MITOCHONDRIAL NAD-DEPENDENT MALIC ENZYME: A NEW REGULATORY ENZYME

Leonard A. SAUER

Departments of Internal Medicine and Molecular Biophysics and Biochemistry, Yale Medical School, New Haven, Conn. 06510, USA

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1. Introduction

Electrophoretic evidence for multimolecular forms of malic enzyme (EC 1.1.1.40, NADP-malic dehydrogenase, decarboxylating) was reported by Henderson in 1966 [1]. Subsequent purification and assay by spectrophotometric and other methods revealed that malic enzyme isoenzymes were present in cytosol and mitochondrial fractions derived from several different animal tissues [2–6]. Of particular importance were the recent findings of Frenkel [7] that the mitochondrial isoenzyme has allosteric properties, and that succinate was a positive modulator. Here, we demonstrate that calf adrenal cortex mitochondria contain an NAD-dependent malic enzyme (EC 1.1.1.39) with regulatory properties. Fumarate was a positive and ATP a negative modulator. The enzyme also reduced NADP but only at about one-quarter of the rate of NAD reduction. These kinetic properties suggest that this enzyme is an important regulator of the rate of malate oxidation.

2. Materials and methods

Calf adrenal glands were obtained from the local abattoir. The glands were removed and chilled in ice within 20 min of the death of the animal. The medullary portion was cut away and the homogenate was prepared [8] from the cortex scrapings. The mitochondrial fraction was obtained by differential centrifugation and the soluble mitochondrial contents were released by sonication [8]. A clear supernatant fluid was obtained following centrifugation of the sonicate for 1 hr at 105,000 *g*. The malic enzymes in the clear supernate were separated by gel filtration at 4°C on Sepharose 6B columns (2.5 X 90 cm) equilibrated with 30 mM Tris-HCl, 0.5 mM EDTA, and 0.1 mM dithiothreitol, all at pH 7.4. The flow rate was 10 ml/hr. Malic enzyme was assayed in 1 ml cuvettes at 340 nm in 50 mM Tris-HCl, 0.2 mM NAD or NADP, 5 or 10 mM Mn ion, 0.1 mM dithiothreitol, (–) malate at the concentration indicated, and an aliquot of either the clear sonicate supernate or effluent from the column. Fumarate, succinate and/or ATP were also present, as indicated. Measurements were performed on a Gilford Model 2000 spectrophotometer with a recorder full scale setting of 0.2 A unit. The rate of pyridine nucleotide reduction was constant and was dependent on (–) malate, Mn ion, and either NAD or NADP. The slow rates (in the presence of ATP or at low malate concentrations) were measured for 15 to 20 min. Velocities are given as $\Delta A_{340}/5$ min/ml enzyme solution or per total volume of fraction from the Sepharose column. Malic dehydrogenase was measured at similar instrument sensitivity settings by following NADH oxidation at 340 nm in 50 mM Tris-HCl (pH 7.4), 2 mM oxaloacetate, 0.1 mM dithiothreitol and an aliquot of enzyme similar to that used in the malic enzyme assays. Reactions rates are presented in the same units given above. All assays were performed at room temp. Protein was determined by the biuret method [9] using bovine serum albumin as standard.

3. Results

Fig. 1 shows the elution profiles of the NAD- and NADP-linked malic enzymes and malic dehydrogenase

