Purification and Properties of 6-Phosphogluconate Dehydrogenase from Rabbit Mammary Gland

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1. 6-Phosphogluconate dehydrogenase from rabbit mammary gland was purified to homogeneity by the criterion of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The molecular weight of the subunit is 52000. The enzyme was purified 150-fold with a final specific activity of 20 μmol of NADP^+ reduced/min per mg of protein and overall yield of 3%. The molecular weight of the native enzyme is estimated to be 104000 from gel-filtration studies. The final purification step was carried out by affinity chromatography with NADP^+ -Sepharese. 2. The K_m values for 6-phosphogluconate and NADP^+ are approx. 54 μM and 23 μM respectively. 3. Citrate and pyrophosphate are competitive inhibitors of the enzyme with respect to both 6-phosphogluconate and NADP^+ . 4. MgCl_2 affects the apparent K_m for NADP^+ at saturating concentrations of 6-phosphogluconate.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) has been purified from sheep liver (Villet & Dalziel, 1969; Dyson *et al.*, 1973), rat liver (Procsal & Holten, 1972), human erythrocytes (Pearse & Rosemeyer, 1974a) and *Candida utilis* (Pontremoli *et al.*, 1961). The studies reported here describe the purification of the enzyme from mammary gland of lactating rabbit together with some kinetic and molecular properties.

In pregnancy and lactation glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase in the mammary gland are involved in NADPH production, which is essential for lipogenesis. It has been shown that during pregnancy and lactation in the rabbit and rat there is a considerable increase in the activity of both enzymes in the mammary gland, with maximum activity in mid-lactation (Gul & Dils, 1969). For this reason the enzyme was prepared from mammary glands of rabbits at the 15th day of lactation.

The object of the purification is to prepare an homologous antiserum to 6-phosphogluconate dehydrogenase so that changes in the amount of the enzyme can be studied in organ culture of mammary gland by immunochemical techniques.

Materials and Methods

Animals

Primiparous New Zealand white rabbits were obtained on the 15th day of lactation from the Joint Animal Breeding Unit, Nottingham School of Agriculture, Sutton Bonington, Leics., U.K.

Materials:

NADP^+ and Combithek protein calibration set containing cytochrome *c* (horse heart), ovalbumin, serum albumin (bovine), catalase (EC 1.11.1.6) and aldolase (EC 4.1.2.13) were purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. 6-Phosphogluconic acid (trisodium salt), dithiothreitol, riboflavin, *NN'*-methylenebisacrylamide, acrylamide, *NNN'*-tetramethylenediamine and sodium dodecyl sulphate were purchased from BDH Chemicals, Poole, Dorset, U.K. Coomassie Brilliant Blue R, Nitro Blue Tetrazolium and phenazine methosulphate were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. DEAE-cellulose (Whatman grade DE-52) and cellulose phosphate (P11) were obtained from W. and R. Balston (Modified Cellulose) Ltd., Maidstone, Kent, U.K. Sephadex G-200 was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. Hydroxyapatite was obtained from Bio-Rad Laboratories, Bromley, Kent, U.K. NADP^+ -Sepharese prepared by the method of Lamed *et al.* (1973) was a kind gift from Dr. Mike Harvey, Department of Biochemistry, University of Liverpool, Liverpool L69 3BX, U.K. All other chemicals were of A.R. grade.

The Amicon and Minicon Ultrafiltrators were obtained from Amicon, High Wycombe, Bucks., U.K. Conductivity measurements were made on a Radiometer Conductivity meter type CDM 2d. All column eluates were monitored by using a Cecil CE212 Variable Wavelength UV Monitor Spectrophotometer at 280nm which was linked to a Servoscribe recorder.

Centrifugation

Centrifugation was carried out in MSE 65 and MSE 18 centrifuges with 8 × 50 ml fixed-angle rotors.

