A RAPID PURIFICATION OF HEPATIC ATP CITRATE LYASE USING BLUE SEPHAROSE

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Received 5 December 1980

1. Introduction

The lipogenic enzyme ATP citrate lyase (EC 4.1.3.8) has been shown to be phosphorylated in response to hormonal stimulation. Insulin and glucagon have been demonstrated to selectively stimulate phosphorylation of hepatocyte ATP citrate lyase [1,2] and insulin has a similar effect on adipocyte ATP citrate lyase [3]. Furthermore, ATP citrate lyase purified from rat mammary gland has been phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase [4]. The methods available for the purification of ATP citrate lyase from liver are either time consuming [5] or utilize hepatocytes as the starting material [1]. Here, we present a rapid method for the purification of ATP citrate lyase starting with intact liver. The method is an extension of that in [1] in that the procedure for hepatocytes is adapted for use with whole liver by using blue Sepharose as an additional purification step. Benzamidine, a protease inhibitor, is used in order to minimize proteolytic degradation of the lyase during purification. The ATP citrate lyase prepared from whole liver using this method is recovered with a slightly higher specific activity and in a better yield than that obtained from hepatocytes [1]. Furthermore, it is completely free from the endogenously generated proteolytic fragment (M_r 57 000) that has been observed as a major contaminant [1].

2. Materials and methods

Anion exchange cellulose (DE 23) was purchased from Whatman, England; Sepharose CL-6B-200 from Sigma and blue Sepharose from Pharmacia Fine Chemicals, Sweden. Coenzyme A, NADH and malate dehydrogenase were from Sigma. Two male rats (~200 g) of a random-bred Wistar strain were starved for 2 days, then fed a high carbohydrate diet ad libitum for 3 days. They were killed by cervical dislocation and the livers removed, washed in 10 mM phosphate buffer (pH 7.5) and homogenized in 100 ml 10 mM sodium phosphate pH 7.5, 0.25 M sucrose, 100 mM NaF, 5 mM Na_2EDTA, 1 mM DTT and 50 mM benzamidine. This step and all subsequent ones were done at 4°C. The homogenate was centrifuged at 43 000 × g_{max} for 70 min and the supernatant was filtered through glass wool and mixed with an equal volume of 10 mM sodium phosphate (pH 7.5), 0.25 M sucrose, 5 mM Na_2EDTA, 1 mM DTT and 50 mM benzamidine. This supernatant was loaded onto a column of DE23 anion-exchange cellulose (5 × 10 cm) pre-equilibrated with 10 mM sodium phosphate (pH 7.5), 50 mM NaF, 5 mM Na_2EDTA, 1 mM DTT and 50 mM benzamidine. After sample application the column was washed, with 250 ml of the DE23 equilibration buffer. The flow through and column washings were collected and combined (DEAE fraction) and solid $(NH_4)_2SO_4$ was gradually added to 55% saturation. After 1 h the suspension was centrifuged at 20 000 × g_{max} for 20 min. The pellets were resuspended in 4 ml 10 mM sodium phosphate (pH 7.5), 100 mM NaF, 5 mM Na_2EDTA, 50 mM Na_2-citrate and 1 mM DTT ($(NH_4)_2SO_4$ fraction). The suspension was applied to a Sepharose CL-6B column (2.5 × 50 cm) pre-equilibrated with the same buffer. The elution buffer was pumped through at a rate of 25 ml/h and 4.5 ml fractions were collected. The peak ATP citrate lyase fractions emerge slightly before a contaminating brown material. The fractions containing ATP citrate lyase (fractions 34–46 inclusive) were combined (Sepharose fraction) and loaded onto a column (1.5 × 7 cm) of blue Sepharose equilibrated with 10 mM sodium phosphate (pH 7.5), 100 mM NaF, 5 mM Na_2EDTA, 50 mM sodium citrate (equilibration buff-

er) and eluted at 1.3 ml/min. The ATP citrate lyase activity binds to this column. After sample application the column was washed with 20 ml equilibration buffer followed by two 10 ml aliquots of the same buffer containing 5 mM NAD. This was followed by a wash with 20 ml equilibration buffer containing 1 mM DTT. No ATP citrate lyase activity was detected in any of the eluates from these washes although considerable amounts of protein were assayed. The ATP citrate lyase activity was eluted by application of equilibration buffer containing 10 mM ATP and 1 mM DTT. Fractions of 5 ml were collected and the ATP citrate lyase activity was recovered with high yield and spec. act. ~7 units/mg protein in fractions 2 and 3. The fraction(s) containing the ATP citrate lyase activity were dialysed overnight against equilibration buffer containing 1 mM mercaptoethanol, then frozen.

