Hexose-6-phosphate Dehydrogenase of Rat Liver Microsomes

ISOLATION BY AFFINITY CHROMATOGRAPHY AND PROPERTIES

Yukinobu Hino and Shigeki Minakami
From the Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812, Japan

Hexose-6-phosphate dehydrogenase was purified from rat liver microsomal fraction more than 500-fold with a 45% recovery using DEAE-cellulose and 2',5'-ADP-Sepharose 4B columns. The purified enzyme appeared to be immunologically and electrophoretically homogeneous and had broad substrate and cofactor specificities. The enzyme activity was not inhibited by p-chloromercuribenzoate.

The purified enzyme was a glycoprotein in nature, having a Stokes radius of about 55 Å, a sedimentation coefficient of about 8.2 s, and an isoelectric point of about 6.4. Minimum molecular weight of the enzyme was about 108,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas the product cross-linked with glutaraldehyde or dimethyl suberimidate had Mr ≅ 220,000, suggesting that the active enzyme existed as a dimer of identical subunits.

Antiserum raised against the purified enzyme inhibited the activity of the solubilized enzyme but did not inhibit the cytosol glucose-6-phosphate dehydrogenase activity. The antigenic sites of the enzyme were latent in intact microsomes. Comparison was also made between the enzymes isolated from untreated and phenobarbital-pretreated animals.

EXPERIMENTAL PROCEDURES

Preparation of Microsomes

Male Wistar strain of rats, weighing 300–350 g, were used. They were starved overnight prior to killing. The animals were killed by bleeding from the carotid artery and the livers excised were thoroughly perfused with ice-cold 1.15% KCl-10 mM EDTA (pH 7.5) and homogenized in 4 volumes of the perfusing medium. Microsomal fraction was prepared and washed with the same medium as described previously (10).

Isolation of Hexose-6-phosphate Dehydrogenase

A typical experiment with three animals was described. Isolation was carried out in cold.

Solubilization by a Nonionic Detergent—The washed microsomal fraction (550 mg of protein) was solubilized by suspending in 10 mM potassium phosphate buffer (pH 7.25) containing 1 mM EDTA and 0.5% (w/v) Emulgen 913 (at the protein concentration of 5.0 mg/ml). The mixture was stirred in ice for 30 min and was centrifuged at 66,000 × g for 90 min to give a clear solubilized supernatant. Essentially all of the hexose-6-phosphate dehydrogenase activity was found in the supernatant fraction.

DEAE-cellulose Column Chromatography—The solubilized supernatant was applied to a DEAE-cellulose column (2.3 × 13 cm) pre-equilibrated with the solubilizing buffer mentioned above, and the column was washed with the same medium to wash out NADPH-cytochrome P-450 reductase completely. The hexose-6-phosphate dehydrogenase activity was then eluted by increasing the phosphate concentration linearly from 10 to 120 mM. The peak fractions of hexose-6-phosphate dehydrogenase activity were pooled (62 ml) and diluted 3-fold by mixing with distilled water.

2',5'-ADP-Sepharose 4B Column Chromatography—The diluted sample was applied to a 2',5'-ADP-Sepharose 4B column (1.1 × 7.5 cm) pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913 and 0.5 mM EDTA. After the column was washed with more than 10 column volumes of 35 mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913 and 0.5 mM EDTA, the hexose-6-phosphate dehydrogenase was eluted with the washing medium containing 0.5 mM NADP. The preparation thus obtained was homogeneous based on the criteria mentioned in the text.

Removal of Detergent and NADP from Purified Preparation—Removal of Emulgen 913 from the purified preparation was achieved by using a DEAE-cellulose column. The purified sample eluted from the affinity column was diluted with an equal volume of distilled water and was applied to a small column of DEAE-cellulose pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.25) containing 0.5 mM EDTA. After the column was extensively washed with the equilibrating buffer until ultraviolet absorption of the effluent was negligible, the enzyme was eluted as a concentrated solution with 300 mM potassium phosphate buffer (pH 7.25) containing 0.5 mM EDTA. The obtained sample was essentially free from the detergent but contained NADP as revealed by an absorption maximum at 296 nm. The detergent-free enzyme had a tendency to aggregate upon dilution with distilled water, so we stored it usually in 50 mM or a much higher concentration of phosphate buffer (pH 7.25). NADP contaminating in the preparation could be removed by gel filtration.

