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Induction of a Peroxisomal Malate Dehydrogenase Isoform in Liver of Starved Rats

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Abstract—The influence of starvation on malate dehydrogenase (MDH) in rat liver was investigated. Native electrophoresis revealed two MDH isoforms in non-starved rats and three isoenzymes in starved rats. After sucrose density gradient centrifugation of cell organelles from liver, MDH activity was detected in the mitochondrial and cytosolic fractions from non-starved rats. However, additional activity was found in the peroxisomal fraction from starved rats. The latter was identified as the electrophoretically new isoform in starved animals. The three isoforms of malate dehydrogenase from hepatocytes were separated and partially purified by chromatography on DEAE-Toyopearl. Several kinetic and regulatory properties of the three isoforms were rather similar. It is suggested that the newly expressed isoform of MDH operates in the glyoxylate cycle of liver peroxisomes of food-starved animals.

Key words: malate dehydrogenase, starvation, glyoxylate cycle, peroxisomes, subcellular location

Malate dehydrogenase (MDH) is an enzyme with several metabolic functions in cells [1]. First, the enzyme is involved in the tricarboxylic acids cycle, which is responsible for the normal operation of cellular respiration. NADH produced during malate oxidation is further processed to ATP in the mitochondrial electron transport chain. Furthermore, malate dehydrogenase ensures energetic intercoupling of cell organelles. In particular, a malate/aspartate counter-transport carries reducing equivalents between cytosol, microbodies, and mitochondria in animals, fungi, and plants. The ability of MDH to form stable protein complexes with aspartate aminotransferase makes possible the utilization of C_4 compounds in biosynthetic processes, including the synthesis of amino acids [2].

Malate dehydrogenase, in cooperation with PEP carboxykinase, can also participate in gluconeogenesis and, as an enzyme of the glyoxylate cycle, in the mobilization of stored fatty acids towards carbohydrates in plants [3]. The glyoxylate cycle was first described in 1957 for microorganisms grown on C_2 substrates like acetate [4]. Its basic function is the condensation of two molecules of acetyl-coenzyme A into the four-carbon acid succinate. The source of acetyl-coenzyme A can be either acetate in microorganisms or acetyl-coenzyme A from β-oxidation of fatty acids in eukaryotes [5]. In addition to bacteria, the operation of the glyoxylate cycle was also established in plants and fungi [6]. The presence of the glyoxylate cycle in animal tissues was often a matter of discussion. In some worms (nematodes and trematodes), the glyoxylate cycle is involved in the transformation of lipids to carbohydrates during the larval stage [7, 8]. The mitochondrial localization of key enzymes of the glyoxylate cycle is different from other eukaryotic organisms [9]. There are some data about both the presence and the absence of this pathway in amphibia, birds, and mammals. In this context, the activity of a key enzyme of the cycle, isocitrate lyase (ICL), was found in histological analysis in renal blades of the toad *Bufo marinus* and in liver of Guinea pigs and chickens [10-12]. However, succinate, which is generally produced in the isocitrate lyase reaction, could not be demonstrated as a product of citrate metabolism in chicken liver when analyzed by HPLC [13]. The presence of the glyoxylate cycle in brown adipose of bears is also discussed [14, 15]. Malate synthase (MS), another

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key enzyme of the glyoxylate cycle, was seen immunochemically in human liver [16].

Experiments by Lebkova have shown that starvation of rats causes transient glycogen formation [17]. In addition, we could demonstrate the concomitant induction of the glyoxylate cycle in liver of starved rats. There were significant activities of malate synthase and isocitrate lyase in hepatocytes after 3-4 days of starvation. Under the same conditions, malate dehydrogenase and citrate synthase increase their activity [18]. It was also demonstrated that the activities of ICL and MS are located in the peroxisomal fraction [19]. Another reason for the induction of the glyoxylate cycle in mammals is diabetes. The injection of alloxan, which simulates sugar diabetes, resulted, like food deprivation, in induction of glyoxylate cycle enzymes [20].

