Kinetic Characterization of Mitochondrial Malate Dehydrogenase from *Dictyostelium discoideum*

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Malate dehydrogenase (MDH; EC 1.1.1.37) from *Dictyostelium discoideum* was purified and characterized. MDH activity from whole cells was purified 275-fold. The mitochondrial and cytoplasmic MDH present co-purified through three ion exchange and affinity chromatography steps. The isoenzymes were barely separable by either disc gel electrophoresis or isoelectric focusing. The purified preparation containing both isoenzymes had a single pH optimum (9.3–9.5) and an apparent molecular weight of 70 000. It exhibited linear kinetics and responded to known inhibitors of MDH, i.e. thyroxine and hydroxymalonate. Michaelis and dissociation constants obtained with this preparation were similar to those obtained with a 10-fold purified mitochondrial MDH.

INTRODUCTION

Dictyostelium discoideum uses cellular protein as its source of tricarboxylic acid cycle intermediates during cell development (Wright, 1964). Kelly *et al.* (1979*b*) developed a steady state model of the tricarboxylic acid cycle which is serving as a basis for development of a transition model of the cycle during differentiation (Wright, 1981). Malate dehydrogenase (MDH; EC 1.1.1.37) catalyses the reversible oxidation–reduction of malate and NAD to oxaloacetate and NADH. The intracellular concentration of oxaloacetate is the lowest of all the tricarboxylic acid cycle intermediates (Kelly *et al.*, 1979*a*). Throughout differentiation it remains 2- to 10-fold lower than the next lowest pools (2-oxoglutarate and acetyl-CoA). MDH may play an important role in limiting the oxaloacetate concentration and, therefore, in controlling flux through the tricarboxylic acid cycle. In order to realistically evaluate the importance of this reaction in the kinetic model, the kinetic constants (Michaelis and dissociation constants) of MDH must be determined.

In mammalian systems MDH is present in the mitochondria, functioning in the tricarboxylic acid cycle, and in the cytoplasm, functioning in the ‘malate shuttle’ (Krebs *et al.*, 1967). Our attempts to purify the mitochondrial enzyme from whole cell extracts of *D. discoideum* resulted in substantial purification of enzyme activity (275-fold) but failed to separate the mitochondrial and cytoplasmic enzymes. The enzyme was partially purified from isolated mitochondria to determine separately the kinetic constants of the mitochondrial enzyme.

METHODS

Enzyme assays. For enzyme purification and characterization, two fluorimetric assays were used. The assay for measuring oxaloacetate reduction contained 0.4 µmol NADH (Na salt), 1.2 µmol sodium oxaloacetate, and

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sodium bicarbonate buffer (100 μ mol, pH 9.5) to a total of 2 ml. When measuring malate oxidation in the presence of NAD, hydrazine/glycine buffer (final concentration 0.4 M-hydrazine, 1 M-glycine, pH 9.5) was used to remove the oxaloacetate as the hydrazine and allow the reaction to proceed against an equilibrium strongly favouring the opposite direction (Hohorst, 1965). The reactions were started by addition of enzyme, and NADH oxidation or NAD reduction was measured using a Perkin-Elmer fluorescence spectrophotometer. One unit of activity is defined as the amount catalysing the oxidation of 1 μ mol NADH min^{-1} at 25 °C.

Enzyme purification from mitochondria. *Dictyostelium discoideum* NC4 (ATCC 24697) was grown with *Escherichia coli* on nutrient agar sheets (Marshall *et al.*, 1970). Aggregation stage cells from 200 sheets (approximately 80 g dry wt) were harvested and washed three times in distilled water. They were subsequently suspended in 50 mM-Tris/HCl buffer (pH 8.0) containing 0.35 M-sucrose, 1 mM-EDTA and 0.2% (w/v) bovine serum albumin. Mitochondria were isolated according to the method of Komuniecki *et al.* (1980). The crude mitochondrial extract was prepared by resuspending the mitochondria in the same buffer without sucrose, freezing once, thawing, and centrifuging at 27000 g for 20 min. This extract contained 0.25 g protein (assayed by the Lowry method using bovine serum albumin as a standard). The protein yield was low, partly because the mitochondrial isolation technique allowed only 10–15% cell breakage. The mitochondrial extract had only one band of activity on a 7% polyacrylamide gel when stained for MDH activity (see below for details).

