A PUTATIVE PRECURSOR OF RAT LIVER MITOCHONDRIAL MALATE DEHYDROGENASE

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1. Introduction

Mitochondria have their own genetic system but the majority of mitochondrial proteins are coded by nuclear DNA, synthesized on cytosolic ribosomes and imported into mitochondria [1,2]. Little is known of the mechanism of import [3–6]. There is some evidence that the mature form or subunits of the mitochondrial matrix proteins aspartate aminotransferase [6–9] and malate dehydrogenase (EC 1.1.1.37) [6,10–12] can be taken up into the matrix of isolated mitochondria. However, a number of mitochondrial matrix proteins including aspartate aminotransferase [13] are synthesized in precursor form [3,5]. Here, we report a putative precursor or rat liver mitochondrial malate dehydrogenase which is synthesized in a reticulocyte lysate primed with either free polysomes or total RNA from rat liver.

2. Materials and methods

5′-AMP Sepharose and protein A–Sepharose CL-4B were purchased from Pharmacia Fine Chemicals (Uppsala). [^{14}C]Formaldehyde and [^{35}S]methionine, each at the highest specific activity available, were obtained from New England Nuclear Canada (Lachine, Quebec). Bovine heart mitochondrial malate dehydrogenase was from Sigma Chemical Co. (St Louis MO) and porcine heart cytosolic malate dehydrogenase was from Research Plus Lab. (Denville NJ).

Rat liver mitochondrial malate dehydrogenase was extracted from mitochondria of Sprague-Dawley rats by sonication in the presence of 2 mM EDTA, 4 mM β-mercaptoethanol, 20 mM sodium phosphate (pH 7.0). The crude enzyme was initially purified and concentrated by $(NH_4)_2SO_4$ fractionation, dialyzed and applied to a 5′-AMP Sepharose 4B affinity column [14] in the sonication buffer. The enzyme was eluted at ~30 μM NADH in a 0–100 μM NADH gradient in the same buffer. Enzyme was precipitated with $(NH_4)_2SO_4$, passed through a Sephadex G100 column to remove minor protein contaminants and then stored as an $(NH_4)_2SO_4$ precipitate. Bovine heart mitochondrial malate dehydrogenase was also purified from minor contaminants by affinity chromatography. Cytosolic malate dehydrogenase from rat liver was purified as in [15].

Antibodies were induced with SDS-denatured [16] rat liver and bovine heart malate dehydrogenase. About 250–500 μg protein was injected both intramuscularly and subcutaneously into rabbits, first with Freund's complete adjuvant and then alone at 2 week intervals. Rabbits were bled 8 days after the third and subsequent injections. The activity of the antisera were assayed by protein A–Sepharose precipitation [17] of [^{14}C]formaldehyde-labelled [18] malate dehydrogenase either native or denatured with SDS and treated with Triton X-100 [16].

Free polysomes [19] and total RNA [20] were isolated from rat liver and used to prime a nuclease-treated reticulocyte cell-free protein synthesizing system [21]. After 60 min incubation, synthesis was stopped by making the lysate 6% in SDS, 6 mM in EDTA and 10 mM in β-mercaptoethanol. Four volumes of 9% Triton X-100 in a buffer containing 140 mM NaCl, 5 mM EDTA and 50 mM Tris–HCl (pH 7.4) was added after 45 min. Protein was immunoprecipitated using protein A–Sepharose and examined by SDS–polyacrylamide slab gel electrophoresis and fluorography [22].

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3. Results and discussion

3.1. *Purification of malate dehydrogenase and antibody specificity*

Affinity chromatography proved an effective method of obtaining pure malate dehydrogenase from rat liver mitochondria. One major band only at 34 500 M_r was observed on SDS–polyacrylamide slab gels by Coomassie brilliant blue staining (not shown), or fluorography of [^{14}C]formaldehyde-labelled enzyme (see below).

Antibody production and specificity were followed by labelling the enzyme by reductive methylation and precipitation of antibody–antigen complexes with protein A–Sepharose. The antibodies against SDS-denatured rat and bovine enzymes were each slightly more effective against the denatured enzyme used to induce them but did not react with native mitochondrial cytosolic malate dehydrogenase (fig.1). The antibodies also specifically immunoprecipitated malate dehydrogenase from an extract of mitochondria from bovine MDBK cells labelled with [^{35}S]methionine [22] (not shown).

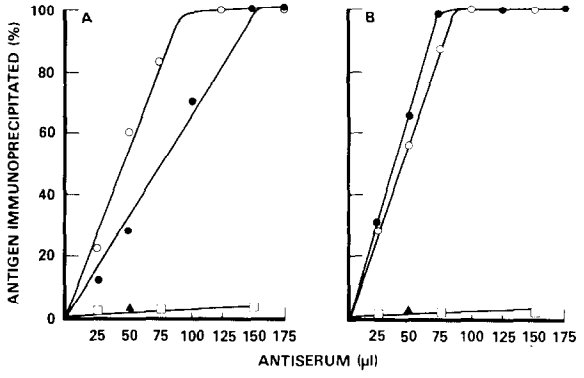

Fig.1. Immunoprecipitation of malate dehydrogenase with antisera against denatured bovine heart (A) and rat liver (B) mitochondrial malate dehydrogenase. Enzymes were labelled with [^{14}C]formaldehyde and 5.5 μg (A) or 3.4 μg (B) of each protein were incubated with increasing volumes of antisera. Native enzymes were dissolved in 140 mM NaCl, 1 mM EDTA, 2 mM β-mercaptoethanol, 50 mM Tris–HCl (pH 7.6) and treated with SDS followed by Triton X-100 [16] to prepare the denatured form. The amount of antigen bound was determined by protein A–Sepharose precipitation of immune complexes. Antigen: (○) denatured bovine; (●) denatured rat; (□) native bovine or rat; (△) denatured cytosolic porcine or rat.

