Purification and Properties of Cytoplasmic and Mitochondrial Malate Dehydrogenases of *Physarum polycephalum*

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Two isoenzymes of malate dehydrogenase (MDH) were demonstrated in plasmodia of *Physarum polycephalum* by polyacrylamide-gel electrophoresis. The more "cathodal" form was uniquely associated with mitochondria (M-MDH) and the other form was found in the soluble cytoplasm (S-MDH). The isoenzymes were separated by acetone fractionation of soluble plasmodial homogenates acidified to pH 5.0. The M-MDH was purified 201-fold by cetylpyridinium chloride treatment, fractionation with ammonium sulfate, gradient elution from sulfoethyl cellulose at pH 6.0, and Sephadex G-100 chromatography. The S-MDH was purified 155-fold by ammonium sulfate fractionation, diethylaminoethyl cellulose chromatography, gradient elution from sulfoethyl cellulose at pH 5.5, and Sephadex G-100 chromatography. The optimal *cis*-oxalacetate concentrations were 0.35 mM for M-MDH and 0.25 mM for S-MDH, and the optimal pH for both isoenzymes was 7.6 for oxalacetate reduction. The optimal L-malate concentrations were 5 mM for S-MDH and 6 mM for M-MDH, and both isoenzymes exhibited an optimal pH of 10.0 for L-malate oxidation. The Michaelis constants of S-MDH and M-MDH served to discriminate between the isoenzymes. The S-MDH was more heat-stable than the M-MDH. High concentrations of oxalacetate and malate inhibited S-MDH more than M-MDH. The isoenzymes were further distinguished by their utilization of analogues of nicotinamide adenine dinucleotide. Many properties of the *Physarum* isoenzymes were similar to those of more complex organisms, especially vertebrates.

In eukaryotic cells, malate dehydrogenase (L-malate: nicotinamide adenine dinucleotide [NAD] oxidoreductase, EC 1.1.1.37; MDH) occurs in at least two isoenzymatic forms. One form is associated with the mitochondria and the other with the cytosol. MDH from animal tissues has been most extensively studied, and the isoenzymes differ in their physicochemical and functional properties.

The Myxomycetes are eukaryotic protists, with affinities in the animal kingdom (9, 21, 33), whose life cycle exhibits a number of differentiating stages (9). Studies on myxomycete MDH can add to our knowledge of the comparative biochemistry of the enzyme. To date, there is a paucity of enzymological data on this intriguing group of organisms.

In this work, the MDH isoenzymes of *Physarum polycephalum* were isolated and purified, and many of their properties were established. The isoenzymes can be distinguished on

the basis of cellular localization, electrophoretic mobility, purification properties, reaction kinetics, and thermostability.

The *Physarum* isoenzymes exhibit many properties similar to those of the analogous isoenzymes of vertebrates.

MATERIALS AND METHODS

Organism and growth conditions. The plasmodial phase of *P. polycephalum* was grown in our semidefined medium (13) excluding ammonium nitrate from the basal salt solution (14). Seven liters of medium, including 3.5 ml of Antifoam 60 silicone emulsion (General Electric, Waterford, N.Y.), was contained in a 9-liter Pyrex bottle. The bottle was cotton-plugged. Included in the plug was a short air-outlet tube and a cotton-plugged air-inlet tube. The apparatus was sterilized by autoclaving for 1 h at 121 C. Sterile hematin (13) was added to the cooled medium, which was then inoculated with 10 ml of presettled microplasmodia.

Humidified air was supplied through a sterile

filtration tube at a rate sufficient to keep the microplasmidia suspended. The bottle was covered with aluminum foil to exclude light (13). Maximal growth yield from exponential (40) cultures was obtained in about 5 days.

Cell harvest. The microplasmidia were allowed to settle for a few min at 4 C, the excess medium was decanted, and the remaining cells and medium were centrifuged at $800 \times g$ for 6 min at 2 C. The surface of the resulting cell paste was gently rinsed with distilled water. Approximately 25 g (wet weight) of cells per liter of medium was obtained.

Cells for mitochondrial isolation were used immediately. Those for enzyme isolation could be stored at -20 C for 1 month without noticeable loss of enzyme activity.

Mitochondrial isolation. Mitochondria were isolated by differential centrifugation in an isolating solution consisting of 0.3 M sucrose, 0.1% bovine serum albumin grade III (BSA), 5.3 mM β -mercaptoethanol (ME), and 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.0. All procedures were performed at 4 C unless stated otherwise.