Assay of 6-phosphogluconate dehydrogenase activity

The enzyme was assayed by the method of Huggins & Yao (1959), based on the method of Glock & McLean (1953), by measurement of the reduction of NADP⁺ at 340 nm in a Pye– Unicam SP.1800 recording spectrophotometer, maintained at 30°C. In the kinetic studies, the enzyme was assayed in a Gilford 240 spectrophotometer at 30°C. In preliminary studies no 6-phosphogluconate-independent NADP⁺ reduction could be measured with tissue extracts, and consequently no correction of enzyme rates was made. One unit of enzyme activity is that which catalyses the reduction of 1 μmol of NADP⁺/min at 30°C.

Protein determinations

Protein concentration was determined by the method of Hübscher *et al.* (1965) or by the method of Lowry *et al.* (1951). Bovine serum albumin was used throughout as protein standard.

Molecular-weight estimations

The molecular weight of the native enzyme was determined by gel filtration on Sephadex G-200 (Andrews, 1965). The column (0.6 cm × 100 cm) was equilibrated in 20 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, and was calibrated with cytochrome *c*, ovalbumin, serum albumin, catalase and aldolase. The molecular weights of the calibration proteins are those given in the Combithex calibration set.

Preparation of particle-free supernatant fractions

All operations were performed at 4°C unless otherwise stated. One rabbit was usually taken for each preparation of the enzyme. Each animal was killed by cervical dislocation and the mammary glands (100–150 g fresh wt.) were removed. Adipose tissue, connective tissue and muscle were dissected from the glands before being cut into small pieces. The tissue was homogenized in approx. 3 vol. of 0.15 M-KCl in an MSE top-drive homogenizer at full speed for 1 min. The homogenate was then strained through double-thickness tubular bandage to remove residual connective tissue and centrifuged for 60 min at 100 000 *g*_{av.}. The particle-free supernatant was filtered through glass-wool to remove any fat.

Ammonium sulphate fraction

Solid (NH₄)₂SO₄ was slowly added with stirring to the particle-free supernatant, at 0°C, to give

194 g/litre (final concn.). The solution was then stirred for 30 min before centrifugation for 15 min at 25 000 *g*_{av.}. (NH₄)₂SO₄ was added to the supernatant to give 378 g/litre (final concn.). After 30 min, the suspension was centrifuged as previously described. The pellet obtained was dissolved in 5 mM-potassium phosphate buffer, pH 6.7, containing 1 mM-EDTA and dialysed overnight against the same buffer.

Ethanol fractionation

MgCl₂ (final concn. 30 mM) was added to the dialysed solution. Ethanol (99%, v/v, stored at –21°C) was slowly added to the stirred solution at 4°C to give 200 ml/litre (final concn.). The suspension was then immediately centrifuged at 4°C for 10 min at 38 000 *g*_{av.}. The supernatant was stirred in an ice-salt bath and ethanol slowly added to give 400 ml/litre (final concn.). The suspension was then stirred for about 30 min, during which the temperature fell to between –8°C and –10°C. This suspension was then centrifuged for 10 min at 38 000 *g*_{av.} at –10°C. The pellet was dissolved in 5 ml of 50 mM-potassium phosphate buffer, pH 8.0, containing 10 mM-EDTA, and diluted to 50 ml with distilled water. Dithiothreitol (final concn. 0.1 mM) was added and the solution was re-adjusted to pH 8.0 with 0.01 M-KOH. The solution was then dialysed against 5 litres of 5 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA and 0.1 mM-dithiothreitol until the conductivity was approx. 0.55 mmhos at 4°C.

DEAE-cellulose chromatography

The dialysed enzyme solution (containing 50 μM-NADP⁺) was applied to a DEAE-cellulose column (2.5 cm × 30 cm) previously equilibrated in 5 mM-potassium phosphate buffer, pH 8.0, containing 1 mM-EDTA, 0.1 mM-dithiothreitol and 50 μM-NADP⁺. The enzyme was eluted with a linear gradient (1 litre) of the same buffer (5–40 mM) containing 1 mM-EDTA, 0.1 mM-dithiothreitol and 50 μM-NADP⁺. Fractions (5 ml) were collected and assayed for activity. Fractions containing enzyme activity were pooled and adjusted to pH 6.5 with a saturated solution of potassium phosphate buffer and dialysed overnight against 20 mM-potassium phosphate buffer, pH 6.5, containing 0.1 mM-dithiothreitol and 10 μM-NADP⁺.

