STUDIES ON THE BIOSYNTHESIS OF MALATE DEHYDROGENASE ISOZYMES IN RABBIT LIVER

Isolation of the *in vivo* labelled isozymes by specific antibodies+

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1. Introduction

The present knowledge on the biosynthesis of mitochondrial proteins indicates that only some of the structural proteins are synthesized within the organelle. Isolated rat liver mitochondria incorporate negligible amounts of ^{14}C-labelled amino acids into the soluble proteins as, e.g., cytochrome *c* and malate dehydrogenase [1]. Gonzáles-Cadavid et al. [2] and Kadenbach et al. [3] showed by *in vivo* pulse-labelling experiments that microsomal cytochrome *c* becomes labelled earlier and to a higher extent than mitochondrial cytochrome *c*. Recently Bingham et al. [4] were able to demonstrate that mitochondrial malate dehydrogenase is one of the proteins synthesized *in vitro* by the microsomal fraction from rat liver.

Using immunoprecipitation techniques we have isolated both isozymes of malate dehydrogenase (mitochondrial = m-MDH, cytosolic = s-MDH) from the soluble protein fractions of microsomes, mitochondria and cytosol. The data reported in the present paper indicate that both isozymes are synthesized at the cytosolic ribosomes and that the mitochondrial isozyme is subsequently transferred into the mitochondria.

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2. Methods

Malate dehydrogenase isozymes were isolated from rabbit heart by ammonium sulfate fractionation, chromatography on DEAE-Sephadex A50 and CM-Sephadex G50 followed by isoelectric focusing. The specific activities of the two enzymes were 414 U/mg for s-MDH and 584 U/mg for m-MDH. The two isozymes proved to be electrophoretically homogeneous. Antisera against these purified isozymes were obtained from sheep. The animals were injected intramuscularly five times with a total of 25 mg protein in complete Freund's adjuvant (Difco). Gamma-globulin fractions were prepared from the sera by sodium sulfate fractionation according to Kekwick [5]. Ouchterlony's double diffusion patterns, equivalence point determinations, and quantitative precipitin analyses were performed as outlined by Kabat and Mayer [6].

Rabbits of the strain 'weisse Russen' weighing 2500–2700 g were injected intravenously with 250 μCi uniformly labelled [^{14}C]leucine (specific activity 342 mCi/mmole) in neutralized isotonic solution. At different time intervals the animals were killed and the microsomal fraction from 40 g of liver was prepared according to Bingham et al. [4]. The isolated microsomes were then extracted with 1% Triton X-100 in 0.15 M NaCl [4]. s-MDH and m-MDH were specifically precipitated in this extract by the respective antisera. Precipitated enzyme activity was determined by measuring MDH activity before and after immunoprecipitation. For determining radioactivity incorporated

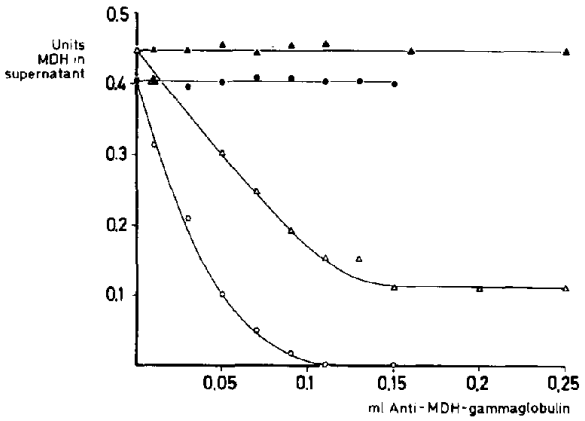

Fig. 1. Quantitative precipitin reactions of purified s-MDH and m-MDH from rabbit heart with Anti-s-MDH and Anti-m-MDH-gamma-globulin fractions from sheep sera. Following completion of precipitation as described under 'Methods' the supernatant fluids were assayed for enzyme activity. With a control serum (results not shown) no significant change in enzyme activity was observed in the supernatant: (○—○—○) m-MDH with Anti-m-MDH; (●—●—●) m-MDH with Anti-s-MDH; (△—△—△) s-MDH with Anti-s-MDH; (▲—▲—▲) s-MDH with Anti-m-MDH.

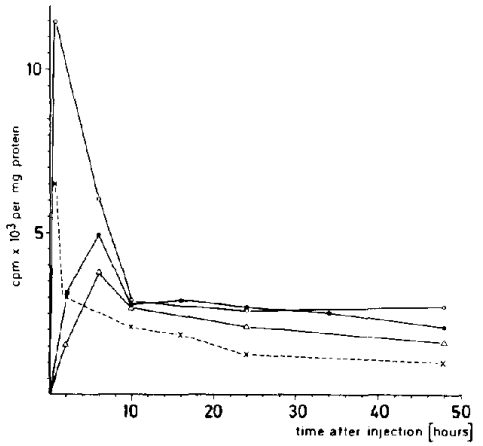

Fig. 2. Kinetics of incorporation of [^{14}C]leucine into liver proteins: (○—○—○) microsomal soluble protein (Triton X-100); (●—●—●) total soluble liver protein (phosphate buffer); (△—△—△) total mitochondrial protein; (X ---X ---X) TCA soluble counts in 100 000 *g* supernatant, as referred to soluble cell protein (cpm X 10^{-1}/mg soluble liver protein).