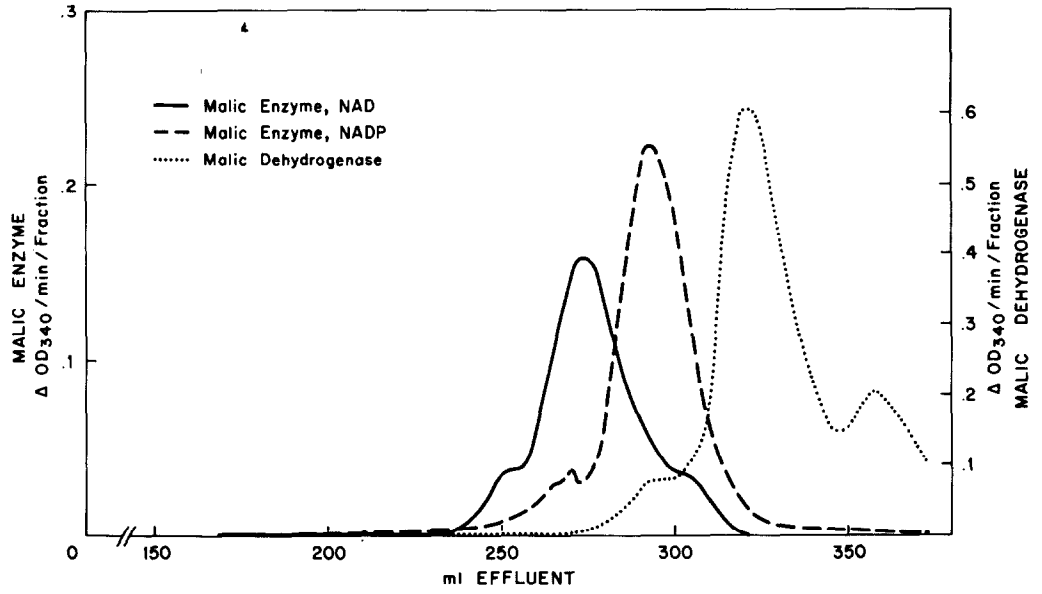

Fig. 1. Separation by gel filtration of the malic enzymes and malic dehydrogenase from a calf adrenal cortex mitochondrial fraction. Thirty mg of calf adrenal cortex mitochondrial fraction were sonicated in 4 ml of 30 mM Tris-HCl, 0.5 mM EDTA, and 0.1 mM dithiothreitol (pH 7.4). After removal of the submitochondrial particles by centrifugation the clear supernate was run in the ascending direction on a 2.5 × 90 cm Sepharose 6B column, as described in Methods. Ten ml fractions were collected for the first 15 hr and 2.2 ml fractions thereafter. The void volume was 152 ml. The specific activity of the NADP-dependent malic enzyme activity in this mitochondrial preparation was 18.2 nmoles NADP reduced/min/mg mitochrondrial protein. This value agrees with the value of 19.5 reported by Simpson and Estabrook [3] for bovine adrenal cortex mitochondria.

which resulted from Sepharose 6B gel filtration of the clear supernate from a sonicated calf adrenal cortex mitochondrial fraction. The first enzyme to appear was a malic enzyme active with NAD. The low NADP-dependent activity of this enzyme was evident as a small peak that preceded the prominent second peak of the strictly NADP-linked malic enzyme. Both of these malic enzymes were separated from malic dehydrogenase. The NAD-linked malic enzyme produced pyruvate and NADH at a molar ratio of one (not shown) and was dependent on malate, NAD, and Mn ion. The kinetic and electrophoretic properties and studies on the intracellular localization of the strictly NADP-dependent malic enzyme will be reported separately (R. Mandella and L.A. Sauer, in preparation).

The NAD-linked malic enzyme showed sigmoid kinetics in plots of activity versus the malate concentration (fig. 2, left). Fumarate (3 mM) converted the reaction to hyperbolic kinetics. The rates of NADH production (at 10 mM malate) in the absence and presence of 3 mM fumarate were 0.172 and 0.22 $\Delta A_{340}/5$ min/ml enzyme solution, respectively. The rates of NADPH production under the same conditions were 0.03 and 0.05 $\Delta A_{340}/5$ min/ml enzyme. Thus, the first peak of malic enzyme activity to elute from the Sepharose column was essentially NAD specific. The effect of increasing concentrations of the positive modulator fumarate (in the presence of 2 mM malate) on NAD-dependent malic enzyme activity is shown in fig. 2 (right side). Maximal activation was observed at about 1–2 mM fumarate. Succinate also served as a positive modulator but was much less effective than fumarate.

ATP inhibited the rate of NADH reduction by the NAD-dependent malic enzyme both in the presence (fig. 3) and absence (not shown) of the positive modulator fumarate. Low levels of ATP gave a marked inhibition. However, the inhibition due to ATP was not complete and at high ATP concentrations the degree of inhibition increased very slowly to a value of about 85% inhibition. When these data were plotted as the reciprocal velocity versus the ATP concentration, as described by Dixon [10], the plots were linear only at low ATP concentrations. At higher ATP concentra-