Analytical and Assay Procedures

Hexose-6-phosphate dehydrogenase activity was measured at room temperature spectrophotometrically by the formation of NADPH or...
Hexose-6-phosphate Dehydrogenase

NADH (1). The standard assay mixture (final volume, 1.0 ml) contained 0.1 M glycine-NaOH buffer (pH 10.0), 1.0 mM glucose 6-phosphate, 0.17 mM NADP, and an enzyme. Emulgen 913 (0.1-0.5%) was included in the reaction mixture when the activity of microsomal fraction was to be assayed. The reaction was started by adding NADP plus glucose 6-phosphate and the formation of NADPH was followed by measuring the absorbance at 340 nm. The absorption coefficient of 6.9 mm⁻¹  cm⁻¹ was used (11). One unit was defined as the activity to produce 1 µmol of NADPH/min under the conditions of this experiment. NADPH-cytochrome P-450 reductase was assayed by the reduction of cytochrome c according to the method of Phillips and Langdon (12). Cytochrome P-450 and nucleoside diphosphatase (inosine diphosphate as a substrate) were assayed by the methods reported by Omura and Sato (13) and Kuriyama (14), respectively. Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as a reference.

Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol was done as described by Laemmli and Favre (16) with the gel of 2-mm thickness. Minimum molecular weight of the enzyme was estimated by comparing the relative mobility with those of standard proteins of known molecular weights. Five subunits of RNA polymerase were used for standards: α (Mr 42,000), β (Mr 140,000), β′ (Mr 180,000). Carbohydrate staining with periodic acid-Schiff reagent was carried out according to the method of Zacharius et al. (17). Electrophoresis was done with a pH gradient of 3.5-10 in the presence of 1% Emulgen 413 containing 0.1 M KC1 and was eluted with the same buffer. The column void volume, and elution volume of a protein.

The sedimentation coefficient was determined on a sucrose linear gradient (4.7 ml, 10 to 30% (w/v) containing 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 M KC1 and was eluted with the same buffer. The column was calibrated with blue dextran 2000 and standard proteins of known molecular weight. Five subunits of RNA polymerase were used for standards: α (Mr 42,000), β (Mr 140,000), β′ (Mr 180,000). Carbohydrate staining with periodic acid-Schiff reagent was carried out according to the method of Zacharius et al. (17). Electrophoresis was done with a pH gradient of 3.5-10 in the presence of 1% Emulgen 413 containing 0.1 M KC1 and was eluted with the same buffer. The column void volume, and elution volume of a protein.

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RESULTS

Isolation of Hexose-6-phosphate Dehydrogenase—We have previously reported the purification of hexose-6-phosphate dehydrogenase from the microsomal fraction of phenobarbital-treated rats. Since then, we tried to improve the isolation procedure and succeeded in isolating the enzyme from untreated rat liver microsomes.

The improved method used a nonionic detergent Emulgen 913 in place of cholic acid for solubilizing the enzyme and omitted a step of aminooctyl-Sepharose 4B column chromatography. The experiment shown in Fig. 1 was done to find the optimal detergent concentration for solubilizing the enzyme. It was found that hexose-6-phosphate dehydrogenase, together with nucleoside diphosphatase, was easily released into the supernatant fraction at lower concentration of Emulgen 913 than were other microsomal protein. Therefore, we used 0.5% (w/v) concentration of the detergent for the solubilization as described under "Experimental Procedures." It is essential to wash exhaustively the microsomal pellet for removing the contaminating glucose-6-phosphate dehydrogenase. Much precaution was also taken to wash out NADPH-cytochrome c reductase from the DEAE-cellulose column prior to the elution of hexose-6-phosphate dehydrogenase.

