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Purification and partial characterisation of NADP$^+$-linked isocitrate dehydrogenase from rat liver cytosol

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NADP$^+$-linked isocitrate dehydrogenase from rat liver cytosol was purified (approximately 135-fold) to apparent homogeneity in 27% yield. The purified enzyme has specific activity of 73 units · mg^{-1}. The native enzyme showed an apparent M_r of 94,000 by gel filtration and was composed of two identical subunits of M_r 45,000 as judged by SDS/PAGE. In isoelectric focusing, a pI value of 5.7 was estimated for the enzyme.

Isocitrate dehydrogenase; Enzyme purification; Rat liver cytosol

1. INTRODUCTION

Both the NAD$^+$- and NADP$^+$-linked isocitrate dehydrogenases are present in mammalian tissues. They catalyse reversible oxidative decarboxylation of isocitrate to α-ketoglutarate as shown below:

Isocitrate + NADP$^+$(P) ⇄ α-ketoglutarate + CO_2 + NAD(P)H

Whilst the NAD$^+$-linked isocitrate dehydrogenase (IDH) is exclusively mitochondrial, the NADP$^+$-linked enzyme is present in both the cytosol and mitochondria. The presence of both the NAD$^+$- and NADP$^+$-linked IDH in mitochondria has raised the question about their relative contribution to the oxidation of isocitrate in the tricarboxylic acid and the subject has caused considerable controversy over the past few years. The original contention that the oxidation of isocitrate in the citric acid cycle for energy production is exclusively catalysed by NAD$^+$-linked IDH [1] is no longer tenable. Moyle and Mitchell [2] have concluded that isocitrate oxidation in rat liver mitochondria proceeds exclusively via NADP$^+$-linked isocitrate dehydrogenase pathway. However, in spite of several other studies, the interrelations and metabolic roles of these two enzymes are yet not fully understood.

The proportional distribution of mammalian NADP$^+$-linked isocitrate dehydrogenase (threo-D_s-isocitrate-NADP$^+$ oxidoreductase (decarboxylating); EC 1.1.1.42) between cytosol and mitochondria is tissue specific. In heart and kidney, the enzyme is predominantly localised in mitochondria whilst in liver, it is largely present in the cytosol [3,4]. These isoenzymes have been shown to be under independent genetic control [5]. The mitochondrial enzyme has been purified from several tissues including bovine heart [6] and pig heart [7]. The bovine heart enzyme has especially been subjected to detailed kinetic and mechanistic analysis [8–12]. Whilst certain differences in the physical and kinetic properties of cytosolic and mitochondrial enzymes have been documented, no detailed studies have been conducted to compare their properties.

The cytosolic enzyme has been previously purified from bovine liver [13] and sheep liver [14]. The former enzyme has been subjected to some kinetic analysis [13,15–17]. In spite of the presumed importance of the cytosolic enzyme, numerous aspects of its action are either poorly understood or not investigated at all; these include its kinetic/mechanistic properties, its precise metabolic function and regulation of the enzyme activity by hormones/nutrients. Furthermore, studies involving the characterisation of the gene encoding the enzyme and control of gene expression have not been hitherto undertaken for the mammalian enzyme. We have recently initiated a broad-based programme to address some of the above issues. In this communication, we report the purification and partial characterisatio of NADP$^+$-linked isocitrate dehydrogenase from rat liver cytosol. We have chosen to study the enzyme from rat because it is an animal more suitable for subsequent detailed metabolic investigations involving either the whole animal or cultured hepatocytes derived from it.

2. MATERIALS AND METHODS

2.1. *Materials*

DL-Isocitrate (sodium salt), NADP$^+$ (disodium salt), dithiothreitol were from Sigma Chemical Co. (Poole, Dorset, UK). Sephadex G25,

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Sephacryl S200, Blue Sepharose, Mono-Q, Molecular weight markers for SDS/PAGE and gel filtration and FPLC equipment were from Pharmacia Fine Chemicals (Uppsala, Sweden). Sartophor electrophoresis system and Servalyt Precotes gels were purchased from Sartorius GmbH (Göttingen, Germany). Sagatal was from May and Baker Ltd. (Dagenham, UK). Other chemicals were of Analar grade and obtained from BDH Ltd. (Poole, Dorset, UK).