The induction of one enzyme of the glyoxylate cycle in rat liver upon starvation raises the question of the concomitant induction of other enzymes of the cycle, possibly in peroxisomes. In this work we wanted to focus on the activity of malate dehydrogenase. So far, isoenzymes of MDH in rat liver have been described for mitochondria and cytosol [21, 22]. We anticipated an additional isoenzyme with different kinetic properties and possibly independent regulation by cell metabolites. Therefore, the present study focuses on the activity of malate dehydrogenase in different organelles of hepatocytes of animals during starvation, the ratio of different malate dehydrogenase isoforms in non-fasting and fasting animals, and the separation of MDH isoforms.

MATERIALS AND METHODS

White laboratory rats (*Rattus rattus* L.) of 180 to 200 g weight were grown on a standard diet and then starved with free access to water for about seven days. Enzyme activity was determined photometrically.

The activity of a malate dehydrogenase was measured at 340 nm by NADH oxidation. The assay medium contained 50 mM Tris-HCl buffer (pH 8.3), 1 mM oxaloacetate, 0.1 mM NADH, and 1 mM $MgCl_2$. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzes the transformation of 1 μmol of NAD^+ in 1 min at 25°C and optimal pH.

The subcellular location of glyoxylate cycle enzymes in rat liver was determined by separating cell organelles by centrifugation in a sucrose density gradient. One gram of rat liver was homogenized in 10 ml of medium containing 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA. The crude extract was centrifuged for 5 min at 2,000*g*. Five milliliters of supernatant were loaded on the top of a linear 50-ml gradient ranging from 20 to 55% sucrose in 50 mM Mops-NaOH, pH 7.8, 1 mM $MgCl_2$, and 0.5 mM DTT. Gradients were centrifuged for 3 h at 100,000*g*. The cross contamination of organelles was determined by marker enzymes, catalase (EC 1.11.1.6) for peroxisomes, fumarate hydratase (FH, EC 4.2.1.2) and succinate dehydrogenase (SDH, EC 1.3.99.1) for mitochondria, and lactate dehydrogenase (LDH, EC 1.1.1.27) for the cytosol. SDH activity was determined at 600 nm by the reduction of the artificial electron acceptor dichlorophenolindophenol, fumarase at 240 nm by the formation or consumption of fumarate, catalase at 230 nm by disappearance of hydrogen peroxide, and LDH at 340 nm by NADH oxidation [3, 23].

Electrophoresis was conducted according to Davis [24] in 15% polyacrylamide gels. The activity of malate dehydrogenase was detected in medium containing 50 mM Tris-HCl, pH 8.3, 50 mM malate, 10 mM NAD^+, phenazine methosulfate (0.5 mg/ml), and 0.5 mg/ml Nitrotetrazolium Blue. The number of isoforms and their electrophoretic motilities were judged by the appearance of reduced tetrazolium precipitates at the bands of MDH isoforms.

To separate MDH isoforms, the enzyme was purified according to the following scheme: 1) homogenization of 1 g of liver in 10 ml of homogenization medium consisting of 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA, and low-speed centrifugation (1,000*g*) for 5 min to remove unbroken tissues and cells; 2) gel filtration of the homogenate on Sephadex G-25 (Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$ for retention of low-molecular-weight molecules; 3) ion-exchange chromatography on DEAE-Toyopearl (Toyo-Soda, Japan). The protein fraction was placed on a column (20 × 1 cm) equilibrated with 10 mM Tris-HCl, pH 7.5. The column was eluted with a linear KCl gradient from 0 to 200 mM in the same buffer.

All experiments were repeated at least three times. Data are given as mean values ± standard deviation. The number of separate experiments is given in parentheses.

RESULTS

Starvation of laboratory rats with free access to water resulted into a 1.6-fold increase in total MDH activity in liver (from 1.25 U/g to 2.06 U/g wet weight) and a 1.5-fold increase in specific MDH activity (to 0.12 U/mg protein) by the fifth day of starvation. By the seventh day of starvation the activity of the enzyme decreased to one half, but the experiment had to be terminated because of attrition of the animals. In liver of non-fasting rats (data not shown), the MDH activity remained at a constant level of about 1.25 U/g wet weight (Table 1).