Emulgen 913 (0.1 mg) was dissolved in 1 ml of 0.1 M potassium phosphate buffer (pH 7.25) with 1% Emulgen 913. The mixture was then diluted with 1.39 ml of a mixture containing 0.1 M Tris-maleate buffer (pH 7.5) and 0.2% Emulgen 913. The reaction was started by adding 5 µl each of 50 mM NADP and 0.3 M glucose 6-phosphate. Some modifications were introduced when necessary.

Chemicals and Other Materials

Cytosol glucose-6-phosphate dehydrogenase was partially purified from the postmicrosomal supernatant fraction of the homogenate according to the method of Matsuda and Yagari (22). Emulgen 913 (polyoxyethylene nonylphenyl ether) and heavy chain of rabbit myosin were kind gifts from Kao-Atlas Co. (Tokyo) and from Dr. K. Yamamoto, Kyushu University School of Medicine, respectively. 2′,5′-ADP-Sepharose 4B was obtained from Pharmacia. RNA polymerase was purchased from Mitsubishi Yuka (Osaka), and DEAE2 was from Whatman Ltd. Bovine plasma cold insoluble globulin was a kind gift from Prof. S. Iwamura, Faculty of Science, Kyushu University. Other chemicals were of reagent grade.

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dehydrogenase. The hexose-6-phosphate dehydrogenase activity was eluted at the phosphate concentration of 40 mM as shown in Fig. 2. Fig. 3 shows the elution pattern of hexose-6-phosphate dehydrogenase from the 2',5'-ADP-Sepharose 4B column. No appreciable activity of hexose-6-phosphate dehydrogenase could be detected in the effluent before the addition of NADP, but on the addition of 0.5 mM NADP, the dehydrogenase activity was eluted as a concentrated solution.

The overall recovery of the activity from the microsomes was about 45% as shown in Table I. The purified preparation was apparently homogeneous based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in Fig. 7. Further evidence for the homogeneity of the purified enzyme came from the Ouchterlony test (Fig. 4, left), showing that a fused single precipitin line was made with microsomes and purified preparation. The purified enzyme from which the detergent and NADP were removed had an absorption maximum at 278 nm and showed no appreciable absorption bands in a visible region. The specific activity measured at pH 10.0 was 3.36 μmol/min·mg of protein, which was 560-fold higher than that of the starting microsomes. From the minimum

Table I
Summary of a purification of hexose-6-phosphate dehydrogenase from rat liver microsomes

<table>
<thead>
<tr>
<th>Steps</th>
<th>Protein</th>
<th>Hexose-6-phosphate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Microsomes</td>
<td>590</td>
<td>3.54 (100)*</td>
</tr>
<tr>
<td>2. Solubilized supernatant</td>
<td>391</td>
<td>3.36 (85)</td>
</tr>
<tr>
<td>3. DEAE-cellulose</td>
<td>26.1</td>
<td>2.19 (62)</td>
</tr>
<tr>
<td>4. 2',5'-ADP-Sepharose</td>
<td>0.47</td>
<td>1.58 (45)</td>
</tr>
</tbody>
</table>

\*% recovery of activity in parentheses.

\*fold purification in parentheses.

molecular weight (see below) and the activity of the purified enzyme reported here, the content in microsomes could be calculated, on a molar basis as well as weight basis, to be approximately 16.5 pmol/mg of protein or 0.179% of total protein.