into total soluble protein, a liver sample was homogenized 4 X 30 sec in a Sorvall Omnimixer and subsequently sonicated 4 X 30 sec with a Branson sonifier in a 9-fold volume of 0.1 M phosphate buffer, pH 7.4. The 100 000 *g* supernatant of this homogenate was also used for immunoprecipitation of the two malate dehydrogenase isozymes. Immunoprecipitation was performed by incubating three aliquots of the samples with the antibodies for 30 min at 22°C. Prolonged incubation did not lead to a further decrease of enzyme activity in the supernatant as was shown by equivalence point determinations. Corrections for nonspecific precipitation were done according to Schimke et al. [7]. The immunoprecipitates obtained were washed three times with 0.15 M NaCl containing 0.5 μmoles leucine/ml. After digestion of the precipitated proteins in 0.5 ml Protosol (NEN Chemicals) radioactivity was determined in a Packard liquid scintillation counter. The toluene scintillation mixture contained 4 g 2,5-diphenyloxazole (PPO) and 0.2 g 2,2′-*p*-phenylen-bis-(5-phenyl-oxazole) (POPOP) per liter.

3. Results and discussion

The antibodies against the two isozymes of malate dehydrogenase used in the immunoprecipitation technique proved to be highly specific. Ouchterlony's double diffusion tests and immunoelectrophoresis revealed only single sharp precipitation lines and no cross-reaction. Identical results were obtained by immunotitration of the enzymes with their corresponding antibodies (fig. 1). The antibody against the mitochondrial isozyme did not precipitate or inhibit the extramitochondrial isozyme, and vice versa. As is evident from fig. 1, the extramitochondrial isozyme was not precipitated completely by the corresponding antibody. The amount of enzyme activity remaining in the supernatant was always 30% of the total activity and could not be diminished by further addition of antibody or prolonged incubation.

At different times after injection of [^{14}C]leucine the isozymes of malate dehydrogenase were precipitated by the specific antibodies from a Triton X-100 extract of the microsomal preparation (i.e. newly synthesized proteins [4], and from the 100 000 g supernatant of the liver homogenate (which contained both

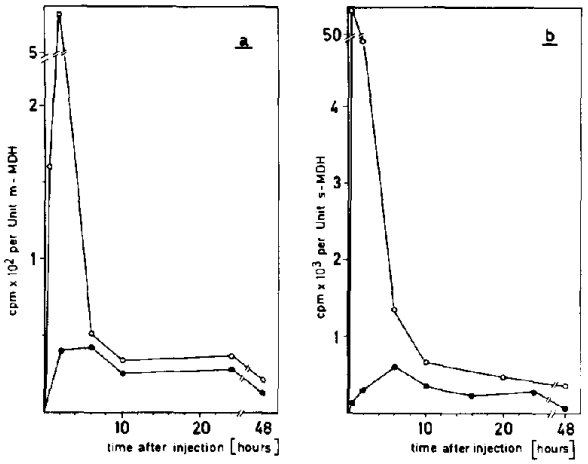

Fig. 3. Kinetics of incorporation of [^{14}C]leucine into the isozymes of malate dehydrogenase: a) mitoc ondrial (•—•—•) and microsomal (o—o—o) m-MDH; b) cytosolic (•—•—•) and microsomal (o—o—o) s-MDH.

extra- and intramitochondrial isozymes). As calculated from enzyme activity determinations before and after immunoprecipitation with the two antisera, the microsomal fraction contains s-MDH as well as m-MDH. The two isozymes are present in the same proportions as in the 100 000 *g* supernatant, i.e. 59 ± 7% s-MDH and 41 ± 7% m-MDH [8].

Incorporation of radioactivity into Triton X-100 extracted microsomal proteins, total mitochondrial protein and total soluble protein of the liver homogenate is given in fig. 2. Fig. 3 shows that s-MDH and m-MDH are labelled earlier and more extensively in the microsomal fraction than in the 100 000 *g* supernatant. Maximal specific radioactivity of s-MDH and m-MDH in the microsomal fraction is observed about 30 min and 120 min respectively after administration of the label. The specific radioactivity of the two isozymes decreases rapidly in the microsomal fraction. After 6 hr it reaches approximately 10% (m-MDH) respectively 2.5% (s-MDH) of the initial maximal values. In contrast, specific radioactivity of both isozymes increases more slowly within the 100 000 *g* supernatant and reaches maximal values after about 6 hr.

These findings suggest that both isozymes are synthesized at the cytosolic ribosomes and that the mitochondrial isozyme is subsequently transferred into the mitochondria. Bingham et al. [4] have already reported synthesis of the mitochondrial MDH *in vitro* by isolated microsomes. The present data indicate that this newly synthesized enzyme may be considered as the precursor of mitochondrial malate dehydrogenase. A similar precursor–product relationship has been reported by Gonzáles-Cadavid et al. [2] and Kadenbach et al. [3] for cytochrome *c*.

As may be seen from fig. 3 the specific radioactivity of s-MDH exceeds that of m-MDH by a factor of approximately 100 in the microsomal fraction and about 10-fold in the 100 000 *g* supernatant. This may be due to various reasons, e.g. different leucine content of the two isozymes, variations of individual pool sizes. In our opinion, different rates of synthesis may at least explain partly the difference in labelling of the two isozymes. Synthesis rate constants calculated according to [9] from degradation rate constants are 6.43 μg per g of liver × hr^{-1} for s-MDH and 2.09 μg per g of liver × hr^{-1} for s-MDH in rabbit liver [10]. Taking into account the rapid decrease of free [^{14}C]leucine [11] (c.f. fig. 2) different rate constants of synthesis should result in a significant difference of labelling of the two isozymes.

Acknowledgements

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