6-Phosphogluconate Dehydrogenase

PURIFICATION AND KINETICS

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A method is described for the isolation and purification of 6-phosphogluconate dehydrogenase from pig liver. The molecular weight is estimated at 83,000 and that of the subunits is 42,000 as determined by gel electrophoresis. The pH maximum is 8.5 in 50 mM glycine/NaOH buffer and from 7.5 to 10 in 30 mM phosphate buffer at 30°C. Magnesium ion is not required for activity and acts as an inhibitor at concentrations above 20 mM. A cellular fractionation study indicates that this enzyme is located almost entirely within the soluble portion of the cytoplasm.

Kinetic studies have been done in 50 mM glycine buffer, pH 8.5, at 30°C. The data are consistent with a sequential mechanism in which NADP⁺ is added first, followed by 6-phosphogluconate, and the products are released in the order, CO₂, ribulose 5-phosphate, and NADPH. The Michaelis constant is 13.5 μM for 6-phosphogluconate. Dissociation constants are 4.8 μM for NADP⁺ and 5.1 μM for NADPH.

The enzyme 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6-phospho-D-gluconate-NADP⁺ oxidoreductase, decarboxylating) catalyzes the reversible oxidative decarboxylation of 6-phospho-D-gluconate to yield D-ribulose 5-phosphate and CO₂, with NADP⁺ being reduced to NADPH (Fig. 1).

In previous studies (1, 2) we have reported the isolation, characterization, and steady state kinetics of glucose-6-phosphate dehydrogenase from pig liver (6). Other isolation and purification studies from mammalian sources include sheep liver (9–11), rat liver (12), and human erythrocytes (13).

The kinetics of the oxidative decarboxylation reaction catalyzed by the enzyme from Candida utilis was studied in 1961, and a random order mechanism was postulated (4). Michaelis constants for NADP⁺ and 6-phosphoglucuronate have been reported for the enzyme from several sources (6, 7, 14). Villet and Dalziel have done kinetic studies of the forward (15) and reverse (16) reactions catalyzed by the sheep liver enzyme, as well as studies of the equilibrium constant and the nature of the carbon dioxide substrate (17). Dissolved CO₂ was found to be the form of carbon dioxide utilized as substrate for the reverse reaction, and contrary to previous results (4), the mechanism was postulated to be ordered sequential, with NADP⁺ being the first substrate bound, CO₂ the first product released, and NADPH the final product released.

MATERIALS AND METHODS

Chemicals—The trisodium salt of 6-phosphogluconic acid, the monosodium salt of NADP⁺, and Sephadex G-200 were purchased from Sigma Chemical Co. Phosphocellulose (Celllex P) was purchased from Bio-Rad Laboratories. Other chemicals used, including standards for molecular weight determination, were purchased from Sigma Chemical Co. Sigma Chemical Co.

Procedures—All spectrophotometric measurements were made on a Gilford DU spectrophotometer connected to a constant temperature bath set at 30 ± 0.01°C. All centrifugation steps were done at 4°C.

Enzyme samples were concentrated by freeze-drying on a VirTis bench-top freeze dryer. All buffers used in the purification procedure contained 1 mM β-mercaptoethanol.

The standard assay procedure was as follows: 2.8 ml of 0.05 M glycine/NaOH buffer, pH 8.5, 0.1 ml of 1.5 mM NADP⁺, and 0.01 ml of enzyme solution were added to a 1-cm cuvette, and the reaction was initiated by the addition of 0.1 ml of 30 mM 6-phosphogluconate. Absorbance readings were recorded after initiation of the reaction. One unit of activity was defined as the amount of enzyme required to form 1 μmol of NADPH/min with both substrates in excess.

Protein concentrations were estimated from absorbance at 280 nm and 260 nm according to the method of Warburg and Christian (18) or by the microbiur method of Goo (19).

All mechanistic studies were performed in 0.05 M glycine/NaOH buffer, pH 8.5, at 30°C. The specific activity of the purified enzyme was 0.4 and the enzyme concentration was estimated to be 0.04 μM in all kinetic experiments. For studies of CO₂ inhibition, varying amounts of a saturated solution of NaHCO₃ were added to the reaction buffer. The concentration of dissolved CO₂ was calculated to be 8.4 mM.

The nomenclature and equations used for analysis of the data were those of Cleland (20–22). Data were computer analyzed using Cleland's programs (23) as described previously (24).