The cells were suspended in nine volumes of isolating solution, homogenized in a Waring Blender at low speed for 30 s, and centrifuged at $600 \times g$ for 10 min. The supernatant fluid was filtered through six layers of cheesecloth and the residue was discarded. The centrifugation and filtration were repeated. The extract was then centrifuged at $5,000 \times g$ for 20 min. The resultant supernatant fluid was discarded and the sediment (mitochondrial fraction) was resuspended in minimal amounts of isolating solution.

This mitochondrial fraction was subjected to repeated cycles of centrifugation at $600 \times g$ for 10 min followed by $2,750 \times g$ for 15 min until little debris was sedimented at $600 \times g$ and no opaque slime was found in the $2,750 \times g$ mitochondrial sediment. After the last $2,750 \times g$ centrifugation, usually the third, the purity of the mitochondrial sample was verified in an electron microscope (unpublished data). The main portion of the sample was suspended in 0.1 M potassium phosphate buffer, pH 7.5, frozen to -20 C, thawed at 4 C, and centrifuged at $48,000 \times g$ for 15 min. The supernatant fluid was then analyzed by enzyme assay and by gel electrophoresis for enzyme activity.

Crude extract preparation. All preparations were at 4 C, and the procedures apply to 60 to 3,000 g (wet weight) of microplasmidia. The cells were suspended in 1.5 volumes by cell weight of solubilizing buffer composed of 0.05 M potassium phosphate, pH 7.5, 0.01 M ME, and 0.01 M diethyldithiocarbamic acid (DIECA; Sigma Chemical Co.).

The crude extract contained the natural yellow pigment of *P. polycephalum*, which is known to be partly phenolic (31). Concern over browning of extracts and binding of this compound to proteins caused the inclusion of DIECA, a phenol oxidase inhibitor (23), in the solubilizing medium. The DIECA not only eliminated browning of extracts but also increased the total enzyme recoveries during purification.

The solution was homogenized in a Waring Blender for 1 min at the low setting and for 30 s at high, by use of 15-s bursts with 10-s stops between bursts. After 30 min, the solution was centrifuged at $16,300 \times g$ for 10 min. The supernatant fluid was decanted, and the sediment was resuspended by homogenization in 0.5 volume of solubilizing buffer. This solution was recentrifuged as described. The two supernatant fluids were combined and recentrifuged. The final supernatant fluid was filtered through nine layers of cheesecloth and all sediment was discarded. Finally, the buffer strength was increased to 0.2 M potassium phosphate, and the total volume was adjusted to two volumes based on the original wet weight. This solution was the crude extract; a 2-ml sample was removed, dialyzed, and analyzed for protein and enzymatic activity.

Acidification of crude extract. The pH of the crude extract was lowered to pH 5.0 by the dropwise addition of 1 M acetic acid to the stirred extract. The pH was monitored continuously. After 20 min, the solution was centrifuged at $16,300 \times g$ for 15 min. The fluid was decanted, and the pellet, resuspended in 0.2 M solubilizing buffer previously adjusted to pH 5.0, was recentrifuged; both supernatant fluids were combined. The washed pellet was discarded. A sample of the supernatant fluid was analyzed for protein and enzyme activity.

With the above procedure, crude extracts prepared from 60 to 1,500 g (wet weight) of cells were conveniently handled. For larger extracts, the acidification procedure was repeated.

Acetone precipitation. All acetone additions were made at 4 C and were calculated as fractional volumes of pH 5.0 supernatant fluids. No more than 1,500 ml of extract was treated at any given time.

Precooled (-10 C) acetone was added to the continuously stirred extract. The extract, during the first acetone addition, was cooled to -5 C in an ethanol-ice bath and maintained at that temperature during fractionation. After each addition, the solution was stirred for 5 min and then held for 20 min. All centrifugations were at $16,300 \times g$ for 15 min at -5 C. Precipitations were obtained with acetone at 0.3-, 0.6-, and 1.0-volume additions. Precipitates were dissolved in minimal amounts of 0.1 M potassium phosphate buffer, pH 7.5, 0.01 M DIECA, and 0.01 M ME with a 50-ml glass-Teflon homogenizer. All samples reserved for analysis were first dialyzed against the same buffer without DIECA.

