AN NAD- AND NADP-DEPENDENT MALIC ENZYME WITH REGULATORY PROPERTIES IN RAT LIVER AND ADRENAL CORTEX MITOCHONDRIAL FRACTIONS

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SUMMARY

The NAD- and NADP-dependent malic enzymes from rat liver and adrenal mitochondrial fractions were separated and partially purified by gel filtration on Sepharose 6B. Two activity peaks were observed. The first contained a malic enzyme capable of reducing either NAD or NADP. This enzyme showed sigmoid kinetics in plots of activity versus the malate concentration. Succinate was an allosteric activator and ATP was a competitive inhibitor of malate. The second peak showed hyperbolic kinetics in plots of activity versus the malate concentration and was unaffected by either succinate or ATP. The relative activities of the two malic enzymes were quite constant in the adrenal mitochondrial fractions. In the liver mitochondrial fractions, the activity of the first peak varied and was sometimes absent while the activity of the second peak was quite constant. The kinetic properties of the first malic enzyme implicate it as an important regulator of malate oxidation.

Multiple molecular forms of malic enzyme (NADP-malic dehydrogenase, decarboxylating, E.C.1.1.1.40) are present in heart, kidney, liver, adrenal cortex and medulla, adipose tissue and brain of several mammals and birds (1,2,3). Current data suggest that the isoenzymes occur as intra- and extramitochondrial forms (1-5). In beef brain, adrenal cortex and heart, the isoenzymes differ kinetically (2-5). The mitochondrial malic enzyme has properties of a regulatory enzyme: plots of the rates of NADPH formation versus the malate concentrations yield sigmoid kinetics (5). Succinate is an allosteric activator. The extramitochondrial isoenzyme, on the other hand, shows Michaelis-Menten kinetics in plots of velocity versus malate and is unaffected by succinate (5).

NAD-dependent malic enzyme has also been observed in mitochondrial fractions. The large granule fraction from Ascaris muscle (6), rabbit and cod ovary and testis (7,8) and beef heart (9) appear to contain an NAD-

linked malic enzyme. The available kinetic data, however, did not suggest a regulatory function for this enzyme (9). In this report we show that mitochondrial fractions from rat liver and adrenal cortex contain two malic enzyme activities. One enzyme is active with both NAD and NADP and the other only with NADP. Moreover, the kinetic properties of the former indicate that the enzyme is an important regulator of malate oxidation.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-200 gm) were used in these experiments. Mitochondrial fractions were prepared by differential centrifugation (10) of homogenates from pooled whole adrenal glands or from the cortex fragments remaining after removal of the medullary portion. Rat liver mitochondrial fractions were prepared by the same method except that the pellet was washed four times to decrease the microsomal contamination. The particles were suspended in 30 mM Tris-HCl, 0.1 mM dithiothreitol (DTT), and 1 mM EDTA, pH 7.4, and sonicated (10). The clear supernatant fluids, called here the mitochondrial soluble fractions, were obtained following centrifugation at 105,000 x g for one hour. Gel filtration of the mitochondrial soluble fraction was performed at 4° on Sepharose 6B columns (2.5 x 90 cm) equilibrated with the above buffer. Malic enzyme activity was assayed at 340 nm in 1 ml cuvettes using a Gilford Model 2000 spectrophotometer. The cuvettes contained 50 mM Tris-HCl (pH 7.4), 0.1 mM DTT, 5 or 10 mM Mn ion, 0.2 mM NAD or NADP, and an appropriate amount of (-) malate, and mitochondrial soluble fraction or eluent from the column. Malic dehydrogenase (MDH) activity was measured by NADH (0.1 mM) disappearance following the addition of 2 mM oxaloacetate to cuvettes containing 50 mM Tris-HCl (pH 7.4), 0.1 mM DTT and sample. Oxaloacetate decarboxylase was determined by measuring the rate of formation of pyruvate from oxaloacetate. Adenylate kinase was determined by measuring the rate of ADP formation from ATP and AMP under the malic enzyme assay conditions. Pyruvate and ADP were measured spectrophotometrically by enzymatic methods (11). All assays were done at room temperature.