To determine the number of MDH isoenzymes in starved and in non-starved rats, we performed electrophoresis of proteins of crude extracts in 15% polyacrylamide gels. Liver of control animals had only two MDH isoforms with R_f 0.08 and 0.14, respectively. However,

Table 1. Induction of malate dehydrogenase in liver during starvation ($n = 6$)

Days of starvation	Total activity, U/g wet weight	Specific activity, U/mg protein
0	1.25 ± 0.05	0.08 ± 0.01
3	1.63 ± 0.09	0.09 ± 0.01
5	2.06 ± 0.08	0.12 ± 0.02
7	1.52 ± 0.10	0.10 ± 0.01

liver from fasting animals showed a new isoform with electrophoretic mobility 0.16 (Fig. 1). To study the distribution of malate dehydrogenase activity in various subcellular compartments, we separated cell organelles in a sucrose density gradient. MDH activity in a gradient with liver of control animals is predominantly associated with the cytosolic and mitochondrial fractions (Fig. 2a). Of 100% activity loaded onto the top of the sucrose gradient, 55% of the activity was recovered in the cytosolic fraction and 20% in the mitochondrial fraction. Less than 10% of the total activity was detected in the peroxisomal fraction. Starvation caused a substantial reallocation of malate dehydrogenase activity to the peroxisomal fraction. Thirty five percent of the MDH activity was found in the fraction containing 43 to 48% sucrose with particulate catalase activity (Fig. 2b).

To study regulation of metabolic processes related to malate dehydrogenase forms with different localization, the chromatographic separation of MDH isoforms from hepatocytes of control and fasting rats was performed. Low-molecular-weight substances were preliminarily removed by gel filtration of a homogenate on Sephadex G-25. Then the enzyme preparation with specific activity of 0.12 U/mg protein (Table 2) was applied on the column with DEAE-Toyopearl. After elution of the protein with a linear KCl gradient, malate dehydrogenase activity was found in two peaks for control animals (Fig. 3). In liver of food-deprived rats a third peak with MDH activity was detected. The specific activities in the three peaks were 2.0, 1.41, and 1.36 U/mg protein for the different isoforms (Fig. 3), corresponding to enrichments of 16.6-, 11.7-, and 11.3-fold, respectively. Electrophoretic analysis showed that isoform MDH1 eluting at 25-40 mM KCl corresponds to the isoform of R_f 0.08, isoform MDH2 eluting at 55 to70 mM KCl corresponds to the isoform of

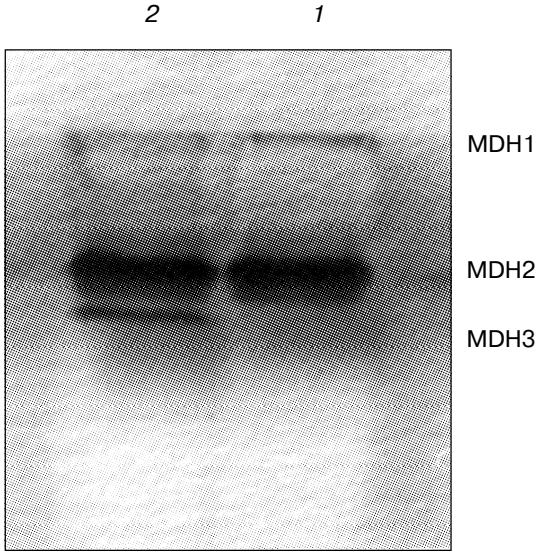

Fig. 1. Electrophoretic separation of malate dehydrogenase isoforms from rat liver in 15% native polyacrylamide gels: *1*) control animal; *2*) fasting animal.