2.2. *Enzyme assay*

$NADP^+$-linked isocitrate dehydrogenase was assayed spectrophotometrically at 25°C by monitoring the production of NADPH at 340 nm. The reaction medium, in a volume of 1.4 ml, contained 33 mM Tris-HCl, pH 7.4, 0.33 mM EDTA, 0.1 mM $NADP^+$, 1.33 mM $MnCl_2$ and 1.3 mM DL-isocitrate. Reactions were initiated by the addition of enzyme. One unit of enzyme forms 1 μmol of NADPH/min at 25°C.

2.3. *Protein determination*

Protein concentration was determined by the Coomassie blue method [18] using serum albumin as the protein standard.

2.4. *Electrophoresis*

The electrophoretic homogeneity of the purified enzyme was established by polyacrylamide gel electrophoresis under denaturing conditions in a conventional vertical slab-gel apparatus by the method of Laemmli [19].

2.5. *Isoelectric focusing*

Isoelectric focusing was performed on Sartorius Sartophor electrophoresis system using Servalyt Precotes gels, pH 3–10, at 4°C according to the manufacturer's instructions.

2.6. *Molecular weight and sub-unit composition*

The molecular weight of the purified native enzyme was estimated by gel filtration [20] using Sephacryl S-200 gel in a 2.6 × 100 cm column with a flow rate of 10–15 ml/h. The enzymes/proteins used as internal standards and their apparent molecular weights by gel filtration were aldolase (158,000), albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700).

The sub-unit molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels and Pharmacia low-molecular weight calibration kit.

2.7. *Enzyme purification*

2.7.1. Preparation of the homogenate

All purification steps were performed at 4°C unless otherwise stated.

In a typical experiment, normal female Wistar rats (approximately 12 weeks old and weighing 200–250 g) fed ad libitum on normal laboratory chow diet were sacrificed by intra-peritoneal injection of 0.3 ml Sagatal (pentobarbitone sodium, 60 mg/ml). The livers were removed and very briefly washed in buffer A (50 mM sodium citrate, 10 mM magnesium sulphate, 0.1 mM EDTA, pH 7.8) to remove blood. The livers were then chopped into small pieces and homogenised in buffer A (1 part tissue:6 parts buffer w/v) using electrically operated Potter-Elvehjem homogeniser. The homogenate was centrifuged at 48,000 × *g* for 20 min. The supernatant which contained the enzyme activity was retained. Control experiments had shown that if the supernatant was further centrifuged at 100,000 × *g* for 30 min, all of the activity was retained in the soluble fraction, suggesting the enzyme was not associated with the microsomal fraction.

2.7.2. Heat treatment

The combined supernatant (in aliquots of 100 ml) was heated to 53°C and the temperature maintained for 10 min in a constant temperature water bath. The enzyme was stable at this temperature and this step achieved ~2-fold purification. The precipitated protein was removed by centrifugation at 48,000 × *g* for 20 min.

2.7.3. Ammonium sulphate fractionation

The supernatant from the previous step was brought to 33% saturation by slowly adding finely ground ammonium sulphate, maintaining pH at 7.5. After gently stirring for 30 min, it was centrifuged at 48,000 × *g* for 30 min. The supernatant was retained and ammonium sulphate was further added to give 58% saturation. After stirring for 60 min, it was centrifuged as described above. The precipitate was taken up in 4–6 ml of buffer B (10 mM sodium citrate, 1 mM magnesium sulphate, 0.1 mM EDTA, 0.33 mM DTT, pH 7.8).

2.7.4. Gel filtration

The sample was applied to a Sephacryl S-200 column (2.6 × 80 cm) previously equilibrated with buffer B. The column was developed at a flow rate of 15 ml · h^{-1}. The active fractions were combined for the next step.

2.7.5. Blue Sepharose (room temp.)

The protein was applied to a Blue Sepharose column (10 × 1.6 cm) equilibrated with buffer B. The column was thoroughly washed until no further absorbance at OD_{280} was detected in the eluate. The protein was eluted with a linear salt gradient (0–1 M NaCl in the equilibration buffer) over 1 h at a flow rate of 50 ml · h^{-1}. The enzyme eluted in the later part of the gradient. The active fractions were pooled and desalted (Sephadex G-25 column, 2.6 × 40 cm) into buffer C (20 mM Tris-citrate, 1 mM magnesium sulphate, 0.1 mM EDTA, 0.33 mM DTT, pH 8.3).

2.7.6. Mono-Q (room temp.)

The desalted protein was subjected to anion-exchange chromatography using Pharmacia fast protein liquid chromatographic (FPLC) system with Mono-Q (HR 5 × 5) in buffer C. The protein was eluted using a linear salt gradient (0–100 mM NaCl) in buffer C over 1 h at a flow rate of 60 ml · h^{-1}. 1 ml fractions were collected. The enzyme activity eluted at 50–60 mM NaCl.