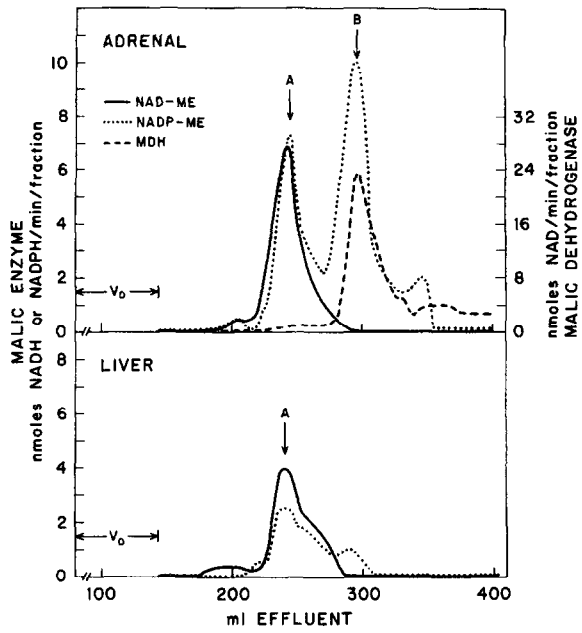

Figure 1. Separation by Gel Filtration of the Malic Enzyme Activities of Rat Adrenal and Liver Mitochondrial Soluble Fractions. Four ml portions of adrenal or liver mitochondrial soluble fractions, derived from 21.3 and 18.9 mg total mitochondrial protein, respectively, were run in the ascending direction on a 2.5 x 90 cm Sepharose 6B column equilibrated with the buffer described in the text. The flow rate was 10 ml/hour. Ten ml fractions were collected through the first 15 hours and 2.5 ml fractions thereafter. The samples were collected into tubes containing 0.5 µmole dithiothreitol. The void volume was 143 ml. Enzymatic assays were performed as described in the text. Sonication, centrifugation and gel filtration gave about a 10 fold purification: The activity of the peak tube for the peak A adrenal enzyme was 30.9 n moles NADPH formed/min/mg protein. The activity of the uncentrifuged sonicate was 3.2 n moles NADPH/min/mg mitochondrial protein.

RESULTS

Figure 1 shows the elution profiles that resulted when mitochondrial soluble fractions from rat adrenal or liver were subjected to gel filtration on Sepharose 6B. The malic enzyme activity of the fraction from the adrenal separated into two distinct peaks. The first peak (A) was composed of both NAD- and NADP-dependent activity. This peak was well separated from MDH, The larger, second peak (B) contained only NADP-linked activity. Identical profiles were observed for mitochondrial soluble fractions prepared from rat adrenal cortex fragments. The malic enzyme activity of the liver mitochondrial soluble fraction also separated into two activity peaks. Peak B

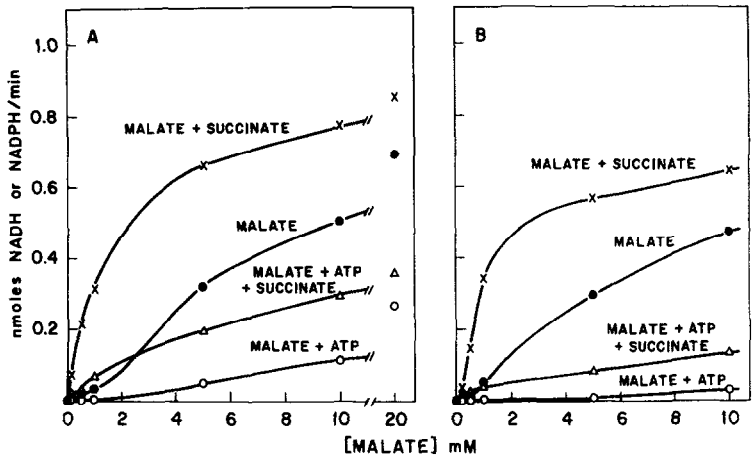

Figure 2. Kinetic Properties of the NAD- and NADP-Dependent Malic Enzyme (Peak A) from Rat Adrenal Mitochondrial Soluble Fractions. Fractions comprising the peak A malic enzymes obtained by gel filtration were pooled and the NAD- and NADP-linked activities measured as described in the text. A. The rate of NADH formation versus the malate concentration. B. The rate of NADPH formation versus the malate concentration. Succinate and ATP, when present, were 10 and 2 mM, respectively. Mn ion was 10 mM with experiments with ATP and 5 mM otherwise. Adenylate kinase activity was not detected in the pooled peak A enzyme preparations.

activity was less than in the adrenal but was clearly discernible in all preparations. Peak A NAD- and NADP-dependent malic enzyme activity, though present in this liver preparation, was found to vary among preparations and was sometimes absent (see below). Elution profiles similar to that shown for liver were observed with rat kidney cortex mitochondrial soluble fractions (not shown). As in liver, kidney peak A NAD- and NADP-dependent malic enzyme activity varied among preparations and was sometimes absent.

