Oxaloacetate Decarboxylases of Rat Liver

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1. Two oxaloacetate decarboxylases of rat liver are described; one is mitochondrial with no metal ion requirement and activity in the alkaline pH region; the other is cytoplasmic with Mn^{2+} or Mg^{2+} requirement and activity around pH 5. 2. A method for the partial purification of the mitochondrial enzyme is described. 3. The apparent K_m of the mitochondrial enzyme is 0.23 mM. 4. Inhibition of the mitochondrial enzyme by substrate, CoA, acetyl-CoA, citrate and phosphoenolpyruvate is described.

The formation of pyruvate by liver preparations has long been known and has been attributed to spontaneous decarboxylation of oxaloacetate (Breusch, 1939; Bessman & Layne, 1950; Krebs *et al.*, 1940). More recently an enzyme catalysing this reaction has been described (Corwin, 1959). Pyruvate formation was observed (Bartley & Dean, 1969) in rat liver mitochondria metabolizing malate, under circumstances that could not easily be explained by spontaneous decarboxylation of oxaloacetate. Subsequently we have observed that mitochondria repeatedly washed with water to remove soluble proteins and incubated at low ionic strengths (8 mM salt) formed pyruvate at rates up to $100 \mu\text{mol/h}$ per g dry wt. Controls with heat-denatured mitochondria showed no measurable increase in pyruvate under these conditions. It was concluded that the formation of pyruvate was enzymic and attempts were made to purify and characterize this activity.

The present paper gives details of the partial purification of an oxaloacetate decarboxylase from rat liver mitochondria together with an account of some of its properties and the distribution within the liver of the pyruvate-producing activity. For comparison the decarboxylase activity of other liver fractions and of kidney, brain and skeletal muscle was briefly investigated with results indicating the presence of a soluble decarboxylase in the cytosol with properties distinct from the mitochondrial enzyme.

Experimental

Animals

These were adult rats of either sex and any weight over 200 g and were of a Wistar strain.

Materials

Lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), NAD^+ , $NADH$, $NADP^+$, CoA, ADP, ATP, AMP, cyclic AMP,

GSH, GSSG, pyruvate and oxaloacetate were from Boehringer Corp. (London) Ltd., London W.5, U.K. Acetyl-CoA was prepared from CoA and acetic anhydride as described by Stadtman (1957) and was purified chromatographically where stated by the method of Jones & Nelson (1968). Other reagents were the purest commercially available.

Tissue fractionation

Mitochondria were prepared from rat livers essentially as described by Werkheiser & Bartley (1957). Livers were removed on to ice before fractionation. The mitochondrial pellet was washed once in sucrose at $120000g\text{-min}$ and once in 0.154 M-KCl. The final pellet was weighed and suspended in 4 vol. of 10 mM-Tris-HCl buffer, pH 7.6. Mitochondria were stored at -15°C .

Purification of mitochondrial decarboxylase

Sonication of mitochondria. The ice-cold mitochondrial suspension (40 g of mitochondria in 280 ml of 10 mM-Tris-HCl buffer, pH 7.6) from 1 kg of liver was pumped through a cylindrical continuous flow cell (20 mm diam. $\times$ 15 mm) attached to an MSE 60W Sonicator with 6 mm diam. probe operated at a setting of 1.2–1.4 A. The cell was clamped so that the probe was 7 mm away from the walls and bottom of the cell. The suspension was pumped at 20 ml/min, so that the mitochondria were passing through the cell (vol. 4.5 ml) for about 12 s. The temperature at the outlet of the cell was less than 10°C and the effluent was collected in an ice-cooled receiver. The material was passed through the cell four times to ensure complete breakage of the mitochondria.

Centrifugation of the sonicated material at $100000g$ for 1 h and assay of the supernatant and pellet showed an equal distribution of activity between the two.

Heat denaturation. The sonicated material (200 ml) was heated in a 1-litre conical flask with constant swirling for 10 min by immersion in a water bath

maintained at 60°C. The final temperature was 58°C. The mixture was rapidly cooled to 0°C and the denatured protein was removed by centrifugation.

