Glucose 6-Phosphate Dehydrogenase from Leuconostoc mesenteroides

PHYSICAL STUDIES*

CHARLES OLIVE$ and H. RICHARD LEVY
From the Biological Research Laboratories, Department of Biology, Syracuse University, Syracuse, New York

SUMMARY

Glucose 6-phosphate dehydrogenase from Leuconostoc mesenteroides, previously isolated in crystalline form (Olive, C., and Levy, H. R., Biochemistry, 6, 730 (1967)), is shown to be essentially homogeneous by disc gel electrophoresis and sedimentation velocity analysis. The weight average molecular weight is 103,700, determined by both high speed and low speed sedimentation equilibrium techniques at pH 4.6, which is the approximate isoelectric point. The partial specific volume was determined to be 0.717 at this pH. The enzyme possesses an absorption spectrum showing a maximum at 280.5 nm with $\varepsilon_{280} = 1.15$ and considerable fine structure in the region of 250 to 270 nm.

The isolation of crystalline glucose 6-phosphate dehydrogenase from Leuconostoc mesenteroides was reported previously (1). In the present paper we describe experiments which demonstrate the enzyme to be essentially homogeneous. Several physical properties, including the molecular weight, partial specific volume, and ultraviolet absorption spectrum, are also reported. Detailed kinetic studies of the enzyme are the subject of an accompanying paper (2).

EXPERIMENTAL PROCEDURE

General Methods—Assays were performed in a Zeiss PMQ II spectrophotometer with the thermostated cell compartment maintained at 25°C. Assay mixtures (3.00-ml final volume) contained the following components: 33.0 mM Tris-HCl, pH 7.8; 3.30 mM glucose 6-phosphate; and either 0.160 mM NADP+ or 2.50 mM NAD+ (neutralized to pH 7). Reactions were initiated with either enzyme or glucose 6-phosphate and followed by noting the increase in absorbance at 340 nm with time.

Protein concentrations were determined from the absorbances at 280 nm and 260 nm (3) or from the extinction coefficient at 280 nm after this was determined.

The enzyme was purified as described previously (1). The following four steps were carried out for us by the Worthington Biochemical Corporation. Cells were grown in 750 liters of medium, harvested, disrupted, and the enzyme was precipitated with ammonium sulfate. Subsequent steps (1) were performed in our laboratory. The enzyme was recrystallized four to six times until it reached a constant specific activity.

Polyacrylamide disc gel electrophoresis was performed using the Buchler Instruments (Poly-Analyt 3-1750) apparatus and the anionic gel system at pH 9.3 as described in the Buchler manual. The gels were stained for protein using the procedure of Chrambach et al. (4) and for enzymatic activity using a variation of the method of Dewey and Conklin (5) in which potassium cyanide was omitted from the staining mixture.

Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium experiments were performed at approximately 20°C in a Spinclo model E analytical ultracentrifuge equipped with a rotor temperature indicator and control (RTIC) system. For the sedimentation velocity experiments a phase plate was used as schlieren diaphragm. For most work a 4° single sector cell with 12-mm optical path was employed. The resulting photographs were measured using a Nikon profile projector equipped with a micrometer stage. Sedimentation coefficients at 20°C and in buffer ($s_{20,w}$) were calculated from plots of $\log x$ versus $t$, according to the method of Schachman (6). These values were converted to $s_{0,0}$ using data for the densities and viscosities of the buffers employed which were obtained from tables given by Svedberg and Pederson (7). For the purpose of extrapolating the sedimentation coefficients to zero protein concentration, a linear plot of sedimentation coefficient as a function of protein concentration was made. The partial specific volume was determined by the method of Edelstein and Schachman (8) in two separate experiments.

Sedimentation equilibrium experiments employed a rotatable light source and Rayleigh interferometric optics (9). A Yphantis cell (10) of 12-mm optical path was used with sapphire windows. A dense liquid, FC43 (10), was used to provide a transparent cell bottom of the proper shape (11). The experimental procedure followed that described by Van Holde (12) and Yphantis (10) for the high speed sedimentation equilibrium method.