Cellulose phosphate chromatography

The enzyme solution was applied to a cellulose phosphate column (2.5 cm × 25 cm) previously equilibrated in 20 mM-potassium phosphate buffer, pH 6.5, containing 0.1 mM-dithiothreitol and 10 μM-NADP⁺. A linear gradient (20–50 mM) of the same buffer (1 litre) was then applied to the column and 5 ml

fractions were collected. Fractions containing enzyme activity were pooled.

Hydroxyapatite chromatography

The enzyme solution (containing $50\mu\text{M-NADP}^+$) was applied to the hydroxyapatite column ($2.5\text{cm} \times 2.5\text{cm}$) previously equilibrated in 35mM -potassium phosphate buffer, pH 6.5, containing 0.1mM -dithiothreitol and $50\mu\text{M-NADP}^+$. The column was washed with 1–2 column volumes of the starting buffer and then with 40mM -potassium phosphate buffer, pH 6.5, containing 0.1mM -dithiothreitol and $50\mu\text{M-NADP}^+$ until the enzyme had been completely eluted. Fractions ($2\text{--}3\text{ml}$) containing the enzyme were pooled.

Concentration of enzyme solution

The enzyme solution was initially concentrated to a small volume with an Amicon Ultrafiltration cell (model 52, PM10 Diaflo membrane). Further concentration to $0.5\text{--}1.0\text{ml}$ was brought about by a Minicon-B15 Macrosolute concentrator.

NADP⁺-Sephacrose affinity chromatography

The concentrated enzyme was applied to an NADP^+ -Sephacrose ($3.2\mu\text{mol}$ of NADP^+/ml) column ($1\text{cm} \times 2\text{cm}$) previously equilibrated in 50mM -sodium phosphate buffer, pH 6.5, containing 1mM -EDTA and 0.1mM -dithiothreitol. The column was thoroughly washed with the starting buffer ($50\text{--}60\text{ml}$) until the E_{280} of the column effluent was below 0.25 and constant. A linear gradient (20ml) of sodium pyrophosphate ($0\text{--}50\text{mM}$) in 50mM -sodium phosphate buffer, pH 6.5, containing 1mM -EDTA and 0.1mM -dithiothreitol was applied to the column. This was followed by additional starting buffer containing 50mM -sodium pyrophosphate until the E_{280} of the column effluent was below 0.2 and constant. Fractions (1ml) containing enzyme activity were pooled and concentrated to $3\text{--}5\text{ml}$. NADP^+ (final concentration $50\mu\text{M}$) was added and the enzyme stored at -21°C . The enzyme is stable for at least 2 months under these conditions.

Criteria of purity

Polyacrylamide-gel electrophoresis. Polyacrylamide-disc-gel electrophoresis was carried out with phosphate buffer, pH 7.4, containing 0.0186M - NaH_2PO_4 and 0.048M - Na_2HPO_4 as running and gel buffers. Gels were prepared from a solution containing 0.5% (w/v) riboflavin, 0.002% (v/v) $\text{NNN}'\text{N}'$ -tetramethylethylenediamine, 4.88% acrylamide and 0.12% NN' -methylenebisacrylamide in the gel buffer. Each solution was polymerized for 30 min in u.v. light from a Kodac Illuminator. Between 10 and $30\mu\text{g}$ of pro-

tein, mixed with glycerol (final concentration 50% , v/v), were layered on each gel. Electrophoresis was carried out with a Shandon analytical polyacrylamide-gel-electrophoresis apparatus at $4\text{mA}/\text{tube}$ for $3\frac{1}{2}\text{--}4\text{h}$ at 20°C .