ATP citrate lyase was assayed as in [6] and 1 unit of activity was that amount which catalysed the oxidation of 1 μmol NADH/min at 30°C. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS) was done in 6 mm tubes according to [7] (running pH 7.2). Protein was precipitated with trichloracetic acid, washed with ethanol, redissolved in 1 N NaOH and then assayed as in [8]. The blue Sepharose can be reused after 4–5 washing cycles of 10 vol. 0.1 M Tris–HCl, 0.5 M NaCl (pH 8.5) then 10 vol. 0.1 M sodium acetate, 0.5 M NaCl (pH 4.5) followed by equilibration with buffer. This can be conveniently performed in a funnel with a fine sinter.

3. Results and discussion

A purification of ATP citrate lyase from whole liver is described in table 1 and fig.1. The ATP citrate lyase activity eluted from the Sepharose CL-6B column in a single symmetrical peak just in front of the protein peak (fig.1). The Sepharose fraction following chromatography on blue Sepharose yields ATP citrate lyase of spec. act. 6.8 units/mg protein with an overall recovery of 21%. A total of 28 units lyase was obtained from 20 g wet wt liver (table 1). Although the ATP citrate lyase activity in the Sepharose fraction is stable for 2 weeks either frozen or at 4°C, the enzyme activity which is eluted from blue Sepharose by 10 mM ATP is unstable, losing 10% of its activity over 48 h at 4°C. However, following overnight dialysis to remove the ATP (section 2) the frozen enzyme is stable for up to 5 days.

Table 1
Purification of ATP citrate lyase from whole liver obtained from fasted re-fed rats

Fraction	Total act. (units)	Yield (%)	Spec. act. (units/mg prot.)	Purification (-fold)
Cytosol	133	100	0.16	1
DE23 fraction	126	95	0.17	1.1
$(NH_4)_2SO_4$ pellet	90	68	0.31	1.9
Sepharose fraction	65	50	0.50	3.1
Blue Sepharose fraction	28	21	6.8	43

ATP citrate lyase was purified from the livers of 2 fasted re-fed rats as in section 2

The blue Sepharose step reproducibly purifies ATP citrate lyase from the Sepharose fraction on both a large scale (50 ml Sepharose fraction on a (1.5 X 7 cm) column of blue Sepharose) and on a small scale (5 ml

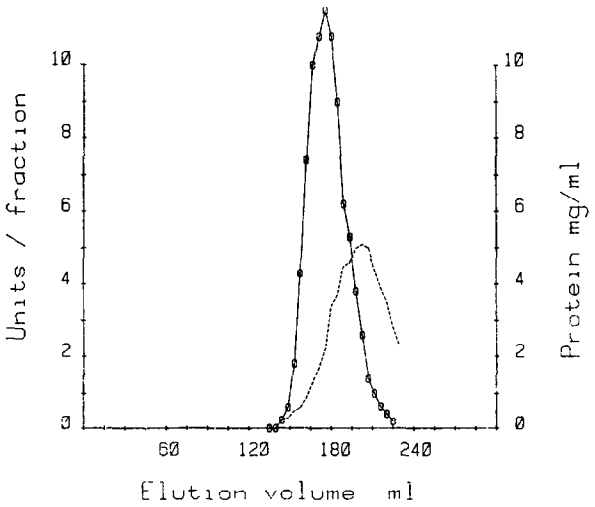

Fig.1. Chromatography of the $(NH_4)_2SO_4$ pellet on Sepharose CL-6B. The $(NH_4)_2SO_4$ pellet (4 ml) was prepared as in section 2 loaded onto a column of Sepharose C1-6B (2.5 × 50 cm). Fractions of 4.5 ml were collected and fractions 34–46 were pooled to form the Sepharose fraction. The continuous line represents ATP citrate lyase activity and the broken line protein concentration.

Table 2
Purification of the Sepharose fraction by chromatography on blue Sepharose