The crude extract was partially purified as follows. Streptomycin sulphate was added to a concentration of 1% (w/v). After the solution had been stirred for 45 min at 0 °C, the resulting precipitate was removed by centrifugation. The supernatant was taken to 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ by slow addition of saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was removed by centrifugation. Sufficient solid $(\text{NH}_4)_2\text{SO}_4$ was then added to bring the supernatant fluid to 90% saturation. The resulting precipitate was dissolved in 50 mM-Tris/HCl buffer (pH 8.0) containing 15% (v/v) glycerol, 1 mM-mercaptoethanol and 1 mM-EDTA and dialysed overnight at 4 °C against the same buffer mixture. The dialysed protein solution was chromatographed on a column (2 $\times$ 20 cm) of DEAE-Sephadex (Pharmacia) equilibrated with the same buffer. The MDH activity did not bind to the column. Fractions of 1 ml were collected and those containing MDH activity were pooled.

Enzyme purification from whole cells. Pre-cultivation stage cells from 50 sheets were harvested in 200 ml 50 mM-Tris/HCl buffer (pH 7.4). The suspension was frozen and thawed three times and then centrifuged at 12000 g for 10 min to remove cell debris. Streptomycin sulphate was added to the supernatant to a final concentration of 1% (w/v). After 45 min at 0 °C, the resulting precipitate was removed by centrifugation. The supernatant was taken to 60% saturation $(\text{NH}_4)_2\text{SO}_4$ by slow addition of saturated $(\text{NH}_4)_2\text{SO}_4$ (300 ml). The precipitate was removed, the supernatant was taken to 90% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ (125 g), and the resulting precipitate (60–90% fraction) was removed. This fraction was dissolved in a standard buffer consisting of 50 mM-Tris/HCl (pH 7.4), 15% (v/v) glycerol, 1 mM-mercaptoethanol and 1 mM-EDTA, and dialysed overnight at 4 °C against the same buffer mixture. The non-diffusible fraction was then chromatographed on a column (2 $\times$ 20 cm) of DEAE-Sephadex which had been equilibrated with standard buffer. The MDH activity did not bind to the column. Fractions containing MDH activity were combined and applied to a 2 $\times$ 5 cm column of Sepharose-Blue (Pharmacia), an affinity material which adsorbs nucleotide-requiring enzymes (Thompson *et al.*, 1975). The column was washed with 75 ml of standard buffer. A linear gradient of NADH (0–3.5 mM in 200 ml of standard buffer) was applied and 9.0 ml fractions were collected and assayed. The activity eluted in fractions 6–13 and peaked at 1.3 mM-NADH. These fractions were pooled and dialysed overnight at 4 °C against standard buffer. The non-diffusible fraction was then chromatographed on a column (2 $\times$ 70 cm) of Agarose A-1.5 (Bio-Rad) which had been equilibrated with standard buffer and 5.0 ml fractions were collected and assayed. The activity eluted in fractions 33–39 as a symmetrical peak. Fractions containing activity were combined and concentrated using an Amicon model 8MC ultrafiltration system. Ultrafiltration, even in the presence of glycerol, resulted in loss of activity at this stage unless bovine serum albumin was included in the sample at a final concentration of about 1 mg ml^{-1} .