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Polyacrylamide gels (10% , w/v) were made as described above, with the addition of sodium dodecyl sulphate (0.1% , w/v, final concentration) to both running and gel buffers. Protein samples were dialysed against water overnight and rotary-evaporated to dryness before heating at 100°C for 5 min in 10mM -sodium phosphate buffer, pH 7.0, containing 2% (w/v) sodium dodecyl sulphate and 2% (v/v) 2-mercaptoethanol. Samples of $10\text{--}30\mu\text{g}$ of protein, mixed with glycerol (50% , v/v, final concentration) and mercaptoethanol (4% , v/v, final concentration) were layered on each gel and electrophoresis was carried out at $8\text{mA}/\text{tube}$ for 4 h at 20°C . Electrophoresis was also carried out with the marker polypeptides derived from haemoglobin, lysozyme, alcohol dehydrogenase, papain and cytochrome c.

Staining for 6-phosphogluconate dehydrogenase activity. Polyacrylamide gels were stained for enzyme activity by immersion in a solution containing 2.7mM -6-phosphogluconate, 0.5mM - MgCl_2 , 0.25mM -phenazine methosulphate, 0.1mM -Nitro Blue Tetrazolium and 0.05mM - NADP^+ (final concentrations) in 0.05M -Tris-HCl buffer, pH 7.4. Each gel was incubated for 1–2 h in the dark at 37°C . The reaction was terminated by washing with 7% (v/v) acetic acid.

Protein staining. Both types of polyacrylamide gels were stained for protein with 0.25% (w/v) Coomassie Blue in methanol–water–acetic acid ($5:5:1$, by vol.). The gels were stained overnight and then destained by repeated washing in an aqueous solution containing 7.5% (v/v) acetic acid, 5% (v/v) methanol and 10% (v/v) trichloroacetic acid.

Experimental and Results

Purification of the enzyme

A typical purification scheme for 6-phosphogluconate dehydrogenase is presented in Table 1. The final specific activity of the enzyme was approx. $22\mu\text{mol}$ of NADP^+ reduced/min per mg of protein, which represents a 150-fold purification of the protein relative to the enzyme in the particle-free supernatant prepared from the mammary gland. The fractionation has been repeated five times with approximately the same final specific activities.

In preliminary experiments large losses in enzyme activity occurred during the initial stages of the purification, particularly in the ethanol fractionation. Loss of activity in the ethanol fractionation was overcome by careful temperature regulation (see the

Table 1. Purification scheme for 6-phosphogluconate dehydrogenase from mammary gland of rabbits on the 15th day of lactation

This is a typical result starting with 120g of mammary gland. Experimental details are given in the text.

Fraction	Volume (ml)	Protein (mg)	Total activity ($\mu\text{mol/min}$)	Yield (%)	Specific activity ($\mu\text{mol/min per mg of protein}$)
1. Particle-free supernatant	188	6075	843	100	0.14
2. 194–378 g of $(\text{NH}_4)_2\text{SO}_4/\text{litre}$	87	2870	682	81	0.24
3. 200–400 ml of ethanol/litre	50	575	368	43.6	0.64
4. DEAE-cellulose eluate	127	74	246	29	3.30
5. Cellulose phosphate eluate	245	4.5	78	9.3	17.30
6. Hydroxyapatite eluate	49	2.9	58	6.8	20.0
7. NADP^+ -Sephadex eluate	27	1.15	25	3.0	22.0

Fig. 1. Affinity chromatography on NADP^+ -Sephadex

After sample (0.45 ml) application, the column was washed with 50–60 ml of 50 mM-sodium phosphate buffer, pH 6.5, containing 0.1 mM-dithiothreitol and 1 mM-EDTA and the enzyme was eluted (1 ml fractions) from the column with a linear gradient of 0–50 mM-sodium pyrophosphate in the starting buffer (20 ml) followed by 30 ml of 50 mM-sodium pyrophosphate in the starting buffer. Enzyme elution commences at the beginning of the linear gradient and is completed by washing as described. The start and finish of the linear gradient are indicated by the arrows. $\circ$, Enzyme activity; —, E_{280} .