pH Optimum—The pH optimum of the activity of purified hexose-6-phosphate dehydrogenase was determined. Fig. 5 shows the activity with NADP and glucose or NADP and glucose 6-phosphate as a function of pH. The pH profile differed depending upon the substrate used and the maximal activity was observed when the pH in the reaction mixture was about 10.2 with glucose 6-phosphate or 7.6 with glucose. Substrate and Cofactor Specificities—Glucose and hexose 6-phosphates could serve as substrates for hexose-6-phosphate dehydrogenase. When the enzyme activity was measured at pH 10.0 using various sugars and their phosphorylated derivatives, their activities relative to that measured with glucose 6-phosphate (1.0 mM, referred to as 100%) were: with NADP-glucose (0.53 m), 19.9%; galactose 6-phosphate (2.7 mM), 78.4%; and with NAD-glucose 6-phosphate, 23.2%; glucose, 92.5%; galactose 6-phosphate, 21.1%. Thus, the activity with NAD was only about 25% of that with NADP when glucose 6-phosphate or galactose 6-phosphate was used, whereas the activity with NADP became 4.6-fold higher than that with NADP when 0.53 m glucose was employed in place of the phosphorylated compound. All the other compounds examined, glucose 1-phosphate (1.1 mM), galactose 1-phosphate (1.3 mM), galactose (33.3 mM), ribose 5-phosphate (3.3 mM), and ribose 1-phosphate (0.8 mM), could not support the reac-
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2566 Hexose-6-phosphate Dehydrogenase was highly specific for NADP and moderately for NAD. All other compounds including potassium ferricyanide (1.1 mM), dichlorophenolindophenol (33.3 μM), coenzyme Q₂ (0.47 mM), cytochrome c (33.3 μM), and cytochrome b₅ (3.0 μM) could not support the reaction (less than 4% of the activity measured with NADP).

Kinetic Parameters—Kinetic parameters determined at two different pH levels are summarized in Table II. The Kₘ for glucose-6-phosphate and galactose-6-phosphate at pH 10.0 were about 10 times higher than that at pH 7.2, whereas those for cofactors did not change greatly even if the pH shifted.

Effect of p-Chloromercuribenzoate—When the effects of p-chloromercuribenzoate on the activities of hexose-6-phosphate and glucose-6-phosphate dehydrogenase preparations were compared at pH 10.0 using NADP and glucose-6-phosphate, the latter was almost completely inhibited by the reagent at 0.5 mM, whereas only 10% of the former activity was inhibitable by the reagent at 1.4 mM.

Physicochemical Properties—An isoelectric point of 6.4 was obtained for the purified enzyme from the electrofocusing results. The minimum molecular weight of hexose-6-phosphate dehydrogenase was estimated to be 108,300 ± 600 (n = 7) by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. The cross-linked product formed by the treatment with glutaraldehyde, on the other hand, had a similar mobility as that of the subunits of cold insoluble globulin co-electrophoresed on the same slab gel as shown in Fig. 6, and an approximate molecular weight of the product could be estimated to be 210,000 to 220,000. Similar results were obtained with dimethyl suberimidate as a cross-linking reagent (not shown). It follows that the active enzyme exists as a dimer of identical subunits at least in the purified preparation. The size of the cross-linked product was not changed by the presence or absence of Emulgen 913 in the enzyme solution.

The Stokes radius of the enzyme was estimated from the results obtained by gel filtration chromatography on a Sephacryl S-300 column. On plotting (−log Kₛₜ)¹/₂ against Stokes radii (23), linear relationships were obtained as reported previously (18), and we have found about 54 Å as the molecular radius of hexose-6-phosphate dehydrogenase. The molecular size was also calculated by means of the following equation proposed by Ackers (24):

\[ Kₛₜ = \left[ 1 - (s/r) \right] \frac{1}{2} 1.104 (s/r) + 2.09 (s/r)² - 0.95 (s/r)³ \]

where \( Kₛₜ \) = distribution coefficient, \( s \) = Stokes radius, and \( r \) = effective pore radius. Assuming that \( Kₛₜ \) equals \( K_m \), the constant \( r \) was determined to be 225.8 using five different standard proteins mentioned under “Experimental Procedures.” This assumption may not seriously change the results (18), and the Stokes radius of the enzyme could be calculated to be 54.8 ± 0.46 Å (n = 3), which was in good agreement with the value obtained by the graphic plotting.