Electrophoresis. Disc gel electrophoresis was performed in acrylamide gels (7% acrylamide, 0.2% bis-acrylamide) at 0–4 °C using a constant current of 2 mA per gel. The reservoir buffer was Tris/glycine (150 mM, pH 8.3). After electrophoresis, the gels were removed from the gel tubes and placed in a foil-covered flask. To stain for enzymic activity, the gels were incubated with 20 ml of a solution containing malate (30 mM), NAD (1.5 mM), phenazine methosulphate (0.3 mM) and MTT tetrazolium (2.5 mM). After incubation (usually 20–30 min) the stained bands were fixed in 7% (v/v) acetic acid.

Isoelectric focusing. Gels (7% acrylamide, 0.2% bis-acrylamide) containing glycerol (12%), ampholytes (2%, pH range 3–10) and riboflavin 5'-phosphate (10 mM) were photopolymerized overnight. Samples for focusing (50 μ l) were mixed with an equal volume of sucrose solution (50%, w/v) containing methyl red (1 mg ml^{-1}). The samples were applied to the gels and overlaid with sucrose (10%) and H_3PO_4 (10 mM) and the catholyte NaOH (10 mM). Runs were made at 0–4 °C at constant voltage (300 V) for at least 6 h. After focusing, the gels were removed from their tubes, and the pH gradient established in the gels during focusing was determined with a surface electrode. Gels were then stained for activity or sectioned and assayed.

Table 1. Purification of malate dehydrogenase from pre-culmination stage cells

Fraction	Volume (ml)	Protein (mg ml ⁻¹)	10 ⁻⁴ × Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Purification factor	Yield (%)
1. Crude extract	210	31.8	1.40	2.0	(1)	(100)
2. Strep. sulphate	200	27.4	1.20	2.2	1.1	86
3. (NH ₄) ₂ SO ₄ (60–90%)	62	14.9	0.58	6.3	3.2	40
4. DEAE-Sephadex	98	0.9	0.40	42.8	21.4	30
5. Sepharose-Blue	63	0.6	0.14	233	116	11
6. Agarose A-1.5	32	0.2	0.11	550	275	8

RESULTS AND DISCUSSION

The MDH activity from mitochondria was purified 10-fold, but only 1.6 mg protein (6.5% yield of MDH activity) remained, making further purification difficult. No NADH oxidase activity, which might interfere with the assay for MDH, was detected. In contrast to the mitochondrial extract, purification of whole cell extracts by the same procedure resulted in a 21-fold purification and a 30% yield (Table 1). Additional purification of MDH activity from whole cells was achieved by Sepharose-Blue affinity chromatography, Agarose A-1.5 chromatography, and concentration with an ultrafiltration system. This procedure resulted in a 275-fold purification of MDH activity (8% yield) containing both major MDH isoenzymes. There were two distinguishable but poorly resolved bands in both polyacrylamide disc gels and isoelectric focusing gels stained for enzymic activity. This behaviour differs from that of MDHs from other sources including chicken heart (Kitto & Kaplan, 1966), *Physarum polycephalum* (Teague & Henney, 1973) and *Saccharomyces cerevisiae* (Hagele *et al.*, 1978). The mitochondrial and cytoplasmic enzymes from these sources were separable by affinity and ion exchange chromatography, separation being achieved because of differences in dinucleotide analogue affinity or charge differences between the isoenzymes (Banaszak & Bradshaw, 1975). Evidently such differences are smaller between the MDH isoenzymes of *D. discoideum*.

The mitochondrial MDH activity was found to have a pH optimum of 9.3–9.5 using Tris/HCl and bicarbonate buffers in assays over a pH range of 7–10. This was much higher than the pH optimum (6.5–7.5) reported from other sources (Flury *et al.*, 1974; Kuan *et al.*, 1979). Both thyroxine and hydroxymalonate inhibited the MDH activity. Thyroxine was 100 times more inhibitory (50% inhibition at 0.06 mM) than hydroxymalonate (50% inhibition at 6.0 mM). Inhibition by these compounds is characteristic of MDHs (Wolff & Wolff, 1957).