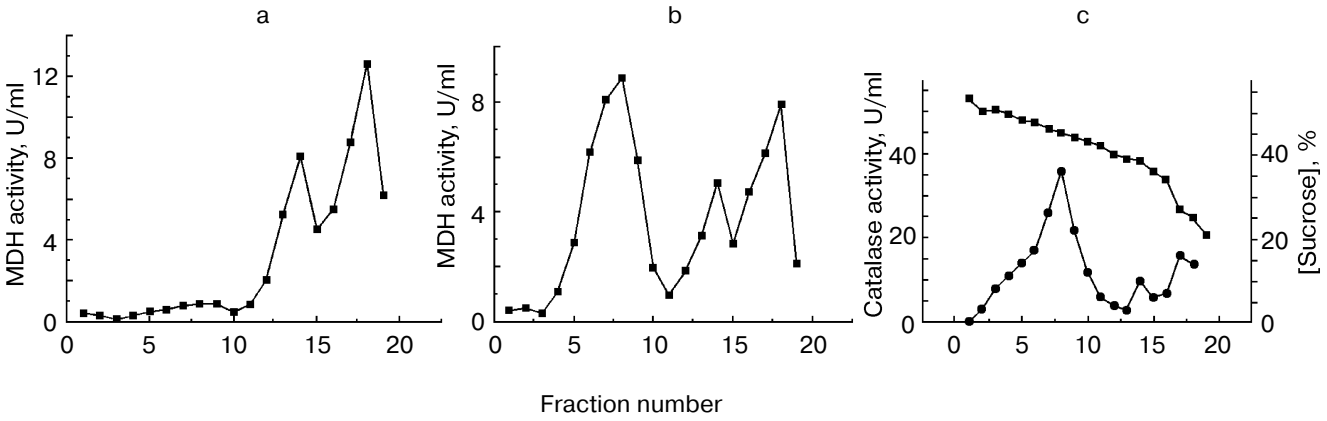

Fig. 2. Distributions of malate dehydrogenase activity in a sucrose density gradient: a) control animal; b) fasting animal; c) concentration of sucrose and distribution of catalase activity for liver of starved rat.

Table 2. Isolation of malate dehydrogenase (MDH) isoforms from liver of fasting rats

Stage	Total protein, mg	Activity		Yield, %	Degree of purification
		total, U/ml	specific, U/mg protein		
Homogenate	99 ± 3	12.5 ± 0.3	0.120 ± 0.002	100	1
Gel filtration on Sephadex G-25	81 ± 2	11.4 ± 0.2	0.140 ± 0.004	91.2	1.2
Ion-exchange chromatography on DEAE-Toyopearl					
MDH1 (25-40 mM KCl)	0.37 ± 0.01	1.75 ± 0.04	4.7 ± 0.1	14.0	39.4
MDH2 (55-70 mM KCl)	0.46 ± 0.01	1.65 ± 0.03	3.6 ± 0.1	13.2	30.0
MDH3 (130-140 mM KCl)	0.25 ± 0.01	1.34 ± 0.03	5.4 ± 0.1	10.7	44.6

R_f 0.14, and MDH3 induced by starvation and eluting at 130-140 mM KCl corresponds to the isoform of R_f 0.16. Analysis of the distribution of MDH activities in organelles of hepatocytes and the ratio among the different MDH forms indicate that MDH3 is located in peroxisomes, MDH1 in mitochondria, and MDH2 in cytosol.

When studying the catalytic properties of the three MDH forms, the cytosolic, mitochondrial, and peroxisomal isoforms each showed positive cooperativity with oxaloacetate. The dependence of reaction rate on substrate concentration for all three isoforms was of sigmoid (Fig. 4a).

For quantitative assessment of the cooperativity, we used an incremental computational method for determination of the Hill factor. In Fig. 4b the dependences of $\log[v/(V_{max} - v)]$ against $\log[S]_0$ are shown. When the Hill factors for the three MDH isoforms were calculated, the values were 2.4 ± 0.1 (n = 3) for mitochondrial, 1.51 ± 0.08 (n = 3) for cytosolic, and 1.57 ± 0.11 (n = 3) for peroxisomal isoform.

All of the MDH isoforms showed classical Michaelis–Menten kinetics for NADH. The K_m values for NADH were 90 μM (mitochondrial MDH1), 58 μM (cytosolic MDH2), and 50 μM (peroxisomal MDH3). Only NADH (not NADPH) serves as a cofactor for all of the isoforms. The rate of the reverse reaction with NADPH instead of NADH was less than 1%, demonstrating that all of the isoforms are highly specific for NADH.