The sedimentation coefficient (\( s_{20,w} \)) was determined by plotting the coefficients of known proteins as a function of the distance migrated during centrifugation. A satisfactory linear relationship was obtained, and about 8.2 s of a sedimentation coefficient of hexose-6-phosphate dehydrogenase could be estimated. The molecular weight was also estimated from the equation:

\[ M_r = 6πηN_A k_B (1 - \bar{v}) \]

where \( \eta \) = viscosity of the medium (assuming 0.01004 g·cm⁻¹·s⁻¹), \( N_A \) = Avogadro’s number, \( a \) = Stokes radius, \( s \) = sedimentation coefficient, \( \bar{v} \) = partial specific volume (assuming 0.725 cm³·g⁻¹), and \( \rho \) = density of the medium (0.9982 cm³·g⁻¹). From the above values, \( M_r = 186,000 \) could be calculated and a fractional ratio (\( k_B/\rho \)) was found to be 1.46, indicating that the enzyme was of a nonspherical shape.

Periodic Acid-Schiff Staining—Fig. 7a shows that hexose-6-phosphate dehydrogenase was stained with Periodic Acid-Schiff reagent. Bovine serum albumin and egg albumin were co-electrophoresed as references.

Inhibition by Antibodies and Latency—The effects of anti-hexose-6-phosphate dehydrogenase IgG on hexose-6-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase were examined and are shown in Fig. 8. About 90% of the hexose-6-phosphate dehydrogenase activity was inhibited by

**Table II**

<table>
<thead>
<tr>
<th>pH in assay mixture</th>
<th>Substrates</th>
<th>Cofactors</th>
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<tbody>
<tr>
<td></td>
<td>Glucose 6-phosphate</td>
<td>Galactose 6-phosphate</td>
</tr>
<tr>
<td>10.0 Kₘ (μM)</td>
<td>25.3</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>7.2 Kₘ (μM)</td>
<td>1.9</td>
<td>1.9</td>
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<td></td>
<td>22.5</td>
<td>22.5</td>
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</table>

Fig. 6. Cross-linking of purified hexose-6-phosphate dehydrogenase with glutaraldehyde. Purified enzyme (4.2 μg) was mixed with 0% (lane 4), 0.02% (lane 5), 0.04% (lane 6), 0.11% (lane 7), and 0.22% (lane 8) glutaraldehyde in a final volume of 0.05 ml containing 30 mM potassium phosphate buffer (pH 7.25). After incubation for 10 min at room temperature, the mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gel was stained with Coomassie brilliant blue R250. The molecular weights of the standard proteins were as follows. Lane 1, reduced form of cold insoluble globulin (α-chain, \( M_r = 220,000 \), β-chain, \( M_r = 212,000 \)); lane 2, RNA polymerase (β, \( M_r = 180,000 \), β, \( M_r = 140,000 \), x, \( M_r = 100,000 \)); lane 3, heavy chain of rabbit muscle myosin (\( M_r = 207,000 \)); lane 9, unreduced form of fibrinogen (\( M_r = 330,000 \)).
The antibodies when a purified preparation was used as an enzyme source, whereas a slightly smaller inhibition (84%) was observed when microsomal fraction was used. The difference between the two may be due to the presence of contaminating glucose-6-phosphate dehydrogenase in the microsomal fraction, since cytosol glucose-6-phosphate dehydrogenase activity was not influenced by the antibodies as shown in the figure.