3.2. *Product of cell-free protein synthesis primed by rat liver free polysomes or total RNA*

Nuclease-treated reticulocyte lysates primed with either free polysomes or total RNA from rat liver were used to examine the synthesis of malate

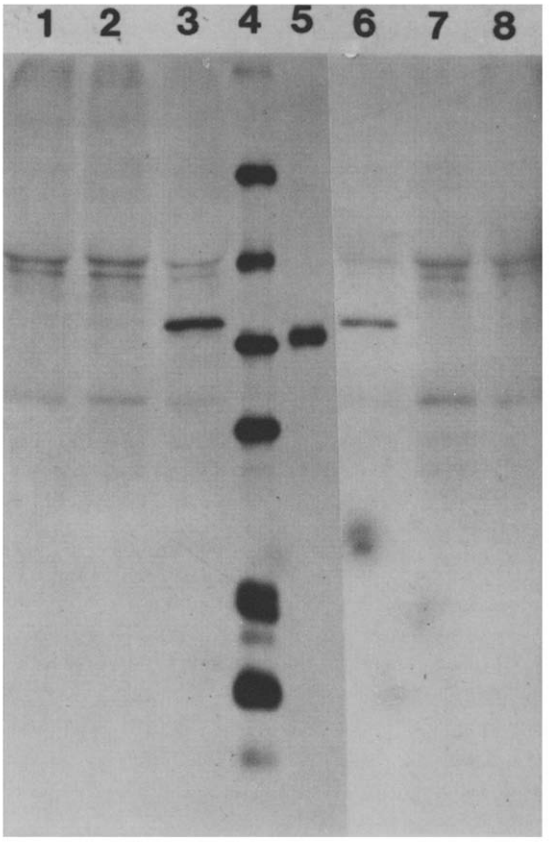

Fig.2. SDS–Polyacrylamide slab gel electrophoresis and fluorography of the immune precipitate of protein synthesized in a reticulocyte lysate in the presence of rat liver free polysomes or total RNA. Polysomes or total RNA at final concentrations in the lysate of 24 A_{260} units/ml and 40 A_{260} units/ml, respectively, were incubated with [^{35}S]methionine at 1 μCi/ml in a final volume of 500 μl for 60 min at room temperature. The lysate was denatured and 10^7 cpm or 5×10^6 cpm of protein synthesized in the presence of polysomes (lanes 1–3) or of total RNA (lanes 6–8), respectively, were incubated with 50 μl antiserum. The figure is the result from one gel but because of the different levels of radioactivity exposure for lanes (1–5) is different from the rest. Lanes: (1,8) pre-immune serum; (2,7) bovine heart malate dehydrogenase antiserum in the presence of 200 μg bovine heart malate dehydrogenase; (3,6) bovine heart malate dehydrogenase antiserum; (4) markers at 12, 17, 26, 34, 43 and 68×10^3 M_r; (5) rat liver malate dehydrogenase labelled with [^{14}C]formaldehyde.

dehydrogenase. Antibody against denatured bovine malate dehydrogenase precipitated <0.1% of the radioactivity incorporated into protein. On analysis on an SDS–polyacrylamide gel a major band some 1500–2000 M_r larger than the mature form of the enzyme was observed using both sources of mRNA with immune (fig.2, lanes 3,6 cf. lane 5) but not with pre-immune serum (lanes 1,8). Some relatively minor bands were also present using both specific and pre-immune serum. Excess non-radioactive denatured bovine heart enzyme prevented recovery of the major band only (lanes 2,7). The same pattern of results was obtained with the antibody against denatured enzyme from rat liver. The results are consistent with the major band being a precursor of malate dehydrogenase. Furthermore, quantitation of the gel showed that 10-times more of the putative precursor could be precipitated by antibody from lysate primed with free polysomes (lane 3) than with total RNA (lane 6). This result is compatible with free but not bound polysomes being the site of synthesis as reported for other matrix enzymes of rat liver mitochondria [23,24].

Mitochondrial malate dehydrogenase appears to be synthesized in a precursor form some 1500–2000 M_r larger than the mature form. Three other rat liver mitochondrial matrix enzymes are known to be made in precursor form: carbamoyl phosphate synthetase, ornithine transcarbamylase and δ-aminolevulinate synthase being 5000–5500 [23–25], 4000 [26,27] and 6000 [28] M_r larger, respectively. The precursor of mitochondrial chicken heart mitochondrial aspartate aminotransferase is 3000 M_r larger [13]. This large variation in the size of the precursor is seen with yeast mitochondrial matrix proteins; for example, the precursors of subunits of F_1 ATPase vary from 2000–6000 M_r larger than the mature forms [29].

The role of the precursor in import of the protein into mitochondria is not known [3–6,30,31]. It has been reported that malate dehydrogenase, a dimeric enzyme [12], can be taken up by isolated rat liver mitochondria either into the matrix [11] or binding to the inner membrane [10] and it can also change its intramitochondrial location [6]. This suggests that the final form of the enzyme or its monomeric subunit has the information for its final localization. These results are not necessarily contradicted by the presence of precursor forms, but may provide insight into the possible role of the precursor for importation of enzymes of the mitochondrial matrix.

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