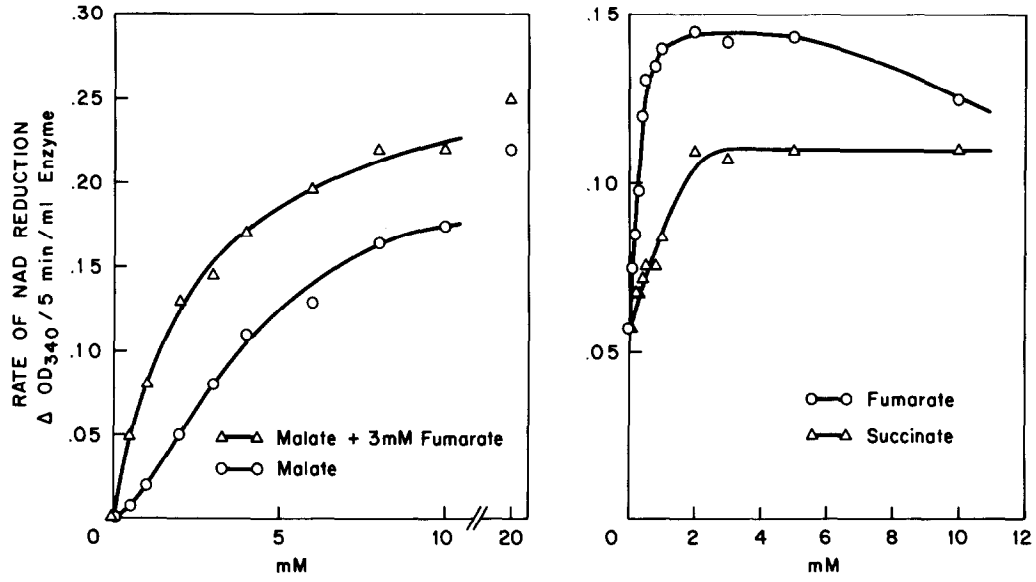

Fig. 2. Kinetic properties of the NAD-dependent malic enzyme. The fractions comprising the ascending portion and the peak of the NAD-dependent malic enzyme activity profile following Sepharose 6B gel filtration (for example, from 250 to 275 ml effluent volume in fig. 1) were pooled. The rate of NAD reduction was measured as described in Methods with increasing (–) malate concentrations in both the presence and absence of 3 mM fumarate (graph on left). The stimulation of NAD-dependent malic enzyme activity due to increasing concentrations of succinate or fumarate is shown on the right. The (–) malate concentration was 2 mM. Each point represents the mean of duplicate measurements.

tions the curves showed an inflection point and then proceeded with a decreased slope (curve concave downward). This incomplete inhibition by ATP is similar to the incomplete inhibitions given by the negative modulators of other regulatory enzymes: for example, the inhibition of homoserine dehydrogenase by L-threonine [11], and the inhibition of L-threonine deaminase by L-isoleucine [12]. These results may be interpreted to mean that ATP inhibits the NAD-linked malic enzyme by interacting at a site other than the active site.

The three single points shown in fig. 3 (above 5 mM ATP) indicate that the rate of NAD reduction at 8 mM malate and 5 mM ATP was dependent on malate and Mn ion. Fumarate was also required to obtain the rate values shown by the curves. The ATP-resistant rate of NAD reduction, therefore, was due to the fumarate-stimulated, Mn ion and malate-dependent malic enzyme. The Mn ion concentrations in the cuvettes were 10 mM (at 2 mM malate) and 15 mM (at 8 mM malate) giving a Mn ion/ATP ratio of 1.5 or greater. The rate of ATP-resistant NAD reduction was not altered by further increases in the Mn ion/ATP ratio.

Double reciprocal plots of the rate of NAD reduction (in the presence of 3 mM fumarate) versus the malate concentration in the presence of 0, 0.4, and 0.8 mM ATP are shown in fig. 4. The inhibition by ATP was competitive with malate. The apparent K_m for malate was increased from 2.4 mM in the absence of ATP to 4.6 and 7.7 mM at 0.4 and 0.8 mM ATP, respectively. In the presence of ATP and low malate concentrations the rates of NAD reduction were faster than expected and these points fell below the double reciprocal plot. This observation is in keeping with the ATP-resistant rate of NADH formation at high ATP/malate ratios (fig. 3).