The activity of hexose-6-phosphate dehydrogenase in microsomal membranes has been reported to be latent (3, 25). We have also observed that the addition of detergent is necessary to reveal the activity. The activity without detergent was only 7.2% of that measured in the presence of 0.27% Emulgen 913. Moreover, we have examined whether the antigenic sites of the enzyme are masked in microsomal membranes. When the microsomes were preincubated with anti-hexose-6-phosphate dehydrogenase IgG without detergent, the remaining activity measured immediately after dilution with Emulgen 913 containing buffer was just the same as that measured after the microsomes were preincubated with detergent without IgG followed by dilution with a mixture containing an equal amount of anti-hexose-6-phosphate dehydrogenase IgG (not shown). We could conclude, therefore, that the antigenic determinants which interact with neutralizing antibodies of hexose-6-phosphate dehydrogenase were masked in microsomes.

Comparison of Hexose-6-phosphate Dehydrogenase Isolated from Untreated and Phenobarbital-pretreated Animals—We have previously isolated the enzyme from phenobarbital-pretreated rats (10), so it seems appropriate to compare it with that isolated from untreated animals (in this paper). Figs. 7b and 4 (right) show that both enzymes had an identical mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and made a fused single precipitin line with antibodies raised against the enzyme of untreated animals. Kinetic properties of the enzyme of phenobarbital-pretreated animals were also similar to those described above. Furthermore, the elution patterns of both enzymes in DEAE-cellulose column chromatography could not be distinguished from each other; in both cases, the peak fraction of the enzyme activity appeared at about 40 mm phosphate concentration. It is highly likely, therefore, that the enzyme of the untreated animals is, if not identical with, very similar to that of the phenobarbital-pretreated animals.

**DISCUSSION**

We present here an affinity method for the purification of hexose-6-phosphate dehydrogenase from rat liver microsomal fraction and its physicochemical properties. Takahashi and Hori have reported the purification of the enzyme using 5′-AMP-Sepharose 4B as an adsorbent, but, as they pointed out, their preparation contained contaminants which were perhaps derived from the outer surface of microsomal membranes. The antibody raised against their purified preparation made extra precipitin lines in Ouchterlony’s test which could be removed by treating the antiserum with intact microsomes (9, 26).

The enzyme of the untreated animals may be very similar to, if not identical with, that of the phenobarbital-pretreated animals (10), because both enzymes behaved similarly in DEAE-cellulose column chromatography, had an identical mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and could not be distinguished from each other immunologically as well as kinetically. The increase of hexose-6-phosphate dehydrogenase activity by phenobarbital is very small when expressed on a basis of microsomal protein (37); hence, it can mainly be ascribed to gross proliferation of endoplasmic reticulum. In our hands, the total activity of hexose-6-phosphate dehydrogenase expressed on unit liver weight increased about three times over that of the untreated control by eight daily injections of the drug (10 mg/100 g of body weight), although the specific activity of the enzyme expressed on a basis of microsomal protein increased only slightly (20–30%) by the same treatment.

The molar activity of the hexose-6-phosphate dehydrogenase can be calculated to be approximately 385 min⁻¹ on the basis of the minimum molecular weight. This value of molar activity is much smaller than those of other pyridine nucleotide-linked enzymes in microsomal membranes: approximately 4,500 min⁻¹ for NADPH-cytochrome c reductase (28) and 41,000 min⁻¹ for NADH-ferricyanide reductase (29); cal-
culated from the data referred to. On the contrary, the contents of the enzyme in microsomes are of the same order as those of the other microsomal pyridine nucleotide-linked enzymes on a molar basis as well as on a microsomal protein basis (30). The enzyme obtained in this report showed a dual nucleotide specificity for NADP and NAD and required hexoses which were phosphorylated at the sixth carbon hydroxyl group. Optimal pH differed depending upon the substrate used. These kinetic properties are quite consistent with previous reports using microsomal fraction (1) or crude enzyme preparations (3, 4) and are quite distinct from those of galactose-6-phosphate dehydrogenase, a cytosol enzyme showing cross-linked product in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cross-linking reagents have successfully been used for analyzing the subunit structure of oligomeric proteins (32, 33). Although the results imply that the native enzyme in the isolated preparation exists as a dimer, it is not clear whether this can be applied to the enzyme in the microsomal membranes in situ.

REFERENCES