Some of the kinetic properties of the adrenal peak A malic enzyme are shown in Figure 2. Plots of activity versus malate concentration with NAD or NADP showed sigmoid kinetics. As described by Frenkel (3,5), succinate was an allosteric activator for the NADP-linked activity. Activation by succinate was also observed for the NAD-dependent activity. ATP decreased the rates of NADH or NADPH formation in either the presence or absence of succinate. In the absence of succinate, 2 mM ATP essentially abolished the malic enzyme activity. Similar concentrations of ADP also inhibited but less

effectively than ATP. AMP had essentially no effect. Combinations of NAD and NADP did not give additive rates (12, 13) except when the pyridine nucleotide levels were less than saturating and the rates were low. NAD was reduced more rapidly than NADP and NADP was a weak competitive inhibitor of NAD reduction. NAD, on the other hand, was a strong competitive inhibitor of NADP reduction (L.A. Sauer, in preparation).

Peak B NADP-dependent malic enzyme gave hyperbolic kinetics in plots of velocity versus the malate concentration and was only slightly affected by either ATP or succinate (not shown). The properties of the peak B enzyme from rat adrenal cortex will be described in a separate report (R. Mandella and L.A. Sauer, in preparation).

The apparent K_m for malate (succinate-stimulated peak A enzyme) was 2.2 and 2.0 mM for the NAD- and NADP-linked activities, respectively. Both peak A and B enzymes produced pyruvate at constant rates with a stoichiometric relationship of 1:1 between pyridine nucleotide reduction and pyruvate formation. Both enzymes were metal dependent. Mn ion gave slightly faster rates than did Mg ion. NADH formation by the peak A enzyme was abolished by the addition of lactic dehydrogenase.

Figure 3 shows that the NAD-dependent peak A activity from rat liver mitochondrial preparations also showed sigmoid kinetics with activation by succinate and inhibition by ATP. A double reciprocal plot of activity (in the presence of 10 mM succinate) versus the malate concentration shows that ATP is a competitive inhibitor of malate. The apparent K_m for malate was 1.1 mM and was increased to 3.1 and 7.3 mM malate by 0.5 and 1.0 mM ATP, respectively. ATP had similar competitive effects on the peak A enzymes from the adrenal (not shown, but see Figure 1).

Since peak A activity was inhibited by ATP and stimulated by succinate and peak B activity was relatively unaffected by these agents, a screening procedure was developed for the estimation of peak A and B malic enzymes in mitochondrial soluble fractions. The procedure used was as follows: The

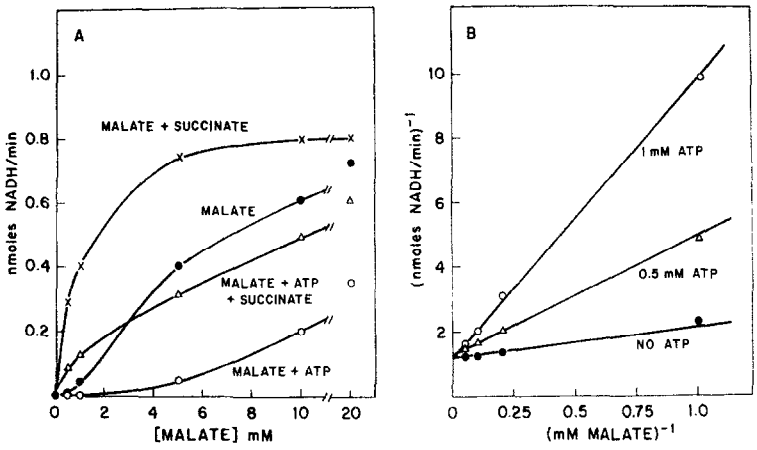

Figure 3. Kinetic Properties of the NAD-Dependent Malic Enzyme from Rat Liver Mitochondrial Soluble Fraction. The measurements were made using a pooled peak A NAD-linked malic enzyme preparation obtained by gel filtration of rat liver mitochondrial soluble fraction. The conditions of the gel filtration and enzymatic assays were as described under Figure 1 and in the text. A. The rate of NAD reduction plotted against the malate concentration. Succinate was 10 mM. ATP was 1 mM. B. Double reciprocal plot of velocity versus the malate concentration at 0, 0.5 and 1.0 mM ATP. The Mn ion/ATP ratio was 10. In the absence of ATP, the Mn ion concentration was 5 mM. Adenylate kinase activity was not detectable in the pooled fractions.