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A low speed sedimentation equilibrium experiment was performed, again using Rayleigh interferometric optics and a short (1.5 mm) solution column in the Griffith cell (13). Rayleigh fringes were measured on the Nikon project scope, and the molecular weight was determined by the method of Nazarian (14) as modified by DiCamelli et al. (15). Corrections relating to D2O density were made by employing a relationship given by Kirschbaum (16). 

$s_{20,w}$ was obtained and from the areas under the peaks it could be estimated that 97% of the protein was in the major band. Samples containing 200 μg of protein were run under identical conditions but stained for enzyme activity using NADP+, NADP+, or a mixture of both. Control gels which were incubated in a staining solution lacking glucose 6-phosphate showed no enzyme activity. The other gels contained one major and three minor bands corresponding to all but the most rapidly moving band observed in gels stained for protein. The latter accounted for less than 0.4% of the total protein present.

Sedimentation-velocity experiments were performed at pH 6.8 and at various initial protein concentrations from 2.2 to 10.2 mg per ml. At each protein concentration the enzyme sedimented as a single component with no perceptible evidence of heterogeneity. Extrapolation of $s_{20,w}$ values to zero protein concentration yielded an $s_{20,w}$ of 6.01 (Fig. 1). The small concentration dependence observed is characteristic of compact, globular macromolecules and does not indicate an association-dissociation equilibrium under the conditions employed (17). A single experiment conducted in the presence of NADP+ (Fig. 1) yielded a value of 5.79 S, whereas 5.72 S was obtained for enzyme at the same protein concentration but without NADP+. Thus there is no evidence for NADP+-induced dimerization of this enzyme, in contrast to such dimerization observed with glucose 6-phosphate dehydrogenases from rat mammary gland (18), human erythrocytes (19-22), and brewers' yeast (23).

Sedimentation velocity studies at pH 4.6 in 0.01 M acetate buffer containing 0.1 M NaCl showed that the enzyme, again, sedimented as a single, sharp peak, with an $s_{20,w}$ value of 6.29. The slight change in sedimentation coefficient between pH 6.8 and pH 4.6 suggests that a small change in shape or hydration occurs over this pH range.

The enzyme employed in these studies thus appears to be over 99.5% pure on the basis of disc gel electrophoresis and sedimentation velocity analysis.

**Partial Specific Volume and Molecular Weight**—Both high speed and low speed sedimentation equilibrium runs were conducted at pH 4.6. This pH was found to be approximate isoelectric point of the enzyme by isoelectric focusing. The partial specific volume, $\bar{\varepsilon}$, was found to be 0.717 ± 0.003 in two separate determinations. An independent determination of $\bar{\varepsilon}$ by DiCamelli et al. (15) gave a value of 0.720. Thus, under these experimental conditions, the $\bar{\varepsilon}$ of glucose 6-phosphate dehydrogenase from *L. mesenteroides* appears to be considerably different than $\bar{\varepsilon}$ values of the glucose 6-phosphate dehydrogenases from brewers' yeast ($\bar{\varepsilon} = 0.744$ at 20°C (24)) or human erythrocytes ($\bar{\varepsilon} = 0.731$, calculated from the amino acid composition (21)).

**RESULTS AND DISCUSSION**

**Homogeneity of Enzyme**—Glucose 6-phosphate dehydrogenase (400 μg) which had been recrystallized to constant specific activity was subjected to polyacrylamide disc gel electrophoresis at pH 9.3 and stained for protein. A single, major band was apparent plus four minor bands. A densitometer tracing was obtained and from the areas under the peaks it could be estimated that 97% of the protein was in the major band. Samples containing 200 μg of protein were run under identical conditions but stained for enzyme activity using NADP+, NADP+, or a mixture of both. Control gels which were incubated in a staining solution lacking glucose 6-phosphate showed no enzyme activity. The other gels contained one major and three minor bands corresponding to all but the most rapidly moving band observed in gels stained for protein. The latter accounted for less than 0.4% of the total protein present.
FIG. 2. Fringe displacements obtained from high speed sedimentation equilibrium experiments with glucose 6-phosphate dehydrogenase in $H_2O$- and $D_2O$-containing buffers. The enzyme was concentrated and dialyzed against 0.01 M acetate buffer, pH 4.6, containing 0.1 M NaCl, and then diluted in the appropriate $H_2O$- and $D_2O$-containing buffers to 0.45 mg per ml. Sedimentation was at 21,740 rpm for 18 hours at 20°. Protein concentration was calculated from the fringe displacement ($Y_N - Y_0$) in millimeters as measured on the microcomparator. The abscissa represents the square of the distance (in centimeters) from the center of rotation. •, $H_2O$ buffer; □, $D_2O$ buffer.

