Studies on the Biosynthesis of Mitochondrial Malate Dehydrogenase and the Location of its Synthesis in the Liver Cell of the Rat

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A method is described for the isolation of mitochondrial malate dehydrogenase from either the whole tissue homogenate or from the microsomal fraction of rat liver. The procedure involves the treatment of the tissue extract with detergent followed by gel filtration and chromatography on Amberlite CG-50 and DEAE-cellulose. The resulting enzyme was homogeneous by the criterion of gel electrophoresis. Incubation of the microsomal fraction from rat liver under the usual conditions for protein synthesis in the presence of [^3H]leucine resulted in the incorporation of ^3H into the mitochondrial malate dehydrogenase when purified as described. The results are taken to indicate that the mitochondrial enzyme is synthesized by the cytoplasmic ribosomes. Possible ways in which the cytoplasmic and mitochondrial forms of malate dehydrogenase reach their final locations in the cell are discussed.

The balance of evidence at present available indicates that only some of the structural proteins of the mitochondria are synthesized within the organelle and that most of the proteins, including those that are soluble, are of extra-mitochondrial origin (see, e.g., Roodyn & Wilkie, 1968; Ashwell & Work, 1970). As an example of a soluble mitochondrial protein the biosynthesis of cytochrome *c* has been studied extensively. From kinetic studies with Krebs ascites-tumour cells Freeman *et al.* (1967) suggested that cytochrome *c* was synthesized extra-mitochondrially. This conclusion was confirmed by kinetic studies on liver, after the injection of ^{14}C -labelled amino acids into rats, by González-Cadavid & Campbell (1967*a,b*) and by Kadenbach (1969, 1970). Further confirmation was obtained by Penniall & Davidian (1968) who used [^3H]aminolaevulinic acid as a precursor for haem.

In their study on the incorporation of ^{14}C -labelled amino acids into protein by isolated rat liver mitochondria Roodyn *et al.* (1962) showed that most of the incorporation was into insoluble protein and there was little into either cytochrome *c* or malate dehydrogenase. We decided to study the synthesis of the latter enzyme for two reasons. First, we hoped to demonstrate the synthesis of malate dehydrogenase by a microsomal preparation and so locate its site of synthesis more certainly than would be possible by kinetic studies. This approach had not been possible with cytochrome *c*, owing perhaps to the covalent attachment of the haem moiety to the apoprotein, which could not be effected by the isolated microsomal fraction. Secondly, it is of interest to study the way in

which the protein moves from the cytoplasmic ribosomes to the mitochondria. In this respect malate dehydrogenase is of particular interest as it exhibits dual location in the cell. In various tissues, including rat liver, there are two major forms of the enzyme, one exclusive to the cytoplasm and one to the mitochondria. These two forms have very different physical and chemical properties, but their substrate specificities are very similar (Thorne *et al.*, 1963; Thorne, 1968; Mann & Vestling, 1968). Thus if both forms of the enzyme are synthesized at the same location, some mechanism must exist for the exclusive movement of one form into the mitochondrion while the other is released into the cytoplasm.

In the present paper we report a method suitable for the purification of rat liver mitochondrial malate dehydrogenase, and its application to a study of the synthesis of the enzyme by isolated liver microsomal preparations.

Materials and Methods

Materials

Chemicals. ATP (disodium salt), GTP (sodium salt), NAD, 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide and tris buffer (Sigma 7-9 grade) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Phosphoenolpyruvate (potassium salt) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. 2-Amino-2-methylpropan-1-ol and *NN,N'*-tetramethylethylenediamine were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. DEAE-cellulose, grade DE23, was from W. and R. Balston (Modified Cellulose) Ltd.,

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Springfield Mill, Maidstone, Kent, U.K. Soluene TM.100 was from Packard Instruments, Middlesex, U.K. Starch, hydrolysed for gel electrophoresis (lot 228-1), was from Connaught Medical Research Lab., Toronto, Canada.

Radiochemicals. L-[4,5-³H]Leucine (specific radioactivity 19.7 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Animals. The rats used in these studies were male, Wistar strain, and were purchased from Tuck and Sons Ltd., Rayleigh, Essex, U.K.

Isolation of the microsomal fraction and preparation of cell sap

Rats were starved overnight, killed by a blow on the head and the livers removed. The livers were minced, disrupted in medium A [0.15M-sucrose–25mM-KCl–10mM-MgSO₄–34.5mM-tris (adjusted to pH 7.8 with HCl at 20°C)] and the microsomal fraction was recovered exactly as described by Ragnotti *et al.* (1969). Cell sap was also prepared and treated with Sephadex G-25 as described in the latter paper.

