Purification and subunit structure of phosphoglycerate dehydrogenase from rabbit liver

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D-3-Phosphoglycerate dehydrogenase (EC 1.1.1.95) was purified from rabbit liver by $(NH_4)_2SO_4$ fractionation, DEAE-Sephacel chromatography, affinity chromatography on AMP–agarose and molecular-sieve h.p.l.c. The purified enzyme was homogeneous as judged by SDS/polyacrylamide-slab-gel electrophoresis. On the basis of molecular-sieve h.p.l.c. and SDS/polyacrylamide-gel electrophoresis, the enzyme is a tetramer composed of subunits of M_r 60000.

INTRODUCTION

Although L-serine plays a key role in cellular metabolism, relatively little is known about the short-term regulation of its biosynthesis *in vivo* in higher organisms. Part of the information that is needed is a correlation of the kinetic and regulatory properties of the enzymes of the L-serine-biosynthetic pathway with the concentrations of their substrates and effectors *in vivo*. We have chosen to use the liver of the rabbit for these studies (Lund *et al.*, 1985) because this tissue contains relatively high activities of the relevant enzymes (Walsh & Sallach, 1966; Lund *et al.*, 1985) and because rapidly frozen liver samples necessary for the determination of the concentrations of the metabolic intermediates *in vivo* can be obtained satisfactorily from the rabbit (Garber & Hanson, 1971; Lund *et al.*, 1985). However, in order to determine the kinetic and regulatory properties of the enzymes of the pathway of L-serine biosynthesis, it is necessary to develop procedures for obtaining highly purified preparations of these enzymes from rabbit liver. In the present paper we describe a procedure for the purification of the first enzyme of the serine-biosynthetic pathway, D-3-phosphoglycerate dehydrogenase, which catalyses the oxidation of the glycolytic intermediate D-3-phosphoglycerate to phosphohydroxypyruvate, utilizing NAD^+ as the electron acceptor.

D-3-Phosphoglycerate dehydrogenase has been purified and characterized from both *Escherichia coli* (Sugimoto & Pizer, 1968; Winicov & Pizer, 1974; Dubrow & Pizer, 1977*a*,*b*) and chicken liver (Walsh & Sallach, 1965; Grant *et al.*, 1978; Grant & Bradshaw, 1978). However, very little information has been available about this enzyme from any mammalian tissue. A method using chromatography on CM-cellulose and DEAE-cellulose was described for the partial purification of D-3-phosphoglycerate dehydrogenase from pig spinal cord (Feld & Sallach, 1975), but no evidence for homogeneity was presented, nor were any properties of the enzyme described. We report that D-3-phosphoglycerate dehydrogenase has been purified to apparent homogeneity for the first time from a mammalian source and present evidence that some of its physical properties differ from those of the bacterial and avian enzymes.

MATERIALS AND METHODS

Materials

Chicken liver D-3-phosphoglycerate dehydrogenase (7 units/mg of protein at 25 °C with D-3-phosphoglycerate as substrate), tricyclohexylammonium salt of phosphohydroxypyruvic acid dimethylketal, disodium salt of NADH (grade III) and other laboratory chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzyme grades of $(NH_4)_2SO_4$ and sucrose were obtained from Schwarz–Mann (Cambridge, MA, U.S.A.). A specially pure grade of SDS manufactured by BDH Biochemicals was purchased from Gallard–Schlesinger (Carle Place, NY, U.S.A.).

Materials for chromatography were obtained as follows: Sephadex G-25 (medium grade) and DEAE-Sephacel from Pharmacia Fine Chemicals (Uppsala, Sweden), AMP–agarose [AMP-(N^6)-hexylamino-agarose] from P-L Biochemicals (Milwaukee, WI, U.S.A.) and TSK G 3000 SW column from Kratos Analytical Instruments (Ramsey, NJ, U.S.A.).

Protein standards for SDS/polyacrylamide-gel electrophoresis were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and for molecular-sieve h.p.l.c. from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Methods

Preparation of 3-phosphohydroxypyruvate. 3-Phosphohydroxypyruvate was prepared from the dimethylketal, purified by ion-exchange chromatography, and quantified as described previously (Lund *et al.*, 1985).

Assay of D-3-phosphoglycerate dehydrogenase activity. The enzyme activity was assayed at 38 °C by monitoring the change in absorbance resulting from the oxidation of NADH in the presence of phosphohydroxypyruvate as described previously (Lund *et al.*, 1985) except that enzyme samples were not desalted. One unit of enzyme activity is equivalent to the oxidation of 1 μmol of NADH/min at 38 °C.