The enzyme was stored in 50% glycerol at –20°C. It is stable up to several weeks under these conditions.

3. RESULTS AND DISCUSSION

3.1. *Purification of isocitrate dehydrogenase*

The procedure used for the purification of the enzyme is summarised in Table I. The method is simple, reproducible and can be completed in 2 working days. The enzyme was purified (approximately 135-fold) to apparent homogeneity. The purification involved heat treatment, ammonium sulphate fractionation, gel filtration, affinity chromatography and anion-exchange chromatography. Inclusion of citrate, a substrate analogue, in all the buffers stabilized the enzyme, especially in the heat treatment step. Gel filtration chromatography which was done using buffer B allowed direct application of the enzyme sample to the Blue Sepharose column. The enzyme binds quite strongly to this matrix and can be eluted at a relatively high salt concentration (0.75–1.0 M NaCl). We normally pooled only those fractions with a specific activity greater than 10 which gave a yield of approximately 80% from this step. It is interesting to note that Balamir [21] has reported that the bovine liver enzyme does not bind to this affinity medium but is merely retarded. This suggests that there may be differences in the physical properties of the enzyme from bovine and rat livers. The enzyme after desalting and change of buffer was applied to Mono-Q, a

Table I

Purification of NADP$^+$-linked isocitrate dehydrogenase from rat liver cytosol

Purification step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
(1) Cytosolic extract	200	630	1,160	0.54	100	1
(2) Heat treatment	200	585	620	0.94	93	2
(3) $(NH_4)_2SO_4$ fractionation	7.8	438	250	1.75	70	3
(4) Gel filtration	21	336	53	6.34	53	12
(5) Blue Sepharose	14.5	243	13	18.7	39	35
(6) Mono-Q	5	170	2.33	73	27	135

Outline of a typical preparation in which 3 rat livers were used. Details are given in section 2.

strong anion exchanger. The enzyme was eluted from this column in one peak and in 5–6 fractions. We normally discarded fraction 1 which contained a minor impurity. Nevertheless, the yield of pure enzyme from this column is approximately 70%.

The enzyme is stable at −20°C for several weeks in 50% glycerol. The enzyme rapidly loses activity if dithiothreitol is omitted suggesting the presence of essential –SH groups for catalytic activity. The enzyme preparation is apparently homogeneous. SDS/polyacrylamide gel electrophoresis of the purified enzyme exhibited only one band when the gel was stained for protein (see Fig. 1). In isoelectric focusing of the purified enzyme, p*I* value of 5.7 was estimated. The specific activity of the purified enzyme was 73 units · mg^{-1}. This value is high when compared with 43 units · mg^{-1} reported for the enzyme from bovine liver [21]. The purified enzyme exhibits no activity when NAD^+ is used as a coenzyme.

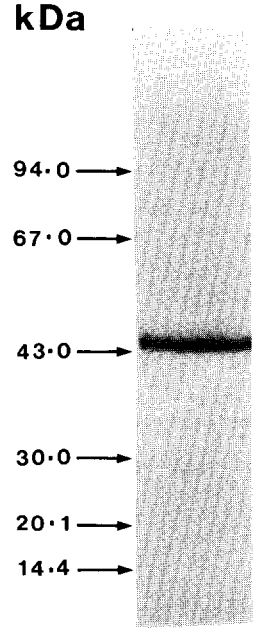

Fig. 1. SDS/polyacrylamide gel electrophoresis of purified isocitrate dehydrogenase (8 μg). Protein size markers, indicated by arrows, were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

3.2. M_r

The molecular weight of the purified enzyme was estimated by gel filtration using Sephacryl S-200. The enzyme eluted in a single symmetrical peak corresponding to an apparent molecular weight of 94,000.

SDS/polyacrylamide gel electrophoresis yielded a single band corresponding to M_r of 45,000 (see Fig. 1). Thus the enzyme appears to be a dimer with physically identical subunits. The subunit and native enzyme molecular weights of 45,000 and 94,000 estimated here are similar to those reported for the enzyme from bovine liver [13]. In contrast, Illingworth and Tipton [14] have found that the pig liver enzyme is a dimer of molecular weight 75,000.

In conclusion, the purification of the enzyme paves the way for detailed kinetic/mechanistic, immunochemical and genetic studies.

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