight, 64,900) of the enzyme.

<table>
<thead>
<tr>
<th>Step</th>
<th>Co-enzyme form</th>
<th>Protein (units/mg)</th>
<th>Volume (ml)</th>
<th>Purification (fold)</th>
<th>Varietal</th>
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<td>Homogenate</td>
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<td></td>
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<td>300</td>
<td>1</td>
<td>1</td>
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<td>3.55</td>
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<td>750</td>
<td>4.4</td>
<td>193</td>
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<td>Triton X-100 treatment</td>
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<td>50</td>
<td>10.9</td>
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<tr>
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<td>DEAE-cellulose ion exchange</td>
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<td>225</td>
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<tr>
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<td>NADPH</td>
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<td>480</td>
<td>950</td>
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</table>
Glucose-6-P Dehydrogenase

67,500) as shown in Fig. 3. The dimer was the major peak and
the only one that catalyzed the reaction with NADP+ and
glucose 6-phosphate.

Finally, electrophoresis according to the method of Weber
and Osborn (28) gave a single subunit band whose mobility was
identical with bovine serum albumin (67,000).

Optimum pH—It has been reported that the activity of this
enzyme from human erythrocytes is a function of ionic strength
and pH (27). We have studied the effect of pH on activity in 50
mM glycine/NaOH and 50 mM Tris/malate/NaOH buffers. A
slow rise in activity is observed with increasing pH to a
maximum at pH 8.5, followed by a gradual decrease in activity.

Ionic Strength—in this study, the ionic strength was varied
at a constant pH of 8.5 in glycine/NaOH buffer. Sodium
chloride was first added to increase the ionic strength. We
observed a marked increase in activity from an ionic strength of
0.01 to 0.5 M, at which point the activity gradually decreased.
When only the glycine/NaOH concentration was increased, the activity was seen to gradually increase until 0.5
M.

Subcellular Fractionation—The results of this experiment
are given in Table II. Unlike glucose dehydrogenase, which is
found in all fractions, glucose-6-phosphate dehydrogenase is
localized almost exclusively in the soluble portion of the cell.

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