Elution condition	Fraction number	Fraction vol. (ml)	Activity (units/ fraction)	Protein (mg/ fraction)	Spec act. (units/mg protein)
Sepharose fraction	1	20	10.5	43.4	0.24
Flowthrough	2	20	Nil[a]	12.6	Nil
Wash with buffer	3	8	Nil	6.7	Nil
Buffer + 5 mM NAD	4	8	Nil	2.7	Nil
Wash with buffer + 1 mM DTT	5	8	Nil	0.9	Nil
Buffer + 1 mM DTT + 10 mM ATP	6	2	Nil	0.06	Nil
Buffer + 1 mM DTT + 10 mM ATP	7	2	Nil	0.06	Nil
Buffer + 1 mM DTT + 10 mM ATP	8	2	2.24	0.33	6.8
Buffer + 1 mM DTT + 10 mM ATP	9	2	4.60	0.62	7.4
Buffer + 1 mM DTT + 10 mM ATP	10	2	2.03	0.50	4.1
Buffer + 1 mM DTT + 10 mM ATP	11	2	1.20	0.34	3.5
Buffer + 1 mM DTT + 10 mM ATP	12	2	0.75	0.26	2.9
Buffer + 1mM DTT + 10 mM ATP	13	2	0.53	0.26	2.1
Buffer + 1 mM DTT + 10 mM ATP	14	2	0.32	0.22	1.5
Buffer + 1 mM DTT + 10 mM ATP	15	2	0.26	0.18	1.4

[a] Below the lower limit of detection of the assay, i.e., <0.05 units/ml sample

Sepharose fraction (20 ml) was loaded onto a 1.5 × 3 cm column of blue Sepharose and eluted under the indicated conditions at a flowrate of ~1 ml/min

on a 1.5 × 1 cm column). Routinely the Sepharose fraction was frozen and when pure ATP citrate lyase was required, an aliquot of the Sepharose fraction was further purified on blue Sepharose.

Table 2 shows a purification (1 of 5 so analysed) of an aliquot of the Sepharose fraction on blue Sepharose using 5 mM NAD followed by 10 mM ATP to elute the enzyme. ATP citrate lyase is eluted from this column by 10 mM ATP with spec. act. 6.9 ± 0.1 units/mg protein (n = 5) and a recovery of ~60%. When 10 mM ATP alone is used for elution, the ATP citrate lyase is recovered with spec. act. 3.4 ± 0.2 units/mg protein (n = 3) and a yield of ~30%. When 5 mM NADH or 5 mM NADPH were used for washings instead of 5 mM NAD, the enzyme was eluted by 10 mM ATP with spec. act. 6.4 ± 0.5 units/mg protein (n = 3) and a yield of ~40%.

The material contained in fraction 9 (table 2) gave a single major protein staining band on 7.5% polyacrylamide gels when 1.5 μg protein was loaded onto the gel (fig.2). This band (using phosphorylase b and egg ovalbumin as markers) had an app. M_r ~105 000, consistent with it being ATP citrate lyase. In some preparations an additional minor band of high M_r was seen. Loading up to 6 μg protein/gel did not cause any further bands to appear (fig.2). The absence of any low M_r bands demonstrated that the ATP citrate lyase activity purified was the intact enzyme totally free from any proteolytic fragments [1]. Polyacrylamide gel electrophoresis of the Sepharose fraction demonstrated several protein bands (fig.2). This is in contrast to the unique protein band observed in [1] on detergent gel electrophoresis of the hepatocyte Sepharose fraction. Presumably the bands seen in the Sepharose fraction here represent non-hepatocyte hepatic proteins.

This method offers advantages over other methods in both its simplicity and the purity of the final product obtained.

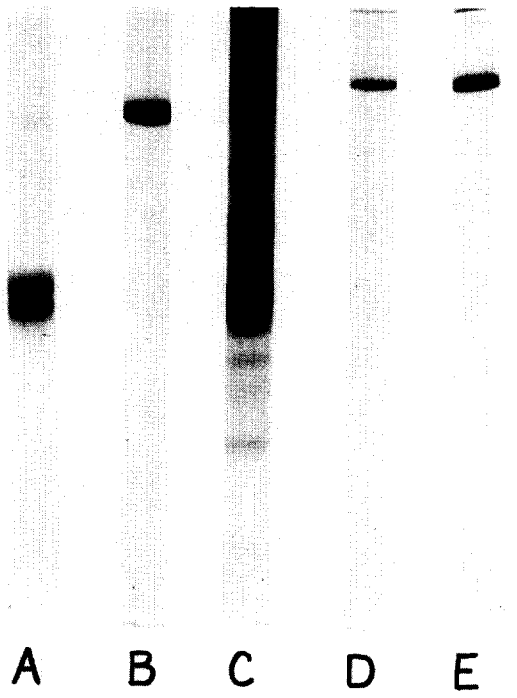

Fig.2. Analysis of ATP citrate lyase by SDS–polyacrylamide gel electrophoresis: (A) egg ovalbumin; (B) phosphorylase b; (C) 12 μg protein Sepharose fraction; (D) 1.5 μg protein from fraction 9, table 2; (E) 6 μg protein from fraction 9. The migration is from top to bottom.

Acknowledgements

This work was supported by a grant from the Medical Research Council of New Zealand. We would like to thank Dr Colin Watts for encouragement during the course of this work.

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