Acetone fractionation. The 130ml supernatant was brought to approximately 75% (v/v) acetone by adding 3 vols. of acetone over a period of 30min. The acetone was pumped through a fine glass nozzle below the surface of the liquid which was constantly stirred at -10°C. The rate of addition was important for reproducible results. The precipitate was collected by centrifugation at 5000g for 15min and the supernatant was discarded. The pellet was dissolved in 25mM-potassium phosphate buffer (pH 7.2), containing 1mM-EDTA, to a volume of 20ml and stored at 0°C.

First $(\text{NH}_4)_2\text{SO}_4$ fractionation. The preparation was brought to 37.5% saturation by addition of 0.6 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution at 0°C. After 30min the precipitate was removed by centrifugation and the supernatant was raised to 52.5% saturation by the addition of 0.32 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ at 0°C. This precipitate was collected by centrifugation at 5000g for 15min and dissolved in the phosphate-EDTA buffer to a final volume of 10ml.

Second $(\text{NH}_4)_2\text{SO}_4$ fractionation. Saturated $(\text{NH}_4)_2\text{SO}_4$ at 0°C (4.3ml) was added and the solution was stirred for 3h at 0°C. The precipitate was removed by centrifugation at 5000g for 10min. A further 2.4ml of saturated $(\text{NH}_4)_2\text{SO}_4$ was added with stirring and the solution stored overnight at 0°C. The precipitate was collected by centrifugation at 5000g for 15min and dissolved in the phosphate-EDTA buffer to a volume of 5ml. This preparation was stored at 0°C.

Chromatography on hydroxyapatite-cellulose. A suspension (105ml) of Whatman standard cellulose powder (14g) in distilled water was stirred with 105ml of hydroxyapatite (30mg/ml) in 1mM-potassium phosphate buffer (pH 6.8), prepared by the method of Bernardi (1971). The mixture was degassed by shaking under vacuum for 15min and immediately poured into a chromatography column. After 1h packing was completed by running buffer through under gravity to give a column of 20cm $\times$ 1.5cm. The column was finally equilibrated overnight at 5°C by pumping 0.5mM-potassium phosphate buffer (pH 6.8) through at 40ml/h.

The enzyme was dialysed against 300ml of 0.1mM- KH_2PO_4 at 5°C overnight; precipitated protein was removed and the supernatant was applied to the column. Elution was at 60ml/h with 45ml of 0.5mM-potassium phosphate (pH 6.8) followed by a linear gradient of potassium phosphate from 0.5 to 250mM (pH 6.8) over 280ml and finally with 100ml of the 250mM buffer. Fractions (5ml) were collected; the enzyme was eluted between 46 and 100ml after commencement of the linear gradient and was precipitated with 60%-satd. $(\text{NH}_4)_2\text{SO}_4$. After

Table 1. *Progress chart for the purification of mitochondrial oxaloacetate decarboxylase*
Enzyme activities are given in $\mu\text{mol}/\text{min}$ per kg of liver at 30°C.

State of purification	Oxaloacetate decarb- oxylase	Malate dehydro- genase	Lactate dehydro- genase	'Malic' enzyme	Pyruvate kinase	Protein (mg)	Oxaloacetate decarboxylase	
							(units/mg of protein)	% recovery
Sonicated mitochondria	21.6	92200	—	—	0	5240	0.0041	100
58°C supernatant	17.9	32400	85	1.55	0	637	0.028	83.0
Acetone fraction	54.9	15300	162	2.13	0	424	0.130	254
First $(\text{NH}_4)_2\text{SO}_4$ fraction	14.7	997	13.6	—	0.82	196	0.075	68.1
Second $(\text{NH}_4)_2\text{SO}_4$ fraction	10.3	31.2	0.56	0.12	0.07	64.5	0.160	47.7
Column eluate	5.7	1.64	0.04	0	0.03	10.2	0.560	26.4

storage overnight and centrifugation the protein was dissolved in 25mM-potassium phosphate buffer (pH 6.8) containing 1mM-EDTA.

A typical progress chart for this fractionation is shown in Table 1.