The pH dependence showed typical optimum curves (not shown) with an optimum at pH 8.5 for the mitochondrial enzyme and at pH 7.8 for both the cytosolic and peroxisomal isoforms.

Divalent metal ions (Mg^{2+} and Mn^{2+}) increased the activity of all three MDH isoforms. At 3 mM concentration, Mg^{2+} increased the cytosolic and mitochondrial MDH activities by 40 and 50%, respectively, and the peroxisomal MDH activity by 15%. Ion concentrations

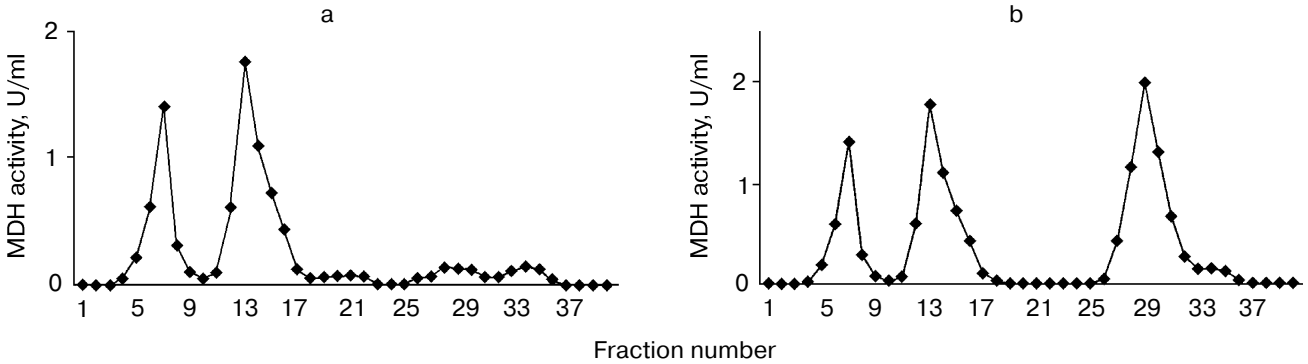

Fig. 3. Profile of MDH activity after elution by a linear KCl gradient (0-200 mM) from DEAE-Toyopearl for liver of control (a) and starved (b) rats.