Materials and Methods section) and losses in subsequent steps were minimized by inclusion of NADP^+ and dithiothreitol in the buffer systems.

Affinity chromatography of the enzyme on NADP^+ -Sephadex resulted in either a small increase in specific activity or even a slight decrease. A typical elution profile from the NADP^+ -Sephadex column is shown in Fig. 1. A high baseline absorbance is shown, which is due to the buffer since the u.v. monitor was calibrated with water.

Comparison of the enzyme preparations after hydroxyapatite and NADP^+ -Sephadex chromatography by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate shows that although there is only a small increase in specific activity of the enzyme after chromatography on NADP^+ -Sephadex several contaminants are removed from the enzyme preparation by this procedure.

Purity of the enzyme

After affinity chromatography, samples of the enzyme preparation were analysed by polyacrylamide-gel electrophoresis and gave a single band when stained for protein or enzyme activity. Analysis of the enzyme preparation by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed one protein band which indicates a single subunit species of molecular weight 52000.

From gel-filtration studies, the molecular weight of the enzyme was estimated to be 104000, which suggests that the native enzyme is a dimer.

Kinetics

Previous work has shown that the activity of 6-phosphogluconate dehydrogenase may vary with concentration of MgCl_2 and ionic strength (Villet & Dalziel, 1969; Procsal & Holten, 1972; Dyson *et al.*, 1973; Pearse & Rosemeyer, 1974a). The results presented in Figs. 2 and 3 show small variations in enzyme activity with varying MgCl_2 and ionic strength

respectively in 8.3 mM-Tris-HCl buffer, pH 7.4, containing KCl.

Fig. 2. Effect of MgCl_2 concentration on 6-phosphogluconate dehydrogenase at constant ionic strength

Enzyme activity was assayed at 1.67 mM-6-phosphogluconate, 0.167 mM- NADP^+ in 8.3 mM-Tris-HCl buffer, pH 7.4, with ionic strength maintained at 0.1 (mol/litre) by KCl. Assays were performed at 30°C.

Fig. 3. Effect of ionic strength on 6-phosphogluconate dehydrogenase

Enzyme activity was assayed as described in the legend to Fig. 2 at 16 mM- MgCl_2 . Ionic strength was changed by varying the concentrations of KCl. Assays were performed at 30°C.

From the double-reciprocal plots shown in Figs. 4 and 5, the K_m for NADP^+ was calculated to be 23 μM and the K_m for 6-phosphogluconate was 54 μM .

Previously it has been shown that many metabolites, including pyrophosphate and citrate, are inhibitors of 6-phosphogluconate dehydrogenase (Dyson & D'Orazio, 1973). The use of pyrophosphate to elute the enzyme from NADP^+ -Sephadex was based on these observations. The results in Figs. 6 and 7 are compatible with competitive inhibition of 6-phosphogluconate dehydrogenase by citrate and

Fig. 4. Double-reciprocal plot of rate of 6-phosphogluconate dehydrogenase activity against NADP^+ concentrations at different 6-phosphogluconate concentrations

Enzyme was assayed in 83 mM-Tris-HCl buffer, pH 7.4, containing 16 mM- MgCl_2 . 6-Phosphogluconate concentrations: 0.83 mM (●); 0.24 mM (■); 0.16 mM (▲). Assays were performed at 30°C.

Fig. 5. Double-reciprocal plots of rate of 6-phosphogluconate dehydrogenase activity against 6-phosphogluconate concentrations at different NADP^+ concentrations

Enzyme was assayed in 83 mM-Tris-HCl buffer, pH 7.4, containing 16 mM- MgCl_2 . NADP^+ concentrations: 0.083 mM (●); 0.025 mM (■); 0.016 mM (▲). Assays were performed at 30°C.