Incubation procedure

The incubation procedure was as follows: In a 250ml flask the following solutions were assembled: L-[³H]leucine (5 ml = 500 μ Ci); 100mM-phosphoenolpyruvate (15ml); 100mM-ATP (2ml); 5mM-GTP (5ml); Sephadex G-25-treated cell sap (20ml); microsomal suspension (40ml); medium A (13ml). For a control incubation, ATP was omitted and the volume of medium A increased accordingly. The microsomal suspension was added and the contents of the flask were thoroughly mixed and divided between five 50ml conical flasks. These flasks were incubated, with constant shaking, in a water bath at 37 $\pm$ 0.3°C for 30min. At the end of the incubation period, the contents of the flasks were pooled, then Triton X-100 was added to a final concentration of 1% and the mixture rapidly frozen for storage overnight at –12°C. The next day the mixture was thawed and centrifuged at 105000g for 60min in an M.S.E. Superspeed 65 centrifuge. The supernatant was used for the isolation and purification of malate dehydrogenase.

Purification of mitochondrial malate dehydrogenase

Initially purification of the enzyme was attempted by the method of Roodyn *et al.* (1962). The method was not very reproducible in our hands, and the method finally adopted was based on that described by Thorne & Dent (1968). This method gives a complete separation of cytoplasmic and mitochondrial malate dehydrogenase in one step by using a column of Amberlite CG-50.

The crude enzyme sample was either the Triton

X-100 supernatant from the microsomal incubations or it was prepared from whole rat liver homogenized in 3 vol. of 0.025M-potassium phosphate buffer, pH 7.0, containing 0.5% (w/v) of Triton X-100. This homogenate was centrifuged at 105000g for 60min to remove the particulate material not solubilized by the Triton X-100. The Triton X-100 supernatant was passed through a column of Sephadex G-25 equilibrated with 0.025M-potassium phosphate buffer, pH 7.0 (the size of the column was such that the sample was equivalent to about 10% of the column volume). This and all subsequent steps were done in a cold-room at 1–5°C.

The Sephadex-treated sample was loaded on a column (1.5cm $\times$ 12cm) of Amberlite CG-50 that had been equilibrated with 0.025M-potassium phosphate buffer, pH 7.0. The resin had previously been pre-treated as described by Hagiwara *et al.* (1958). The fraction that passed through the column unbound contained, among other proteins, the cytoplasmic malate dehydrogenase. The column was washed with a large volume (2 litres) of the 0.025M-phosphate buffer and the protein was eluted with 0.2M-potassium phosphate buffer, pH 7.0. This eluted a deep-red fraction (25 ml) containing the mitochondrial enzyme. The eluate was passed through a column (2.5cm $\times$ 25cm) of Sephadex G-25 equilibrated with 0.02M-tris-HCl buffer, pH 8.3 at 20°C. The eluate was dialysed overnight against the same tris buffer. The fraction was then loaded on a column (1.5cm $\times$ 12cm) of DEAE-cellulose (Whatman DE23, pretreated as recommended in the Whatman Technical Bulletin) equilibrated with 0.02M-tris-HCl buffer, pH 8.3, which was also the eluting buffer. Most of the proteins remained bound to the DEAE-cellulose in a red band but the mitochondrial malate dehydrogenase passed through unretained in a colourless fraction.

Electrophoresis of mitochondrial malate dehydrogenase

The purity of the isolated enzyme was determined by gel electrophoresis either with polyacrylamide gel or starch. The polyacrylamide-gel system was similar to the pH 8.9 system of Davis (1964). The spacer and sample gels used by Davis (1964) were not employed, 50–100 μ l of enzyme sample (containing 5% sucrose plus a trace of Bromophenol Blue) being layered directly on the top of the running gel. The samples were run at 4mA/tube (350–400V) until the Bromophenol Blue band reached the bottom of the tube (usually 45–75min) and sometimes the samples were run for a longer time, as the malate dehydrogenase has a low mobility. Gels were stained for protein (Chrambach *et al.*, 1967) or for malate dehydrogenase activity (Davidson & Cortner, 1967).

Starch-gel electrophoresis was performed by the method of Fine & Costello (1963) by using a gel

15cm × 15cm, 5mm thick. The enzyme sample was applied to a strip of Whatman 3MM chromatography paper, which was then inserted in a slit in the gel. The electrophoresis was done in the cold-room at 15mA for 2–3h, until a Bromophenol Blue band (applied with the sample) reached the anode end of the gel. The gel was stained for malate dehydrogenase activity by the method used for the polyacrylamide gels.