NADP-dependent enzymes were assayed at 4 mM malate in the presence of 1 mM ATP and then at 4 mM malate in the presence of 10 mM succinate. The activity with ATP and malate was considered to represent the peak B enzyme, and the activity with malate and succinate to represent both peak A and B enzymes. Peak A activity was obtained by difference. The results of several determinations are shown in Table I. The adrenal peak A and B NADP-dependent activities were quite constant among preparations. In the liver, the peak A activities were variable. This agreed with our gel filtration experiments in which liver peak A activity varied and was occasionally absent.

DISCUSSION

The above data indicate that rat adrenal and liver mitochondrial fractions contain an NAD- and NADP-dependent malic enzyme with allosteric properties. The similar kinetics of activation and inhibition, the competitive phenomena between the pyridine nucleotides and the identical elution positions occupied during gel filtration support the idea that the peak A

TABLE I

NADP-Dependent Malic Enzyme Activities in Rat Adrenal and Liver Mitochondrial Soluble Fractions[a]

	Malic Enzyme Activity (n moles NADPH/min/mg mitochondrial protein)		
	Total	Peak B	Peak A
Adrenal	11.4	8.2	3.2
	8.7	4.9	3.8
	8.8	4.9	3.9
	8.0	4.8	3.2
	10.0	6.3	3.7
Liver	2.6	1.6	1.0
	1.9	1.7	0.2
	3.6	1.6	2.0
	2.5	1.6	0.9
	3.3	1.9	1.4

[a]The method of preparation of the rat adrenal and liver mitochondrial fraction supernates and the malic enzyme assay procedure used is as described in the text. The activities are given in terms of total mitochondrial protein (includes matrix and membrane protein).

activities reside in the same macromolecule. It is attractive to consider that both peak A and B malic enzyme activities are mitochondrial. We have demonstrated that the rate of malate-dependent pyruvate formation by rat adrenal mitochondrial fractions is stimulated by oxidative phosphorylation and is inhibited by KCN, rotenone and inhibitors of the phosphate-malate exchange diffusion carriers (13). Also, the extramitochondrial cell sap does not contain an NAD-dependent malic enzyme (unpublished results). The regulatory kinetic properties of the peak A NAD-linked activity suggest that this enzyme functions primarily to form NADH for oxidative phosphorylation. The peak A and B malic enzymes may prove to be similar to the mitochondrial

isocitric acid dehydrogenases, i.e., an NAD-dependent allosteric form and a NADP-linked form with hyperbolic kinetics. However, an intramitochondrial location for the peak A and B malic enzymes, though likely, cannot be unequivocally described on the basis of these data. Experiments are in progress to determine the exact intracellular location of these interesting enzymes.

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REFERENCES

1. Brdiczka, D., and Pette, D., Eur. J. Biochem., 19, 546 (1971).
2. Simpson, E.R., and Estabrook, R.W., Arch. Biochem. Biophys. 129, 384 (1969).
3. Frenkel, R., J. Biol. Chem. 246, 3069 (1971).
4. Frenkel, R., Arch. Biochem. Biophys. 152, 136 (1972).
5. Frenkel, R., Biochem. Biophys. Res. Comm. 47, 931 (1972).
6. Papa, S., Cheah, K.S., Rasmussen, H.N., Lee, I.Y., and Chance, B., Eur. J. Biochem. 12, 540 (1970).
7. Mounib, M.S., and Eisan, J.S., Comp. Biochem. Physiol. 43B, 393 (1972).
8. Mounib, M.S., and Eisan, J.S., Endocrinology 91, 1375 (1972).
9. Davis, E.J., Lin, R.C., and Chao, D.L. in Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria (Mehlman, M.A., and Hanson, R.W., eds.) p. 211, Academic Press, New York (1972).
10. Sauer, L.A., and Mulrow, P.J., Arch. Biochem. Biophys. 134, 486 (1969).
11. Estabrook, R.W., and Maitra, P.K., Anal. Biochem. 3, 369 (1962).
12. Sauer, L.A., Endocrinology, Suppl. 88, A-90 (1971).
13. Sauer, L.A., and Park, R., Biochemistry, 1972 (in press).