CPC precipitation. A 10% solution of cetylpyridinium chloride (CPC; Sigma Chemical Co.) in water at room temperature was added dropwise to the 10 C extract of the 0.6-volume acetone precipitation fraction with constant hand stirring until no observable precipitate formed. The solution was immediately filtered through double gauze milk filters (Kendall Co., Walpole, Mass.) and then was centrifuged at $16,300 \times g$ for 30 min to remove any remaining sediment.

Ammonium sulfate precipitation. Amounts of ammonium sulfate (enzyme grade; Schwartz/Mann) required to achieve desired final concentrations were determined from a solubility table (10).

Ammonium sulfate precipitates were dialyzed against 0.005 M potassium phosphate buffer, pH 7.5, containing 0.001 M ME.

Column chromatography on cellulose absorbents. Both *o*-diethylaminoethyl cellulose (DEAE-cellulose) and *o*-sulfoethyl cellulose (SE-cellulose) were obtained from BioRad (Richmond, Calif.). The celluloses were prepared for chromatography by the method of Hsieh and Vestling (16) and were stored at 4 C.

Information for proper cellulose chromatographic conditions was obtained from batch-treating samples of extracts with various types of cellulose at various conditions of pH and ionic strength.

The cellulose absorbent (twice the estimated required capacity) was contained in a column with a length to width ratio of approximately 10:1. All chromatography was done at 4 C.

For mitochondrial MDH purification, columns of SE-cellulose were initially equilibrated with 0.03 M potassium phosphate buffer, pH 6.0, containing 0.001 M ME. The ammonium sulfate-fractionated MDH (dialyzed against the same buffer) was applied at a concentration of 13 mg of protein/g of cellulose, and the column was washed with the same buffer. The enzyme was then eluted with a linear gradient extending from 0.03 to 0.08 M potassium phosphate buffer, pH 6.0, containing 0.001 M ME. The total gradient volume was 21 ml/g of cellulose.

The equilibration buffer for DEAE-cellulose columns was 0.002 M potassium phosphate, pH 7.5, containing 0.001 M ME. The ammonium sulfate-fractionated supernatant MDH, dialyzed against the same buffer, was applied and eluted with the same buffer.

Supernatant MDH was further purified on SE-cellulose columns equilibrated with 0.008 M citrate-potassium phosphate buffer, pH 5.5, containing 0.001 M ME. After applying the enzyme, the column was washed with the same buffer. The enzyme was eluted with a linear gradient of 0.008 to 0.05 M citrate-phosphate buffer, pH 5.5, containing 0.001 M ME. The volume of the gradient was 24 ml/g of cellulose.

Protein concentration of the column effluent was monitored by following absorption at 280 nm in a Beckman DU-2 spectrophotometer.

Gel filtration chromatography. Sephadex G-100 superfine (Pharmacia) was contained in a column, 90 by 1.5 cm, and equilibrated with 0.005 M potassium phosphate, pH 7.6, containing 0.001 M ME. The final bed dimensions were 70 by 1.5 cm. The sample was applied in a volume not exceeding 3% of the total bed volume and a protein concentration of not less than 3 mg/ml. Fractions of 2 ml were collected, and protein was monitored by absorption at 280 nm. The void volume was 35 ml.

MDH assays. All assays were done at 25 C by following spectrophotometrically either the reduction of NAD or the oxidation of reduced NAD (NADH₂) at 340 nm.

For the NADH₂ oxidation assay, the reaction mixture consisted of 0.39 μ mol of NADH₂, 0.9 μ mol of *cis*-oxalacetate (OAA), sufficient enzyme to cause a decrease in absorbancy of 0.05 to 0.15 per cm per min,

and sufficient 0.1 M potassium phosphate buffer, pH 7.6, to obtain a final volume of 3.0 ml. The NAD reduction assay mixture consisted of 1.23 μ mol of NAD, 19.5 μ mol of L-malate, enough enzyme to cause an increase in absorbancy of 0.05 to 0.15 per cm per min, and sufficient 0.075 M sodium glycinate buffer, pH 10.0, for a final volume of 3.0 ml.

Assays were also performed substituting D-malate for L-malate and nicotinamide adenine dinucleotide phosphate (NADP) for NAD.