Assay of malate dehydrogenase

The enzyme was assayed spectrophotometrically by measuring the reduction of NAD^+ by malate at 340nm (Lowry, 1957), in a Hilger–Gilford recording spectrophotometer, with a constant-temperature water-bath at 25°C. One unit of malate dehydrogenase activity was the amount giving an increase in E_{340} of 2.09/min. This is equivalent to a reduction of $1\mu\text{mol}$ of NAD^+ /min, assuming ϵ at 340nm of $6.27 \times 10^3 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for NADH.

Determination of protein

Protein in effluents from columns was determined by measuring the extinctions at 280 and 260nm, the amount of protein being given by the formula: protein concentration (mg/ml) = $1.55 E_{280} - 0.76 E_{260}$ (Warburg & Christian, 1941, as modified by Layne, 1957).

Where a precise measurement of protein content for calculation of specific enzymes or specific radioactivity was required, this was done by the method of Lowry *et al.* (1951) as modified by Campbell & Sargent (1967).

Measurement of specific radioactivity

Protein samples (usually 0.5 or 1.0ml) were taken in quadruplicate: two for protein determination (as described above), two for radioactivity determination. To each of the samples for radioactivity determination was added an equal volume of 2M-NaOH; the samples were then kept at room temperature for 5min to hydrolyse RNA (Ragnotti *et al.*, 1969). An amount of trichloroacetic acid (50%, w/v) equal to the original volume was added slowly with constant mixing and the samples were kept at approx. 4°C for at least 1h to allow the precipitate to form. The samples were then collected on individual glass-fibre discs (Whatman GF/A, 2.1cm diameter) by using a water-suction pump and a Millipore sintered-glass disc holder. The precipitates were washed on the discs by flushing with three portions (5ml each) of trichloroacetic acid (5%, w/v), followed by 3 × 5ml of 'fat solvent' (chloroform–ether–methanol, 2:1:1, by vol.). The discs were placed in scintillation vials and dried in an oven at 80°C for 15min to remove the last traces of solvent. As the proteins were ^3H -labelled they were solubilized by the addition of 1.5ml of Soluene

TM.100 plus 0.1ml of water. The capped vial was then left overnight at room temperature. Scintillation fluid (10ml), composed of 2,5-diphenyloxazole (4g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (100mg/l) in toluene solution, was added and the samples counted for radioactivity in a Beckman LS-200B liquid-scintillation counter. The efficiency of counting ^3H radioactivity was individually determined for each vial by 'spiking' with an internal standard of 37000 d.p.m. of [^3H]uridine: efficiencies were between 8 and 22% depending on the quantity, and colour, of protein in the vial. The background count for ^3H was 23–25 c.p.m.

Results

Purification of mitochondrial malate dehydrogenase

Thorne & Dent (1968) reported a method for the complete separation of the cytoplasmic and mitochondrial enzymes by using Amberlite CG-50. This was taken as the starting point for the method of purification described in the Materials and Methods section. The method is based on the fact that whereas the mitochondrial enzyme is retained by a column of Amberlite CG-50, the cytoplasmic enzyme passes through the column unabsorbed. The 0.2M-potassium phosphate buffer, pH 7.0 eluted a fraction containing the mitochondrial enzyme, which was further purified on a column of DEAE-cellulose equilibrated with the tris buffer. Most of the proteins in the extract were bound as a red band to the DEAE-cellulose, but the mitochondrial malate dehydrogenase was eluted unbound. The purity of the eluate was determined by polyacrylamide gel electrophoresis. Only one protein band, apart from that at the origin, was stained with Coomassie Blue; the band moved towards the anode, and its position corresponded to that of the band that we stained for malate dehydrogenase activity in the duplicate gel. This was taken therefore as evidence for the purity of the preparation of mitochondrial malate dehydrogenase.

The enzyme activity of samples before and after chromatography on Amberlite and after DEAE-cellulose was checked by starch-gel electrophoresis. The Amberlite chromatography removed a large proportion of the material migrating to the anode and the DEAE-chromatography completely removed it, so it is therefore assumed that all the cytoplasmic malate dehydrogenase was removed by these methods.

The purification of the mitochondrial enzyme and the yields at different steps are summarized in Table 1.

Biosynthesis of mitochondrial malate dehydrogenase by an isolated microsomal fraction

An isolated microsomal fraction from rat liver was incubated with [^3H]leucine, and at the end of the

Table 1. *Summarized purification steps for malate dehydrogenase: typical values for specific activity and yield*

Experimental details are given in the text.