FIG. 3. Ultraviolet absorption spectrum of glucose 6-phosphate dehydrogenase. The sample cuvette contained 1.64 mg of salt-free enzyme (see text) in 0.09 M Tris-KCl buffer, pH 7.2, total volume 3.0 ml. The blank cuvette contained buffer only. Sedimentation was at 10,580 rpm for 3 hours at 20°. The enzyme (2.0 mg per ml) was dialyzed against the same buffer (containing $H_2O$) used in the high speed runs. The data were plotted in a manner analogous to those shown in Fig. 2 and also yielded straight lines. An apparent weight average molecular weight of 103,700 was calculated from the high speed and low speed centrifugations (Table I). DiCamelli et al. (15), in an independent determination for the same enzyme, obtained a value of 103,530. Thus the molecular weight of $L$. mesenteroides glucose 6-phosphate dehydrogenase is very similar to the monomer molecular weights calculated from ultracentrifugation experiments for glucose 6-phosphate dehydrogenases from brewers' yeast (24), Candida utilis (20), rat liver (20), rat mammary gland, and human erythrocytes (22). The molecular weight of 120,000 previously reported for the $L$. mesenteroides enzyme (1) was an approximate value based on two gel filtration experiments.

Absorption Spectrum—The enzyme showed no light absorbance in the visible range (350 to 800 nm). The ultraviolet absorption is shown in Fig. 3. The maximum absorbance occurs at 280.5 nm. The extinction coefficient at this wave length and in 0.09 M Tris-HCl, pH 7.2, is 1.15 for a 0.01 M solution. The ratio of absorbances at 280 nm to 260 nm is 1.88. Other maxima are seen at 253, 259, 266, and 269 nm, corresponding closely with the known absorption maxima of phenylalanine (27). Although these peaks are small, they were always observed.

Kawahara, Wang, and Talalay (28) observed fine structure (of considerably greater intensity) in the same region of the absorption spectrum of 3-ketosteroid isomerase, and concluded that the absence of tryptophan in that enzyme allowed the absorption maxima of phenylalanine and tyrosine, normally obscured by the more strongly absorbing tryptophan, to be observed. The amino acid composition of $L$. mesenteroides glucose 6-phosphate dehydrogenase has not been determined, but one might predict, on the basis of the absorption spectrum, that the tryptophan content will prove to be low relative to phenylalanine.

REFERENCES
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3. WARBURG, O., AND CHRISTIAN, W., Biochem. Z., 310, 384 (1941).
6. SCHACHMAN, H. K., IN S. P. COLOWICK AND N. O. KAPLAN

Table I

<table>
<thead>
<tr>
<th>Method</th>
<th>Protein concentration</th>
<th>$M_w$</th>
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<tbody>
<tr>
<td>High speed (21,740 rpm)</td>
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<td>104,200</td>
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<td></td>
<td>0.45</td>
<td>105,900</td>
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<tr>
<td></td>
<td>0.33</td>
<td>102,200</td>
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<tr>
<td></td>
<td>0.22</td>
<td>109,000</td>
</tr>
<tr>
<td>Mean</td>
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<td>105,300 ± 3700</td>
</tr>
<tr>
<td>Low speed (10,580 rpm)</td>
<td>2.0</td>
<td>102,200 ± 3600c</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>103,700</td>
</tr>
</tbody>
</table>

a Yphantis (10).

b Nazarian (14) and DiCamelli et al. (15).
c Variation refers to results from measuring several plates taken at various times after reaching equilibrium.
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