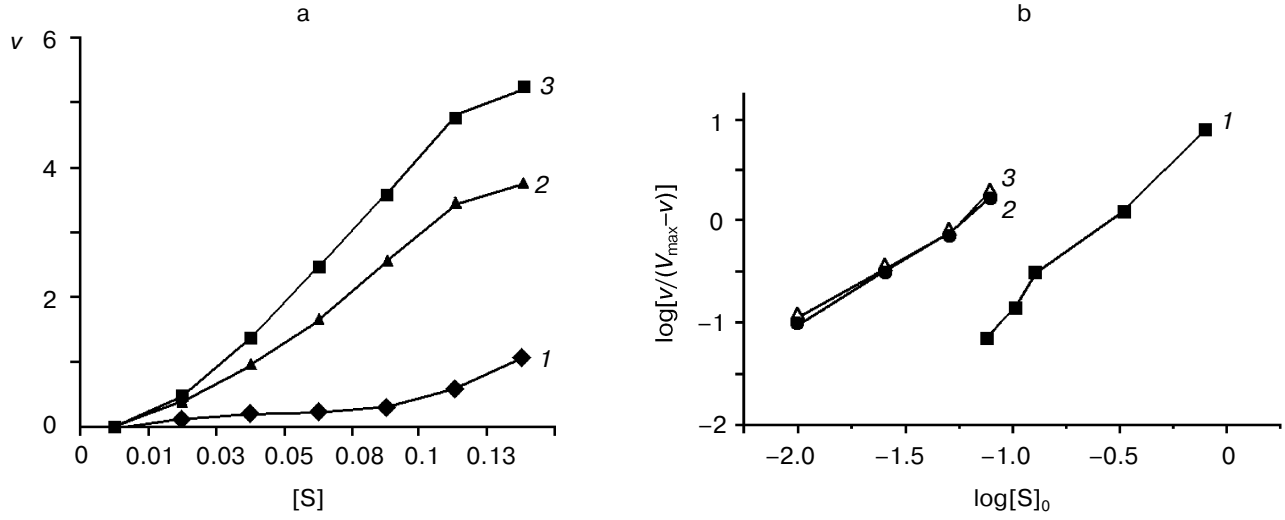

Fig. 4. Dependence of MDH activity on oxaloacetate concentration: a) substrate (oxalacetate) dependence of MDH activity; b) the dependence $\log[v/(V_{max} - v)]$ versus $\log[S]_0$ for incremental determination of the Hill factor; *1*) mitochondrial MDH1; *2*) cytosolic MDH2; *3*) peroxisomal MDH3.

higher than 3.5 mM Mg^{2+} inhibited the enzyme. Three millimolar Mn^{2+} enhanced the peroxisomal MDH activity by 20%, the mitochondrial enzyme by 30%, and did not influence the cytosolic enzyme.

Among other compounds, succinate and citrate had inhibiting effects on all of the isoforms. The inhibition constants as determined by the Dixon method are summarized in Table 3. Nucleotides had a dual influence on the activity of the enzyme. Thus, 300 μM ADP activated mitochondrial and cytosolic MDH by 10-15%, but further increase of its concentration caused inhibition of all of the malate dehydrogenase forms. ATP at concentrations up to 1 mM did not greatly influence the activity of either isoform.

Table 3. Influence of cellular metabolites on the activity of the mitochondrial, cytosolic, and peroxisomal MDH isoforms from hepatocytes of starved rats ($n = 3$)

Effector	Inhibition constant (K_i), mM		
	mitochondrial isoform	cytosolic isoform	peroxisomal isoform
Succinate	4.00 ± 0.08	3.50 ± 0.10	3.80 ± 0.07
Citrate	3.20 ± 0.06	2.60 ± 0.04	3.00 ± 0.06
ADP	4.90 ± 0.10	6.70 ± 0.16	7.30 ± 0.20
AMP	5.40 ± 0.09	7.50 ± 0.22	7.80 ± 0.15

DISCUSSION

Adaptation to starvation has a strong influence on the general metabolism of rats. Here we have demonstrated that the activity of MDH in rat liver participates in these adaptive reactions through the expression of a new MDH isoform in the peroxisomes of hepatocytes. The increase in the activity of the enzyme in fasting animals is apparently related to the mobilization of reserve nutrients to glycogen. After the beginning of starvation, glycogen is formed in rat liver, the most relevant resource for the glycogen formation being lipid reserves [25]. The β-oxidation pathway of fatty acid degradation has been shown to operate both in mitochondria and in peroxisomes of hepatocytes [5]. In this context, it should be pointed out that the maximum induction of malate dehydrogenase activity was correlated with the induction of a new isoform in peroxisomes. In a previous investigation, it was established that starvation induces key enzymes of the glyoxylate cycle, i.e., malate synthase and isocitrate lyase, in peroxisomes of rat liver [19]. The remaining enzymes of the glyoxylate cycle, citrate synthase, aconitate hydratase, and malate dehydrogenase are the same activities as in the TCA cycle. The occurrence of additional isoenzymes and the clarification of their localization in higher animals are not trivial problems because the localization of the glyoxylate cycle was shown to be exclusively mitochondrial in Ascarides [9], while plants and fungi develop specific isoenzymes in glyoxysomes (as shown for MDH [26] and citrate synthase [27]) and in the cytosol (as shown for aconitase [28]).

The induction of a new MDH isoform in addition to the constitutively expressed mitochondrial and cytosolic isoforms was shown by ion-exchange chromatography on DEAE-Toyopearl and by electrophoresis and activity staining in polyacrylamide gels under native conditions. The catalytic properties and the inhibitory effects of metabolites are all very similar among the three isoforms. Possibly, induction of the glyoxylate cycle upon starvation leads to intensive production of succinate, and subsequently oxaloacetate, which can then enter into gluconeogenesis or biosynthesis of amino acids.

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