Only initial rates were used in calculations; an enzyme unit was defined as that required to oxidize or reduce 1 μ mol of coenzyme per min. Enzyme specific activity was defined as enzyme units per milligram of protein.

pH optima. The effect of varying the pH of the reaction mixture was determined by use of the routine MDH assays. For OAA reduction, the buffers were constructed between pH 5.5 and 8.5 by titrating 0.1 M monopotassium phosphate with 0.1 M dipotassium phosphate. For malate oxidation, buffers were varied between pH 8.0 and 11.5 by titrating 0.075 M glycine with 0.075 M NaOH.

Substrate optima. Appropriate dilutions of neutralized potassium oxalacetate and potassium L-malate were used in the standard MDH assays.

K_m determinations. The apparent Michaelis constant (*K_m*) values were calculated from initial velocities by the double reciprocal plot method in conjunction with the least square method for determination of the best straight line.

NAD analogues. The following NAD analogues were obtained from P-L Biochemicals (Milwaukee, Wis.): 3-acetylpyridine adenine dinucleotide (APAD), 3-acetylpyridine hypoxanthine dinucleotide (APHD), 3-pyridinealdehyde adenine dinucleotide (PAAD), and thionicotinamide adenine dinucleotide (TNAD). The analogues were used in the usual coenzyme concentration (0.41 mM) in the assay with L-malate at concentrations of 6.5 and 1 mM. The manufacturer's observed molar extinction coefficients at the wavelength of maximal absorption were used to determine reaction rates.

Thermostability. The thermostability of the isoenzymes was determined at concentrations of 1 enzyme unit/ml by (A) incubating the enzyme for 15 min at 25, 45, 50, 55, 60, and 70 C, and (B) incubating the enzyme at 50 C and removing samples every 5 min for a total of 30 min, and then every 10 min for an additional 30 min. All samples were plunged immediately into an ice bath after incubation and assayed for enzyme activity.

Electrophoresis. Polyacrylamide-gel disk electrophoresis was performed on the isoenzymes by use of 7% acrylamide separating gels and a buffer pH of 9.5. The chemical formulation for this process was that outlined by Canalco (1965 Data Sheet, Canal Industries, Bethesda, Md.).

The gels were stained for MDH activity by the tetrazolium technique outlined by Canalco. All gels were measured and the *R_f* values were calculated by dividing the distance the enzyme traveled from the top of the gel (cathode) by the distance the tracking dye traveled.

Concentrating protein solutions. Protein solutions were concentrated at 4 C with an ultrafiltration apparatus (Amicon Corp., Lexington, Mass.) and by dialysis under negative pressure.

Dialysis and water. Dialysis tubing was cleaned by boiling in 2% sodium carbonate for 10 min, rinsing in distilled water, and boiling in 0.2% ethylenediaminetetracetate. The tubing was finally rinsed in distilled water.

Glass-distilled water was used throughout this work.

Protein analysis. Protein concentrations were determined by the Lowry method as previously described (13).

RESULTS

MDH isoenzyme localization. Polyacrylamide-gel electrophoresis of crude extracts revealed two bands demonstrating MDH activity (Fig. 1A): a more cathodal band with an average R_f of 0.22 (0.20 to 0.24) and a more anodal band with an average R_f of 0.38 (0.36 to 0.40).

Gel electrophoresis of soluble proteins ob-

tained from extracts of isolated mitochondria has consistently shown only the cathodal band (Fig. 1B). This slower band (cathodal) is therefore designated mitochondrial MDH (M-MDH). The faster band could not be localized with any particulate fraction and is designated supernatant MDH (S-MDH).

Enzyme purification. Purification procedures are summarized in Table 1. The data are based on an initial 1,000 g (wet weight) of microplasmodia.

Acidification and acetone fractionation of crude extract. The M-MDH precipitated at pH 4.4 and S-MDH at 4.0 under the given conditions, but the stability of both forms was reduced. Both isoenzymes were stable at a pH of 5.0. The ionic strength of the phosphate buffer influenced the amount of protein and MDH occurring in the pH 5.0 precipitate. In the presence of 0.2 M potassium phosphate buffer, the pH 5.0 precipitate was found to contain about 40% of the total protein but only about 6%

FIG. 1. Polyacrylamide-gel electrophoresis of soluble proteins stained for MDH activity. All gels were 7% polyacrylamide run at pH 9.5 at 4 C. Each contains 0.5 enzyme unit as measured by OAA reduction. The bottom band in each case is the tracking dye. The O indicates the origin; the + indicates the anode. The gels are: A, crude extract; B, mitochondrial isolation; C, purified M-MDH; D, purified S-MDH.