Assay of mitochondrial oxaloacetate decarboxylase

The assay was carried out in two steps. Samples were withdrawn from a single incubation mixture at different times up to 15min and deproteinized (see below). Oxaloacetate and pyruvate were then measured spectrophotometrically by the addition of NADH, malate dehydrogenase and lactate dehydrogenase. Attempts to develop a continuous spectrophotometric assay failed.

The basic reaction mixture contained 20mM-potassium phosphate buffer, pH 7.2, 2mM-EDTA, 4mM-arsenite, 2mM-oxaloacetate and sufficient enzyme to give a rate of pyruvate formation of up to $0.1 \mu\text{mol/min per ml}$. The arsenite was added to inhibit further oxidation of pyruvate by pyruvate dehydrogenase and was omitted with preparations after the acetone fractionation step. When crude preparations were assayed HClO_4 (0.5M final concn.) was used for deproteinization followed by neutralization with $0.25\text{M-K}_2\text{CO}_3$. A 0.2ml portion of the supernatant after centrifuging, 0.3ml of 0.5M-potassium phosphate buffer, pH 7.6, 0.015ml of 10mM-NADH and water to 1ml were placed in a cuvette and the extinction at 340nm measured. Malate dehydrogenase and lactate dehydrogenase ($1 \mu\text{l}$ of each) were added in that order with sufficient time between for a constant extinction to be reached. Oxaloacetate and pyruvate concentrations were calculated by using a molar extinction coefficient for NADH of $6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 340nm. When purer preparations (after the acetone stage) were used it was sufficient to stop the reaction with HCl instead of HClO_4 . In this case the sample ($0.1\text{ml} + 0.1\text{ml}$ of 1M-HCl) was neutralized by addition of 0.4ml of 0.5M- Na_2HPO_4 . The whole neutralized sample then received 0.03ml of 10mM-NADH and water to 2ml and was assayed as above. The small amount of protein present did not interfere. Incubation volume, sample size and sampling times were varied as circumstances required.

Assay of 'supernatant' decarboxylase activity

The method was similar to that for the mitochondrial enzyme except that the phosphate buffer was replaced by 20mM-sodium acetate buffer (pH 5.0, final concn.) and EDTA was replaced by either 0.1mM- MnCl_2 (final concn.) or 1mM- MgCl_2 .

Controls for assays of both types of decarboxylase

Oxaloacetate decarboxylation rates are affected by various metal ions and amino acids (Bessman & Layne, 1950). When crude preparations or 'supernatant' enzymes were assayed non-enzymic rates were estimated for each assay by inclusion of a control in which the enzyme preparation was previously heated at 100°C for 10min. For the mitochondrial enzyme control rates were never more than 20% of the total rates of active fractions and were often much less so that errors in control rates are at acceptable levels. For assay of 'supernatant' enzyme, control rates were commonly 50% or more. Values for 'supernatant' enzyme must therefore be interpreted with caution. When assays were performed with the purified mitochondrial enzyme the non-enzymic rate was calculated from the rate constant of decarboxylation (see below) under assay conditions.

Other assays

Other enzyme activities were assayed by the following methods: malate dehydrogenase, Ochoa (1953); lactate dehydrogenase, Kaplan & Ciotti (1961); 'malic' enzyme (EC 1.1.1.40) and pyruvate kinase, Taylor *et al.* (1967); glucose 6-phosphate dehydrogenase, Glock & McLean (1953); succinate dehydrogenase, Pennington (1961), with tetrazolium as acceptor.

All enzyme assays were performed at 30°C . All activities are expressed as EC units (μmoles of substrate transformed/min).

Results

Spontaneous decarboxylation of oxaloacetate

The rate of spontaneous decarboxylation under the standard assay conditions for the mitochondrial enzyme was a linear function of oxaloacetate concentration with a first-order rate constant of $2.16 \times 10^{-3} \text{ min}^{-1}$. The spontaneous decarboxylation rate varied with pH and composition of the medium (Figs. 1b and 5b).