Kinetic constants were determined using 10-fold purified mitochondrial MDH, and MDH from whole cells purified as far as the Sepharose-Blue step (116-fold). Initial velocity studies, with each substrate being used as the fixed or variable substrate, were done to determine Michaelis (K_m) and dissociation (K_i) constants. Lineweaver–Burk plots of the results of each set of experiments (e.g. a set of plots of reciprocal velocity versus reciprocal of NADH concentration for several fixed oxaloacetate concentrations) resulted in several families of straight lines. These, and re-plots of the y intercepts of the lines versus the reciprocals of the fixed substrate concentrations gave the K_m and K_i values listed in Table 2 (Cleland, 1963). Malate concentrations used were 10.0, 5.0, 2.0 and 1.0 mM; NAD concentrations were 2.0, 1.0, 0.5, 0.25 and 0.1 mM. Oxaloacetate concentrations used were 0.25, 0.19, 0.125, 0.1 and 0.06 mM; NADH concentrations were 0.05, 0.04, 0.03, 0.02 and 0.01 mM.

The whole cell MDH preparation, although containing two different isoenzymes, gave linear Lineweaver–Burk plots, and the values for K_m and K_i were similar to those for the mitochondrial MDH preparation (Table 2). The linearity of the reciprocal plots from initial velocity studies with MDH from whole cells suggested that it contained either (a) isoenzymes with very similar constants, or (b) isoenzymes with very different constants such that only the

Table 2. *Kinetic constants for MDH purified from whole cells and mitochondria*

Kinetic constant*	Mitochondrial MDH† (mM)	Whole cell MDH‡ (mM)
K_m NADH	0.04	0.03
K_m oxaloacetate	0.27	0.17
K_{iq} NADH	0.04	0.04
K_m malate	1.33	5.0
K_m NAD	0.10	0.14
K_{ia} NAD	0.31	0.11

* Nomenclature of Cleland (1963).

† Enzyme purified as far as DEAE-Sephadex chromatography step (10-fold).

‡ Enzyme purified as far as Sepharose-Blue chromatography step (116-fold).

major isoenzyme was detected. It is probable that the presence of isoenzymes with kinetic constants differing by a factor of 2 or more would have resulted in non-linear reciprocal plots. The purified enzyme had a single pH optimum (9.3–9.5). The apparent molecular weight of this MDH, as determined by gel filtration on a calibrated Sephadex G-200 column (1.5 × 70 cm), was 70000. The activity chromatographed as a single symmetrical peak, and there was no evidence of heterogeneity. MDHs from a variety of eukaryotic sources all have similar molecular weights (Banaszak & Bradshaw, 1975; Murphy *et al.*, 1967).

Since the MDH purified from whole cells behaved as a homogeneous enzyme, and the kinetics of the mitochondrial and whole cell MDH preparations were comparable, the more highly purified whole cell MDH was used in product inhibition studies to obtain evidence regarding the enzyme kinetic mechanism. Plots were made of reciprocal velocity versus either oxaloacetate concentration or NADH concentration at a fixed unsaturated concentration of the second substrate, with either 0, 0.025, 0.050 and 0.075 mM-NAD or 0, 2.0, 5.0 and 10.0 mM-malate. The variable substrate concentrations were those used for determining kinetic constants (see above). The fixed NADH concentration was 0.5 mM; the fixed oxaloacetate concentration was 1.25 mM. In all cases the inhibition was found to be uncompetitive. This pattern of inhibition was consistent with an iso-ordered bi-bi reaction mechanism (Cleland, 1963). This mechanism has been determined by Heyde & Ainsworth (1968) for bovine heart MDH and by Raval & Wolfe (1962) for the porcine heart enzyme. Kinetic experiments are not sufficient to determine the order of substrate binding. However, an increase in fluorescence of the coenzyme was noted when enzyme was added in stoichiometric amounts. This indicated that NADH could combine with the free enzyme suggesting that the NADH/NAD pair were the first to bind to and the last to be freed from the enzyme (Heyde & Ainsworth, 1968), which is generally thought to be the case for the dehydrogenases (Banaszak & Bradshaw, 1975).

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