Purification—Pig liver fresh from the slaughterhouse was cut into approximately 2-cm cubes. Then 75-g amounts were added to 150 ml of 0.2 M phosphate buffer, pH 7.0, and homogenized in a Waring Blender for 90 s. The pH of the resulting homogenate was lowered to 5.5 with approximately 10% acetic acid and then stored at 4°C with occasional stirring for at least 2 h. The solution was then centrifuged at 12,000 × g for 30 min. The residue was discarded and (NH₄)₂SO₄ was added to the supernatant to 25% saturation (17.5 g/100 ml) with gentle stirring. This solution was allowed to stand for 45 min at 4°C, then centrifuged at 12,000 × g for 15 min. The precipitate was discarded, and (NH₄)₂SO₄ was again added (14 g/100 ml) to bring the solution to 45% saturation. After standing for 45 min at 4°C, the solution was centrifuged at 12,000 × g for 15 min. The supernatant was discarded, and the precipitate was redissolved in a minimum
volume of 0.2 M phosphate buffer, pH 7.0.
The enzyme solution was then heated in a water bath at 55° for 5 min, after which it was immediately chilled and centrifuged at 12,000 x g for 15 min. The supernatant was added in 5-ml batches to a column (2.5 x 45 cm) of Sephadex G-200 equilibrated with 0.005 M phosphate buffer, pH 7.0. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min. The 15-min fractions of approximately 3 ml each were collected. The most active fractions eluted from this column were combined and lyophilized to dryness. The resulting powder was redissolved in 5 ml of 0.2 M phosphate buffer, pH 7.0, and again passed down the same Sephadex G-200 column under the same conditions.

The most active fractions from the second Sephadex G-200 run (5 to 10 ml) were added to a Cellex P column equilibrated with 0.005 M phosphate buffer, pH 6.0, and the column was washed with about 10 ml of the same buffer. The protein was then eluted from the column by washing with successively higher concentrations of buffer, a stepwise gradient from 0.005 to 0.2 M being created by adding 0.005 M, 0.01 M, 0.05 M, and finally 0.2 M phosphate buffers at pH 6.0. The flow rate was about 0.6 ml/min, with 5-min fractions of 3 ml each being collected. The enzyme eluted from the Cellex P column was stored in solution at 4°.

Although an accurate assay of the homogenate is not possible due to the turbidity of the solution, the acid treatment gives a 2- to 5-fold increase in specific activity with very little loss of total activity. Extracts not acid-treated remain turbid even after the ammonium sulfate precipitate is redissolved, and separation on Sephadex G-200 is much poorer. The specific activity of the final Cellex P sample is 0.4 unit/mg. A typical set of results is given in Table I.

Subcellular Fractionation—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 (25) as described previously (1). Both 6-phosphogluconate dehydrogenase and glucose dehydrogenase were assayed in the clear supernatants of the various fractions. Glucose dehydrogenase (26) was assayed with glucose (33.3 mM) and NAD (50 PM) as substrates in 0.06 M glycine buffer (1 mM EDTA) at pH 10, 30°.

**Effect of Ionic Strength**—Since 6-phosphogluconate dehydrogenase isolated from other sources shows a marked dependence on ionic strength (10, 13, 14), the effects of ionic strength and of buffer concentration on the purified pig liver enzyme have been studied. The enzyme activity rises steadily with increasing glycine concentration up to 0.1 M and then remains constant, with no inhibition observed up to 0.5 M glycine. When the ionic strength is varied using NaCl, the activity rises to a maximum at 0.05 M, and then falls off, with considerable inhibition above 0.1 M. The specific activity of the final Cellex P sample is much poorer. The specific activity of the final Cellex P sample is 0.4 unit/mg. A typical set of results is given in Table I.