Purity of the mitochondrial enzyme preparation

Ultracentrifuge studies on the purified enzyme in 25mM-potassium phosphate buffer, pH 7.2, at 3mg/ml and 25°C showed two peaks of roughly equal proportion with uncorrected *s* values of 4.8 and 6.76S. Decarboxylase activity was found only in the faster-sedimenting peak.

Substrate specificity

The mitochondrial enzyme did not decarboxylate malate, acetoacetate or 2-oxoglutarate. The decarb-

Fig. 1. Effect of pH on (a) mitochondrial oxaloacetate decarboxylase and (b) spontaneous decarboxylation of oxaloacetate

In both (a) and (b) conditions were as for the assay of mitochondrial oxaloacetate decarboxylase except for the inclusion in (b) of bovine serum albumin (0.15mg/ml) and the use of a wide-range buffer containing glycylglycine, KH_2PO_4 and H_3BO_4 each at a final concn. of 13mM. NaOH was added to give the desired pH. The pH of incubation mixtures was verified by pH meter.

oxylation of oxaloacetate proceeded to virtual completion. The presence of pyruvate in amounts up to 50% of the oxaloacetate concentration did not influence the reaction rates.

pH optimum

The activity of the purified mitochondrial enzyme increased with increasing pH over the range pH 6.0–pH 9.5 whereas the non-enzymic rate fell over this range (Fig. 1).

Effect of oxaloacetate concentration

The mitochondrial enzyme showed Michaelis-Menten kinetics with oxaloacetate concentrations up to about 0.3mM, above which substrate inhibition became apparent (Figs. 2a and 2b). A double-reciprocal plot of these values indicated a K_m value of about 0.23mM.

Fig. 2. Effect of oxaloacetate concentration on the activity of mitochondrial oxaloacetate decarboxylase (a) Plot of v against oxaloacetate concentration; (b) Lineweaver-Burk plots of the same results.

Fig. 3. Inhibition of mitochondrial decarboxylase by CoA, acetyl-CoA, citrate and phosphoenolpyruvate. Assays were performed under standard conditions as stated in the Experimental section. $\circ$, CoA; $\square$, acetyl-CoA; $\blacksquare$, acetyl-CoA purified chromatographically; $\triangle$, citrate; $\blacktriangle$, phosphoenolpyruvate. The arrows indicate the concentrations of inhibitors giving 50% inhibition.

Inhibition studies

CoA inhibited the mitochondrial enzyme (giving 50% inhibition at about 0.05 mM); acetyl-CoA was equally effective.

Citrate and phosphoenolpyruvate were weak inhibitors (giving 50% inhibition at about 4.5 mM and 28 mM respectively) (Fig. 3). Unlike that described by Corwin (1959) our mitochondrial decarboxylase was not inhibited by 8-hydroxyquinoline.

Effect of KCl concentration on the mitochondrial enzyme

The activity of the mitochondrial enzyme was diminished by addition of KCl to the assay medium as shown in Fig. 4.

Distribution of decarboxylase activity

Activity was assayed for both supernatant and mitochondrial enzymes in nuclei, microsomal frac-

tions, mitochondria and supernatant fractions prepared from rat liver by differential centrifugation (Alberti & Bartley, 1963). All but the supernatant fraction were sonicated before assay. Succinate dehydrogenase and glucose 6-phosphate dehydrogenase were used as mitochondrial and supernatant markers respectively. The results (Table 2) show the presence of two enzymes, one mitochondrial and one in the cytosol. The activity of the latter was seven-fold higher with a pH optimum of about 5 (Fig. 5). Kidney contained a similar amount of the mitochondrial enzyme to that in liver, but muscle and brain had negligible activity.

Fig. 4. Effect of KCl concentration on the activity of mitochondrial decarboxylase

In the absence of added KCl the incubation medium had an ionic strength of 0.04.

Fig. 5. Effect of pH on (a) supernatant oxaloacetate decarboxylase and (b) spontaneous decarboxylation of oxaloacetate

In both (a) and (b) conditions were as for the assay of supernatant oxaloacetate decarboxylase with 0.1 mM-Mn²⁺, except for the pH of the acetate buffer and the inclusion in (b) of bovine serum albumin (7.5 mg/ml). The pH of incubation mixtures was verified by pH meter.