Purification of D-3-phosphoglycerate dehydrogenase. Livers were obtained, immediately after the animals were

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killed, from male New Zealand rabbits weighing 1000–1500 g. Livers were briefly soaked in 0.25 M-sucrose and then either processed immediately or frozen at −80 °C. All procedures were performed at 0–4 °C unless otherwise indicated. The pH of all buffers was adjusted at 25 °C.

Rabbit livers were homogenized as described by Fleischer & Kervina (1974). The homogenization buffer was 0.25 M-sucrose/10 mM-Hepes/KOH buffer, pH 7.5, containing 1 mM-dithiothreitol and 1 mM-phenylmethanesulphonyl fluoride. The dithiothreitol and phenylmethanesulphonyl fluoride were added to the buffer just before homogenization. The homogenate was filtered and then centrifuged at 48000 ***g*** for 90 min. The supernatant was carefully decanted and the pellet was discarded.

$(NH_4)_2SO_4$ (16.4 g/100 ml of supernatant) was slowly added with stirring to the supernatant. After the last addition of $(NH_4)_2SO_4$, the mixture was stirred for 30 min and then was centrifuged at 32000 ***g*** for 20 min. The supernatant was collected and $(NH_4)_2SO_4$ (11.7 g/100 ml of supernatant) was slowly added with stirring. The mixture was stirred and the precipitate was collected by centrifugation as described above and frozen at −80 °C.

The $(NH_4)_2SO_4$-fraction pellets were suspended in 10 ml of buffer A (50 mM-Hepes/KOH buffer, pH 7.2, containing 1 mM-EDTA, 1 mM-dithiothreitol and 1 mM-phenylmethanesulphonyl fluoride) and dialysed for 16 h against 4 litres of the same buffer. Denatured protein was removed by centrifugation and the supernatant was applied to a column (2.7 cm × 38 cm) of DEAE-Sephacel equilibrated with buffer A. The column was washed with 100 ml of buffer A, and the enzyme activity was eluted with a 1000 ml linear gradient of 0–400 mM-KCl in buffer A. Starting with application of the sample, fractions (8.5 ml) were collected at a flow rate of 35 ml/h. The active fractions were precipitated with $(NH_4)_2SO_4$ (39.8 g/100 ml of solution) as described above and stored at −80 °C.

A column (2 ml bed volume) of AMP–agarose was equilibrated with buffer B (20 mM-Hepes/KOH buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-dithiothreitol). The $(NH_4)_2SO_4$-fraction pellets from the previous step were suspended in buffer B, and the $(NH_4)_2SO_4$ was removed by gel filtration of the enzyme suspension through a column of Sephadex G-25. Only those protein-containing fractions that were free of $(NH_4)_2SO_4$ (as judged by Nessler's reagent) were pooled and applied to the AMP–agarose column. The column was washed with 12 ml of buffer B and then the enzyme was eluted with a 14 ml linear gradient of 0–0.8 mM-phosphohydroxypyruvate and 0–0.4 mM-NADH in buffer B. The column was washed with 10 ml of 1 M-KCl in column buffer. Starting with application of the sample, fractions (0.8 ml) were collected at a flow rate of about 40 ml/h. The active fractions were precipitated by the addition of $(NH_4)_2SO_4$ (43.6 g/100 ml of pool) with stirring. After being stirred for 30 min, the suspension was divided into four equal portions and the precipitates were collected in four tubes by centrifugation and stored at −80 °C. The enzyme was stable in this form for at least 6 months.

H.p.l.c. was performed at room temperature with a Laboratory Data Control (Milton Roy) high-pressure liquid chromatograph equipped with a 200 μl sample loop on the injector, a post-injector guard column (0.75 cm × 10 cm) packed with TSK G 3000 SW gel, and a TSK G 3000 SW molecular-sieving column (0.75 cm × 60 cm). One of the $(NH_4)_2SO_4$-fraction pellets from the AMP–agarose column was dissolved in buffer C (20 mM-sodium phosphate buffer, pH 7.2, containing 0.1 M-Na_2SO_4 and 1 mM-dithiothreitol) to a final volume of 200 μl and injected into the column system that had been equilibrated with degassed buffer C. Fractions (1.0 ml) were collected on ice at a flow rate of 0.5 ml/min. The purified enzyme was stored in small portions at −80 °C.

SDS/polyacrylamide-gel electrophoresis and determination of the subunit M_r. Samples were reduced with 80 mM-dithiothreitol and treated with 2% (w/v) SDS. The samples were heated in a boiling-water bath for 3 min immediately after the addition of 2% SDS and then analysed by electrophoresis in slab gels containing 10% acrylamide and 0.1% SDS (Laemmli, 1970). The subunit M_r was determined from a standard curve obtained by plotting the relative mobilities of standard proteins (bovine serum albumin, M_r 68000; catalase, M_r 58000; ovalbumin, M_r 43000; fructose-bisphosphate aldolase, M_r 40000; chymotrypsinogen, M_r 25000) against $\log M_r$.