**Table I: Purification scheme and results**

| Step            | Activity | Protein | Volume | Specific activity | Purification | Yield |
|-----------------|----------|---------|--------|------------------|--------------|-------|
|                 | units/ml | mg/ml   | ml     | units/mg         | -fold        | %     |
| 1. Homogenization| 0.40     | 115     | 1800   | 0.0035           | 100          | 100   |
| 2. Acid treatment| 0.34     | 47.1    | 1800   | 0.0072           | 2.06         | 2.06  |
| 3. Ammonium sulfate fractionation| 1.26 | 108 | 436 | 0.012 | 1.61 | 3.31 | 90 90 |
| 4. Heat treatment| 1.20     | 60.7    | 322    | 0.016            | 1.34         | 4.40  |
| 5. Sephadex G-200 | 0.17     | 1.89    | 1420   | 0.090            | 5.74         | 25.6  |
| 6. Cellex-P      | 0.12     | 0.30    | 1750   | 0.41             | 4.56         | 117   |

**Characterization**

**Effect of pH**—The pH of maximum enzyme activity in glycine/NaOH buffer is 8.5; in phosphate buffer, the activity is maximal and nearly constant from pH 7.5 to pH 10.0. pH activity profiles in Tris and bicarbonate buffers gave results similar to those in glycine/NaOH. The enzyme of the activity in all of the above buffers at pH 8.5 and 50 mM was essentially identical. Finally, activity measurements in 5 mM buffer and 0.1 M NaCl gave identical results for all of the above buffer systems.

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**Effect of Magnesium Ion**—Malic enzyme and isocitrate dehydrogenase, enzymes which catalyze similar oxidative decarboxylation reactions, show an absolute requirement for Mg²⁺ or Mn²⁺ (15). Although 6-phosphogluconate dehydrogenase from other sources has not been found to require divalent ions, its activity is affected by Mg²⁺ (12, 15). This enzyme lost no activity on dialysis overnight against 10 mM EDTA. Presumably, divalent ions are not required for activity. The effect of added MgCl₂ on the reaction gave results similar to those obtained in the ionic strength study. To determine if there was a specific effect of Mg²⁺ above that due to the increase in ionic strength, a series of samples of identical ionic strength were prepared, in one case using NaCl to vary the ionic strength. In the other MgCl₂, Mg²⁺ caused a slight activation at concentrations up to 20 mM, and strong inhibition at higher concentrations.

**Molecular Weight Studies**—Molecular weight and subunit size were measured using gel electrophoresis. A molecular weight of 83,000 (±3,000) was obtained on 7% gels using the method of Ornstein (27) and Davis (28). Standards used included yeast alcohol dehydrogenase (150,000), horse liver alcohol dehydrogenase (80,000), and bovine serum albumin (67,000). Sodium dodecyl sulfate electrophoresis according to the method of Weber and Osborn (29) gave a single subunit band of molecular weight 42,000 (±3,000). Standards used included bovine serum albumin (67,000), horse liver alcohol dehydrogenase (41,000), yeast alcohol dehydrogenase (36,000), and lactate dehydrogenase (35,000).

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

**Kinetic Equations**

The majority of previous studies (15, 16, 30) of oxidative decarboxylation enzyme reactions are consistent with the basic reaction mechanism being Ordered Bi-Ter, with NADPH being the first substrate bound, NADPH the last product released, and CO₂ the first product released. This mechanism is shown in schematic form in Equation 1 (below). The data from all kinetic studies were fitted to the equations for the
above mechanisms, and any deviations were then accounted for by alterations of this mechanism.

In all equations used in this work, \( v \) is the measured initial velocity, \( V \) is the maximum velocity for the forward reaction, and \( K_m \) is the equilibrium constant. \( A, B, P, Q, \) and \( R \) are concentrations, \( K_a, K_B, K_P, K_Q, \) and \( K_R \) are Michaelis constants, and \( K_{ap}, K_{bp}, K_{ip}, K_{ip}, \) and \( K_{ir} \) are dissociation constants for NADP\(^+\), GlcA-6-P, \( \text{CO}_2 \), Rbu-5-P, and NADPH, respectively.

The steady state rate equation for the mechanism in Equation 1 can be derived by analysis of the King-Altman (32) patterns. The resulting equation can be simplified to an initial velocity equation for an Ordered Bi-Ter mechanism.

\[
v = \frac{V}{K_a K_B + \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{AB}}{AB}}
\]

Equation 2 is written in reciprocal form for analyzing data with either \( A \) (NADP\(^+\)) or with \( B \) (GlcA-6-P) as the variable substrate.