4. Discussion

The presence in mammalian tissues of an NAD-dependent malic enzyme with regulatory properties is, to our knowledge, a new finding. Based on the above experiments and an earlier report [13], we can now state that regulatory NAD-dependent malic enzymes are present in rat liver and kidney, and in both rat and calf adrenal cortex. Calf adrenal cortex is the most ac-

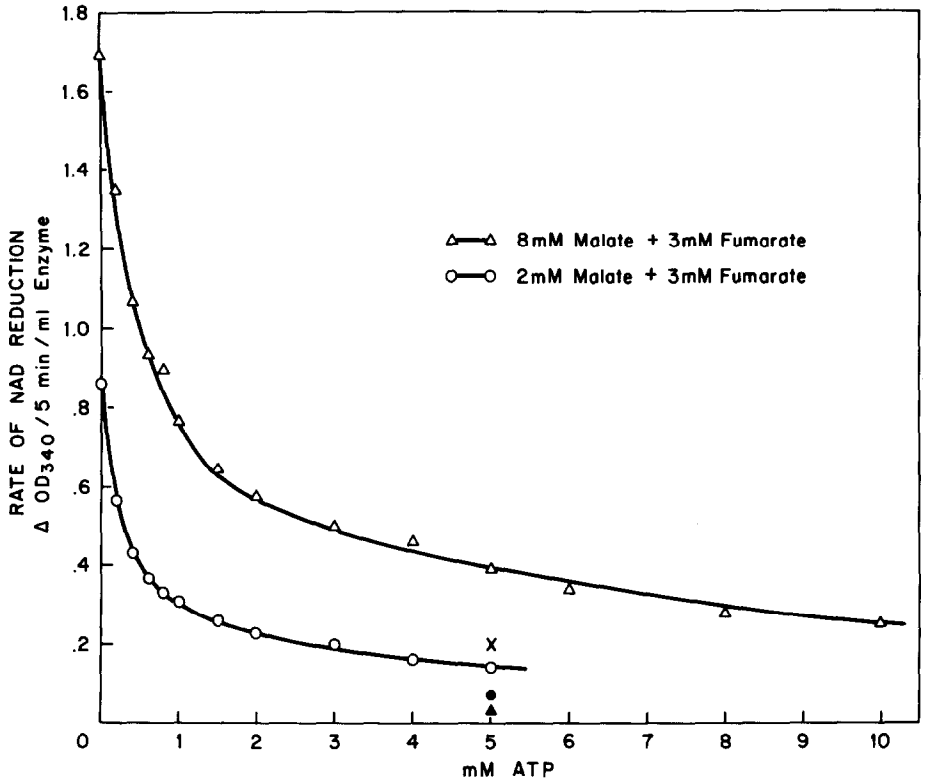

Fig. 3. The effect of ATP on the rate of NAD reduction by the fumarate-activated NAD-dependent malic enzyme. The complete assay mixture contained 30 mM Tris-HCl (pH 7.4), 3 mM fumarate, 2 (lower curve) or 8 mM (upper curve) (–) malate, 10 (lower curve) or 15 mM (upper curve) Mn ion, 0.2 mM NAD, 0.1 mM dithiothreitol, an aliquot of enzyme, and ATP, as indicated. The rate of NAD reduction was measured as described in Methods. The single values lying above the 5 mM ATP point represent measurements done in the complete mixture containing 8 mM (–) malate and 5 mM ATP, except that either Mn ion (▲), malate (●), or fumarate (X) were omitted. These rate values should be compared to the rate measured in the complete mixture at 5 mM ATP (Δ-Δ). Each value represents the mean of duplicate assays.

tive source of enzyme that we have yet found. The specific activity (in the presence of 4 mM malate and 10 mM succinate) was 17.6 nmoles NADH formed/min/mg mitochondrial protein (mean value for 3 mitochondrial preparations). This is about twice the specific activity of the enzyme in rat adrenal cortex mitochondrial fractions. The calf adrenal cortex enzyme reacted with NADP only very slowly and was essentially NAD specific. It differed from the rat enzyme which reduced NADP at rates about one-third to one-half as fast as the rate of NAD reduction. The rat adrenal cortex enzyme, however, had a smaller apparent K_m for NAD (relative to NADP) and in mixtures of the two coenzymes NAD was reduced preferentially [14]. Functionally, therefore, the rat adrenal cortex NAD- and NADP-dependent malic enzyme behaved as an NAD-dependent dehydrogenase.