Determination of the native enzyme M_r. The M_r of the purified enzyme was estimated from its retention time in a molecular-sieving column with the h.p.l.c. system described above. The column was calibrated by chromatographing 200 μl of a mixture containing bovine thyroglobulin (M_r 670000), bovine γ-globulin (M_r 158000), chicken ovalbumin (M_r 44000), horse myoglobulin (M_r 17000) and vitamin B-12 (M_r 1350). A standard curve was constructed by plotting the retention time of each protein against $\log M_r$.

RESULTS AND DISCUSSION

Enzyme purification

Although D-3-phosphoglycerate dehydrogenase has been purified to homogeneity from one other vertebrate tissue (chicken liver; Grant *et al.*, 1978), it was necessary to develop a completely new purification procedure for the purification of this enzyme from rabbit liver, because our initial studies revealed that the two enzymes have substantially different net charges. The enzyme from chicken liver is a basic protein with pI 8.95, and its failure to bind at pH 7.6 to the DEAE-cellulose anion-exchange resin was used as the basis of the first chromatography step in the purification procedure reported by Grant *et al.* (1978). In contrast, the enzyme from rabbit liver appears to be an acidic protein, since it was tightly adsorbed on the DEAE-Sephacel anion-exchange resin at relatively low pH values. Therefore, after the $(NH_4)_2SO_4$-fractionation step described in the Materials and methods section, the dialysed fraction was chromatographed on a DEAE-Sephacel column. Because the D-3-phosphoglycerate dehydrogenase activity was tightly adsorbed on the resin at pH 7.2 and was not eluted along with most of the protein when the column was washed with buffer, there was a substantial increase (6-fold) in the specific activity of the enzyme pool obtained on elution of the column with a salt gradient (Table 1).

Table 1. Purification of D-3-phosphoglycerate dehydrogenase

For experimental details see the Materials and methods section. Protein was determined by the procedure of Bradford (1976) with bovine serum albumin as a standard, except that the homogenate was assayed by the procedure of Lowry *et al.* (1951).

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)	Recovery (%)
Homogenate	5052	391.5	0.08	100
$(NH_4)_2SO_4$ fraction	1200	254.6	0.21	65
DEAE-Sephacel pool	121	160.6	1.32	41
AMP–agarose pool	3.1	111.0	35.8	28
H.p.l.c. molecular-sieve pool*	1.14	92.8	81.3	24

* One-fourth of the AMP–agarose pool was applied to the molecular-sieve column at one time.

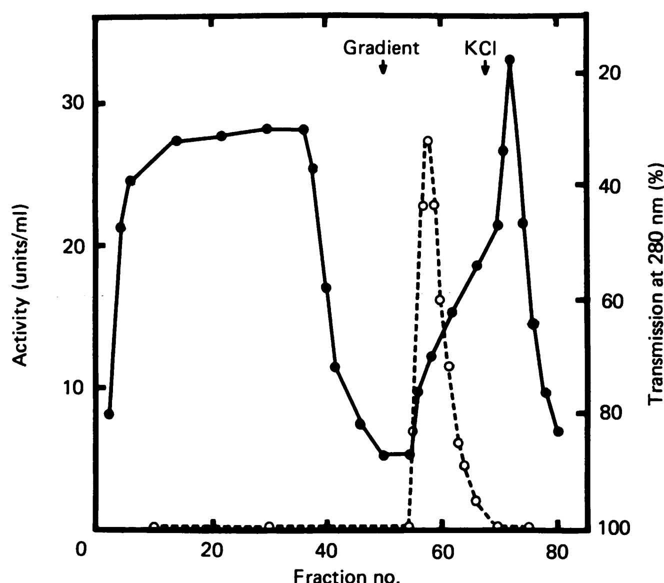

Fig. 1. Affinity chromatography of D-3-phosphoglycerate dehydrogenase

The pool obtained from the DEAE-Sephacel column was concentrated by $(NH_4)_2SO_4$ precipitation, desalted by gel filtration on Sephadex G-25, and then applied to an AMP–agarose column. Starting at fraction 50, the enzyme activity (○) was eluted by a linear gradient of 0–0.8 mM-phosphohydroxypyruvate and 0–0.4 mM NADH in the column buffer; at fraction 68, the column was washed with 1 M-KCl in column buffer. Transmittance at 280 nm (●) was measured with an LKB Uvicord II u.v. absorptiometer.