\[
\frac{1}{V} = \frac{K_a}{V_1} \left( 1 + \frac{K_a}{A} \right) \left( 1 + \frac{K_B}{B} \right) \left( 1 + \frac{K_{AB}}{AB} \right)
\]

\[
\frac{1}{V} = \frac{K_B}{V_1} \left( 1 + \frac{K_B}{B} \right) \left( 1 + \frac{K_A}{A} \right)
\]

\[
\frac{1}{V} = \frac{P}{V_1} \left( 1 + \frac{P}{A} \right) \left( 1 + \frac{Q}{B} \right) \left( 1 + \frac{R}{AB} \right)
\]

\[
\frac{1}{V} = \frac{Q}{V_1} \left( 1 + \frac{Q}{B} \right) \left( 1 + \frac{R}{A} \right)
\]

\[
\frac{1}{V} = \frac{R}{V_1} \left( 1 + \frac{R}{A} \right)
\]

Both of these equations are linear equations, and a Lineweaver-Burk (33) plot of reciprocal velocity versus reciprocal concentration of the variable substrate will yield a family of intersecting straight lines whose slopes and intercepts are a function of the reciprocal concentration of the nonvaried substrate.

Cleland (21) described how product inhibition studies can be used to verify enzyme kinetic mechanism and help establish the order of substrate binding and product release. Initial velocity equations for product inhibition studies are Equations 5 through 10 (see below).

**RESULTS AND DISCUSSION**

**Forward Reaction**—For studies of the forward reaction, initial velocity measurements were made at six concentrations of GlcA-6-P for each of six different concentrations of NADP\(^+\). The data were computer fitted to Equation 2. A plot of reciprocal velocity versus reciprocal concentration of GlcA-6-P (Fig. 2) gives a series of straight lines which intersect at a common point as predicted by Equation 2. This result shows that the mechanism is a sequential one in which all substrates are bound before any products are released. The Michaelis constants are 13.5 (+1.0) \( \mu \)M for NADP\(^+\) (\( K_a \)) and 29.2 (+1.0) \( \mu \)M for GlcA-6-P (\( K_b \)). The dissociation constant for the E·NADP\(^+\) complex (\( K_{ap} \)) was 4.8 (±0.8) \( \mu \)M, and the maximum velocity,
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**FIG. 2.** Initial velocity pattern with GlcA-6-P as the varied substrate at 30°. GlcA-6-P varied from 10 to 500 μM, NADP varied from 7.5 to 100 μM, E₀ is 0.04 μM, 50 mM glycine/NaOH buffer, pH 8.5.

$V_\text{max}$ was 0.0493 (±0.0006) μM/min. These values are in the same range as those determined for the sheep liver enzyme (15).

**NADPH Inhibition**—For NADPH product inhibition studies with NADP⁺ as the variable substrate, the GlcA-6-P concentration was held constant at 0.50 mM. The NADP⁺ concentration was varied between 10 and 100 μM for each of three levels of NADPH. The results fit Equation 9 which predicts the linear competitive inhibition shown in Fig. 3. $K_i$ was calculated from the slope of a linear slope versus NADPH concentration replot (not shown) and is 5.6 μM.

For NADPH product inhibition studies where GlcA-6-P was the variable substrate, the NADP⁺ concentration was held constant at 50 μM. A plot of the data is shown in Fig. 4 and the inhibition is noncompetitive as predicted by Equation 10. The slope and intercept replots are parabolic at CO₂ concentrations above 1 mM. This suggests that CO₂ combined with some form(s) of the enzyme in a dead-end fashion in addition to its product inhibition due to combination with the EQR form.

**CO₂ Inhibition**—Results of studies of CO₂ inhibition on this enzyme give the inhibition patterns predicted by Equations 5 and 6 for simple product inhibition. The inhibition is noncompetitive; however, the slope and intercept replots are parabolic at CO₂ concentrations above 1.0 mM. This suggests that CO₂ combined with some form(s) of the enzyme in a dead-end fashion in addition to its product inhibition due to combination with the EQR form.

**Conclusion**—All of the results obtained in the kinetic studies are consistent with the proposed Ordered Bi-Ter mechanism. The inhibi-
Another possible mechanism is one in which CO₂ and ribulose 5-phosphate are released (or add) in a random sequence, whereas NADP⁺ 6-phosphogluconate, and NADPH add or release in order. In such a mechanism with ribulose 5-phosphate or CO₂, as an inhibitor, and either substrate variable, noncompetitive inhibition is predicted. This is not the case with the observed ribulose 5-phosphate inhibition which is clearly uncompetitive.

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