Fig. 6. Inhibition of 6-phosphogluconate dehydrogenase by citrate

Enzyme activity was assayed in 83 mM-Tris-HCl buffer, pH 7.4. (a) Concentration of NADP^+ was 0.167 mM; (b) concentration of 6-phosphogluconate was 1.67 mM. Citrate concentrations: 3.3 μM (■); 20.8 μM (○); 53 μM (▲). (●), No citrate. Assays were performed at 30°C.

pyrophosphate at both 6-phosphogluconate and NADP^+ sites.

A comparison of Figs. 4, 6(b) and 7(b) shows that the K_m values for 6-phosphogluconate dehydrogenase with respect to NADP^+ are 23 μM , 67 μM and 74 μM respectively. The latter two values were measured in the absence of MgCl_2 and from this it appears that MgCl_2 has an effect on the K_m of the enzyme with respect to NADP^+ . In contrast there appears to be no change in the K_m value of the enzyme for 6-phosphogluconate (see Figs. 3, 6a and 7a) in the presence or absence of MgCl_2 .

Discussion

The methods used in the purification of 6-phosphogluconate dehydrogenase from mammary gland are essentially similar to those used in the purification of the enzyme from other sources. One basic distinction is that the enzyme from mammary gland would not bind to CM-cellulose under conditions where binding occurs with the sheep liver enzyme (Villet & Dalziel, 1969). It was therefore necessary to develop alternative methods for the purification of the mammary-gland enzyme. The final step of the purification was

carried out by affinity chromatography. This resulted in little change in the specific activity of the enzyme but had the desired effect of removing several minor contaminants, to give a homogeneous preparation by the criterion of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

NADP^+ (final concentration 50 μM) and dithiothreitol (final concn. 0.1 mM) were included in the buffers after the ethanol fractionation. The NADP^+ concentration was lowered to 10 μM for cellulose phosphate chromatography, otherwise between 50 and 70% of the enzyme would not bind to the cellulose phosphate.

It appears from staining for enzyme activity after polyacrylamide-gel electrophoresis that 6-phosphogluconate dehydrogenase from rabbit mammary gland occurs as a single isoenzymic form, in contrast with the human erythrocyte enzyme, which is reported to have three isoenzymic forms (Pearse & Rosemeyer, 1974a). Glucose 6-phosphate dehydrogenase has been shown to exist in two isoenzymic forms in the mammary gland of the rat. During pregnancy and lactation, a third isoenzymic form appears which is not present in the unstimulated tissue (Richards & Hilf, 1972).

Fig. 7. Inhibition of 6-phosphogluconate dehydrogenase by pyrophosphate

Enzyme activity was measured in 83 mM-Tris-HCl buffer, pH 7.4. (a) Concentration of NADP^+ was 0.167 mM; (b) concentration of 6-phosphogluconate was 1.67 mM. Pyrophosphate concentrations: 0.83 μM (■); 5.2 μM (▲); 13.3 μM (○). (●), No pyrophosphate. Assays were performed at 30°C.

The molecular weight of the subunits was shown to be 52000 by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The native enzyme has a molecular weight of 104000 as measured by gel filtration. These results agree well with the estimate of 104000 for the molecular weight of the human erythrocyte enzyme (Pearse & Rosemeyer, 1974b) and 102000 for the molecular weight of the rat liver enzyme (Procsal & Holten, 1972). From the results presented here it appears that 6-phosphogluconate dehydrogenase from rabbit mammary gland is a dimer.

The K_m values of the enzyme from mammary gland are of the same order as those reported by other workers. However, direct comparisons are difficult owing to the differences in the conditions in which measurements have been made. The enzyme appears to be competitively inhibited by pyrophosphate and citrate with respect to both 6-phosphogluconate and NADP^+ (Figs. 6 and 7).

From the results shown in Figs. 4, 5, 6 and 7, it appears that MgCl_2 has an effect on the apparent K_m for NADP^+ , at saturating 6-phosphogluconate concentrations, which results in an approximately threefold increase in K_m . It appears from this that

MgCl_2 may affect NADP^+ binding to the enzyme. This effect is not seen on the K_m for 6-phosphogluconate.

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