The special functions that an NAD-linked malic enzyme might play in cellular metabolism remains to be elucidated. The kinetic properties of the adrenal cortex enzyme, however, suggest an important role in malate metabolism. In intact adrenal mitochondria, the rate of malate decarboxylation was increased during state 3 respiration and by uncouplers of oxidative phosphorylation and was inhibited by rotenone or KCN [9]. NADH produced via malic enzyme, therefore, would appear to be accessible to the NADH dehydrogenase of electron transport. Further studies on the role of this enzyme in mitochondrial metabolism are in progress.

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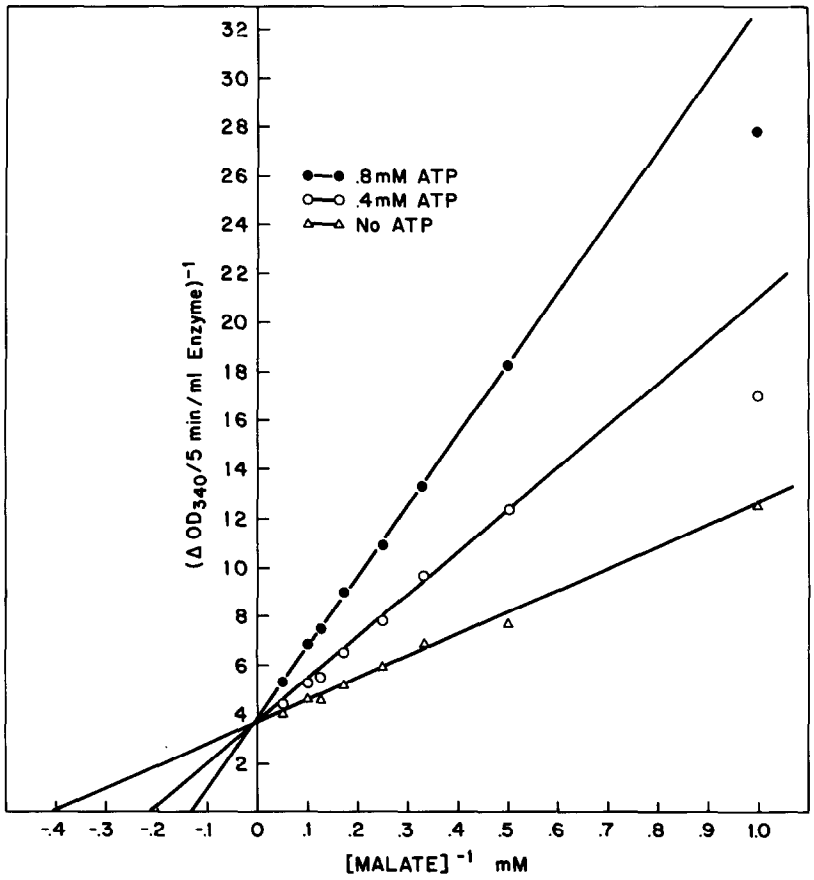

Fig. 4. Double reciprocal plot of the rate of NAD reduction versus the malate concentration at 0, 0.4 and 0.8 mM ATP. The assay mixture was as described in Methods. Mn ion was 10 mM.

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References

[1] N.S. Henderson, Arch. Biochem. Biophys. 117 (1966) 28.
[2] D. Brdiczka and D. Pette, European J. Biochem. 19 (1971) 546.
[3] E.R. Simpson and R.W. Estabrook, Arch. Biochem. Biophys. 129 (1969) 384.
[4] R. Frenkel, J. Biol. Chem. 246 (1971) 3069.
[5] R. Frenkel, Arch. Biochem. Biophys. 152 (1972) 136.
[6] M.S. Mounib and J.S. Eisan, Endocrinology 91 (1972) 1375.
[7] R. Frenkel, Biochem. Biophys. Res. Commun. 47 (1972) 931.
[8] L.A. Sauer and P.J. Mulrow, Arch. Biochem. Biophys. 134 (1969) 486.
[9] L. Szarkowska, L. and M. Klingenberg, Biochem. Z. 338 (1963) 674.
[10] M. Dixon, Biochem. J. 55 (1953) 170.
[11] P. Datta and H. Gest, J. Biol. Chem. 240 (1965) 3023.
[12] J.P. Changeux, Cold Spring Harbor Symp. Quant. Biol. 26 (1961) 313.
[13] L.A. Sauer, Biochem. Biophys. Res. Commun. 50 (1973) 524.
[14] L.A. Sauer, Federation Proc. 32 (1973) 576 Abs.
[15] L.A. Sauer and R. Park, Biochemistry 12 (1973) 643.