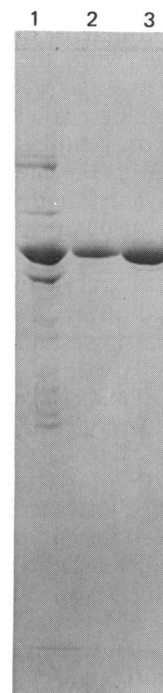

Fig. 2. SDS/polyacrylamide-gel electrophoresis of D-3-phospoglycerate dehydrogenase

Samples were reduced with dithiothreitol, heated with 2% SDS, and then analysed by electrophoresis in a slab gel containing 10% acrylamide and 0.1% SDS. Lane 1, sample of AMP–agarose pool (Fig. 1). Lane 2, sample of TSK G 3000 SW pool dialysed against 20 mM-Hepes buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-dithiothreitol. Lane 3, sample of TSK G 3000 SW pool concentrated and dialysed against 20 mM-Hepes buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-dithiothreitol.

AMP–agarose was tested as a possible biospecific affinity-chromatography step because many dehydrogenases bind to this affinity matrix and are specifically eluted by their substrates (Mosbach, 1974; Harvey *et al.*, 1974). D-3-Phosphoglycerate dehydrogenase from rabbit liver was tightly adsorbed on AMP–agarose but was specifically eluted by a gradient containing low concentrations of its substrates, phosphohydroxypyruvate and NADH (Fig. 1), resulting in a 27-fold increase in the specific activity (Table 1). When the column was washed with KCl, a sharp peak of 280 nm-absorbing material was eluted just after the peak of enzyme activity, this being due to the elution of NADH from the column; no protein bands were detected when fractions from this peak were analysed by polyacrylamide-gel electrophoresis. In contrast, when the fractions containing D-3-phosphoglycerate dehydrogenase activity were pooled and analysed by SDS/polyacrylamide-slab-gel electrophoresis, one major protein-staining band was observed, together with a number of minor bands (Fig. 2, lane 1).

The final step in the purification procedure was h.p.l.c. on the molecular-sieve gel TSK G 3000 SW. As judged

by SDS/polyacrylamide-slab-gel electrophoresis, this step successfully removed the contaminating protein bands from the AMP–agarose pool (Fig. 2, lanes 2 and 3). Attempts were made to determine whether the enzyme preparation also appeared to be homogeneous on non-denaturing polyacrylamide gels; however, all of the protein-staining material remained at the top of non-denaturing 7% polyacrylamide gels runs at pH 8.3 or at pH 4.0. The most likely explanation for this result is that the purified enzyme aggregates under the conditions used in non-denaturing electrophoresis and forms a high-M_r complex that cannot penetrate 7% polyacrylamide gels. It is noteworthy that a similar result was also observed for the D-3-phosphoglycerate dehydrogenase from chicken liver (Grant *et al.*, 1978).

Determinations of native enzyme and subunit M_r values

The M_r of the native enzyme was estimated from plots comparing the retention time of the purified enzyme on molecular-sieve h.p.l.c. with the retention times of protein standards. The average M_r value calculated from two separate experiments was 247000. The D-3-phosphoglycerate dehydrogenase subunit M_r was assessed from plots comparing the mobility of the purified enzyme on SDS/polyacrylamide-slab electrophoresis with the mobilities of protein standards. An average subunit M_r of 60100 ± 400 (S.E.M.) was calculated from results obtained in four separate experiments.

Since the enzyme had an M_r of 247000 in the native state and migrated as a single band on SDS/polyacrylamide-gel electrophoresis with a subunit M_r of 60000, it appears that the D-3-phosphoglycerate dehydrogenase purified from rabbit liver is a tetramer. The D-3-phosphoglycerate dehydrogenases purified from both *E. coli* (Winicov & Pizer, 1974) and chicken liver (Grant & Bradshaw, 1978) are also tetramers, but the subunit M_r values are approx. 40000 instead of 60000. Although it is possible that the smaller subunit size of the *E. coli* and the avian enzymes is the result of proteolysis, it seems unlikely in the case of the avian enzyme because proteinase inhibitors (leupeptin and phenylmethanesulphonyl fluoride) were used in early stages of th purification (Grant *et al.*, 1978).

This is the first time that D-3-phosphoglycerate dehydrogenase has been purified to apparent homogeneity from a mammalian tissue, and the observation that the mammalian enzyme has a subunit M_r that is significantly larger than those of both the *E. coli* enzyme and the avian enzyme raises interesting questions about the evolution of D-3-phosphoglycerate dehydrogenase.

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