Table 2. Enzyme activities in rat liver fractions

Activities are expressed as μmol/min per g wet wt. of liver at 30°C. Values in parentheses are percentages of totals.

	Supernatant	Mitochondria	Microsomal fraction	Nuclei
Glucose 6-phosphate dehydrogenase	1.11 (93.5)	0.045 (3.8)	0.031 (2.6)	0.0013 (0.1)
Succinate dehydrogenase	0.0033 (1.0)	0.115 (35.2)	0.097 (29.7)	0.112 (34.3)
Decarboxylase (supernatant-type assay)	0.587 (97.5)	0.0025 (0.4)	0 (0)	0.012 (2.1)
Decarboxylase (mitochondrial-type assay)	0 (0)	0.056 (64.4)	0.023 (26.4)	0.008 (9.2)

Discussion

Oxaloacetate decarboxylases from plant and microbial sources are typically enzymes with a pH optimum of 5–6 and divalent metal ion requirement, Mn^{2+} being most effective (Utter, 1961). A similar enzyme has been extracted from cod muscle (Kosicki, 1968). A decarboxylase from rat liver mitochondria has been described by Corwin (1959) with a pH optimum of 7.5 and showing Mg^{2+} activation which was antagonized by Mn^{2+} .

Our results (Table 2) show that rat liver contains two enzymes having different pH optima and metal requirements. The supernatant enzyme had a pH optimum of 5 and requires Mn^{2+} or Mg^{2+} for full activity and the mitochondrial one had an alkaline pH optimum with no metal requirement.

In addition to decarboxylases there are several enzymic routes which can account for the formation of pyruvate from oxaloacetate. (1) Malate dehydrogenase + 'malic' enzyme; (2) phosphoenolpyruvate carboxykinase + pyruvate kinase; (3) glutamate-oxaloacetate transaminase + glutamate-pyruvate transaminase. Pathways (1) and (2) are mainly extramitochondrial. The results of Table 1 and the substrate requirements exclude these pathways from our work; spontaneous decarboxylation was allowed for.

The mitochondrial enzyme seems to be associated with the membrane fraction since sonication only solubilized about 50% of it. This is reinforced by the necessity to use an acetone treatment to obtain good fractionation with $(NH_4)_2SO_4$, suggesting association with lipid.

Corwin (1959) reported a K_m value of 0.11 mM for the mitochondrial decarboxylation of oxaloacetate which compares with our value of 0.23 mM (Fig. 2). However, this is some 100-fold higher than the expected mitochondrial oxaloacetate concentration (Gumaa *et al.*, 1971). The K_m value for oxaloacetate may perhaps be lower *in vivo*.

The normal amounts of CoA and its derivatives in the mitochondria are given as 1.72–1.95 nmol/mg of protein by Garland *et al.* (1965), enough to cause severe inhibition. Both CoA and acetyl-CoA inhibited with equal effect, although the enzyme from *Pseudomonas ovalis* Chester is inhibited by acyl-CoA derivatives but not by CoA (Horton & Kornberg, 1964). The normal amount of citrate is given by Gumaa *et al.* (1971) as 100 nmol of mitochondrial

citrate/g of liver, the liver containing 0.21 ml of mitochondrial water/g dry wt. This is equal to 2.5 mM which gives about 40% inhibition. The highest salt concentration in mitochondria may be about 0.4 molal (Bartley *et al.*, 1969) which would inhibit considerably (Fig. 4). The natural concentrations of these substances and the K_m value would appear to result in the enzyme having little activity *in vivo*. It is possible that the decarboxylation of oxaloacetate is not the true function of this enzyme *in vivo* and that it has some additional function or participates in some coupled membrane process. We have observed that swelling of mitochondria in media of low ionic strength (to leach out soluble constituents) resulted in a loss of phosphoenolpyruvate synthesis and an increase in pyruvate formation.

On the present evidence no physiological role can be assigned to the supernatant enzyme.

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