Stage	Specific activity (units/mg of protein)	Yield (% of total units of malate dehydrogenase activity in starting materials)
1. Triton X-100 supernatant (from incubation)	0.2	(100)
2(a). Material passing through Amberlite CG-50 column unbound (containing cytoplasmic enzyme)	0.3	75
2(b). Material eluted from Amberlite CG-50 column by 0.2M-phosphate, pH 7.0 (containing mitochondrial enzyme)	3.0	18
3. Material passing DEAE-cellulose column in 0.02M-tris buffer, pH 8.3	40.0	5

Table 2. *Isolation of mitochondrial malate dehydrogenase after incubation of rat liver microsomal fraction*

Incubation (final concentration, 2.9mg of particulate protein/ml) was for 30 min with 500 μ Ci of [3 H]leucine, either in complete medium, or without ATP. Carrier (3.28 units of malate dehydrogenase) was added in the ratio 108mg of carrier/26mg of fraction 5 for 'Complete' and 97mg of carrier/22mg of fraction 5 for 'No ATP'.

Fraction	Specific radioactivity (d.p.m./mg of protein)		Specific enzyme activity (units/mg of protein)	
	Complete	No ATP	Complete	No ATP
1. Total protein of mixture after incubation	208 150	85 600	0.19	0.18
2. Triton X-100 supernatant	55 900	34 550	0.22	0.16
3. Fraction 2 after passing through Sephadex G-25	20 455	12 790	0.22	0.15
4. Material not bound to Amberlite column	21 400	6 315	0.29	0.30
5. Eluate from Amberlite column	5 898	1 686	0.66	0.61
6. Pooled fraction 5 plus unlabelled carrier	1 358	585	2.65	2.62
7. Material passing DEAE-cellulose column	1 015	405	20.7	18.2

incubation mitochondrial malate dehydrogenase was purified from the mixture. The incubation mixture was divided into two halves, which were completely identical with the exception that ATP was omitted from one of the mixtures. The results of this experiment are given in Table 2. Non-radioactive carrier enzyme was added after the Amberlite-column stage of the purification. This carrier consisted of a preparation from whole rat liver taken through the purification procedure as far as the Amberlite-column stage. The addition of carrier was necessary to maintain a sufficient bulk of protein for ease of physical handling during the final stages of the purification. The protein with malate dehydrogenase activity thus obtained had a significant amount of radioactivity. The purity of the final product of the purification was checked by polyacrylamide-gel electrophoresis and was found to consist of a single band of protein corresponding to the band of malate dehydrogenase activity. Because of the small amount of protein being

handled it was not possible to demonstrate radioactivity in the protein after electrophoresis. Omission of the energy source from the incubation mixture caused a large fall in the amount of radioactivity incorporated into the protein. The reason that the incorporation in the absence of ATP did not fall to zero may be attributed to the fact that an unwashed microsomal fraction was used in which there would be a low endogenous concentration of ATP remaining. It was considered that to have washed the microsomal fraction by resedimentation would have had a deleterious effect and could have decreased the incorporating efficiency to a value where it might not have been possible to detect incorporation into a specific product.

Discussion

The chromatographic procedures described proved very convenient for the separation of the cytoplasmic

and mitochondrial forms of malate dehydrogenase. The resulting mitochondrial enzyme appeared to be homogeneous and satisfactory for biosynthetic studies. The results demonstrate that the synthesis of the mitochondrial enzyme is effected by the microsomal fraction. In other experiments (R. W. Bingham & P. N. Campbell, unpublished work) we have confirmed the work of Roodyn *et al.* (1962) who showed that isolated mitochondria are unable to effect the synthesis of mitochondrial malate dehydrogenase. As one must assume that the cytoplasmic form of malate dehydrogenase is also synthesized by the cytoplasmic ribosomes, one must postulate the existence of some mechanism to direct the two forms of the enzyme to their unique intracellular locations. It is tempting to suggest that the destination of the two forms is determined by their relative physical and chemical properties.

The cytoplasmic enzyme is anionic and has a substantial net charge as shown by its electrophoretic mobility. In contrast, the mitochondrial enzyme is slightly basic and has a rather low net charge at neutral pH values, as shown by its low mobility during electrophoresis. This difference also applied to the two forms of malate dehydrogenase found in various other organisms and tissues (see e.g., Thorne *et al.*, 1963; Thorne, 1968; Mann & Vestling, 1968). It is noteworthy that cytochrome *c* is also a basic protein.

The mitochondrial phospholipids are apparently synthesized on the endoplasmic reticulum (Wilgram & Kennedy, 1963; McMurray & Dawson, 1969; Jungalwala & Dawson, 1970) and it has been suggested that they migrate as a protein-phospholipid complex. Kadenbach (1968) has already suggested that the soluble mitochondrial enzymes are transferred as such a complex and certainly a basic protein would more readily complex with phospholipid. Even if this were not so the proteins transferred might well have to have such properties to enable them to complex with the phospholipids of the outer membrane of the mitochondrion. It would, therefore, be interesting to examine the properties of other mitochondrial enzymes, especially those that exhibit a dual location in the cell.

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