TABLE 1. Summary of the isolation and purification procedures for M-MDH and S-MDH^a

Fraction	Total enzyme units ^b	Total protein (mg)	Sp act ^c	Purification factor ^d
Crude extract	185 × 10 ³	37,000	5	1
M-MDH				
0.3 to 0.6 vol acetone	120 × 10 ³	2,000	60	12
CPC ^e	66 × 10 ³	254	260	52
(NH ₄) ₂ SO ₄	59.4 × 10 ³	216	275	55
SE-cellulose	31 × 10 ³	34	912	182
G-100 Sephadex	17.1 × 10 ³	17	1,007	201
S-MDH				
0.6 to 1.0 vol acetone	46.3 × 10 ³	1,715	27	5.4
(NH ₄) ₂ SO ₄	33.3 × 10 ³	476	70	14
DEAE-cellulose	22.5 × 10 ³	122	184	37
SE-cellulose	16.8 × 10 ³	27	622	124
G-100 Sephadex	10.6 × 10 ³	13.7	774	155

^a Based on an initial 1,000 g (wet weight) of microplasmodia.^b As measured by oxalacetate reduction at 25 C in 0.1 M potassium phosphate, pH 7.5.^c Units per milligram of protein.^d Specific activity of each fraction/specific activity of crude extract.^e Cetylpyridinium chloride.

of the MDH activity. The pH 5.0 supernatant fluid was then fractionated with acetone, resulting in separation and partial purification of the isoenzymes (Table 1).

CPC treatment and (NH₄)₂SO₄ precipitation of M-MDH. The acetone-precipitated M-MDH was treated with CPC to eliminate the viscous material occurring in the fraction. There was a 40 to 50% loss of activity with this procedure.

The enzyme was then precipitated with (NH₄)₂SO₄, and the 70 to 90% saturation fraction contained the bulk of the enzyme activity.

SE-cellulose chromatography of M-MDH. The (NH₄)₂SO₄-precipitated M-MDH was further purified by chromatography on SE-cellulose. A typical elution pattern is shown in Fig. 2. The bulk of the M-MDH eluted at 0.052 M phosphate buffer, and the elution range was between 0.047 M and about 0.07 M buffer.

Gel filtration of M-MDH. The SE-cellulose-purified M-MDH was finally chromatographed on Sephadex G-100 superfine. Only one major protein fraction was observed (eluting between 40 and 60 ml of effluent); this peak coincided with the enzyme activity.

(NH₄)₂SO₄ fractionation and DEAE-cellulose chromatography of S-MDH. The acetone-precipitated S-MDH was fractionated with (NH₄)₂SO₄, and the active soluble fraction precipitated between 58 and 69% saturation. The sample was then chromatographed on a DEAE-cellulose column; a typical elution pattern is shown in Fig. 3.

SE-cellulose chromatography of S-MDH. The S-MDH fraction from the DEAE-cellulose

column was next chromatographed on SE-cellulose. A typical elution pattern is shown in Fig. 4.

The S-MDH began eluting at about 0.014 M buffer, and 90% of the enzyme eluted by the time the gradient reached 0.023 M buffer. The maximal elution occurred at about 0.018 M buffer.

Gel filtration of S-MDH. The SE-cellulose-purified S-MDH was finally chromatographed on Sephadex G-100 superfine. The enzyme fraction eluted between 40 and 52 ml of effluent.

Enzyme stability. During isolation and purification of the isoenzymes ME was necessary to prevent enzyme inactivation. For example, if 0.01 M ME was omitted during acidification, about 10% of the enzyme was inactivated.

The purified samples were stable for up to 2 months at 0 C in a 0.05 M potassium phosphate buffer, pH 7.5, containing 0.001 M ME.

Gel electrophoresis of purified isoenzymes. Samples of the purified isoenzymes were subjected to electrophoresis and stained for enzyme activity. The M-MDH (Fig. 1C) demonstrated one band with an average *R_f* of 0.25. The S-MDH (Fig. 1D) exhibited one band with an average *R_f* of 0.48 (0.46 to 0.5).

Gel electrophoresis, followed by protein staining, revealed a purity of at least 90% for both isoenzymes, since there were only a few trace bands of contaminating protein. All of the following experiments employed these separated and purified isoenzymes.

Optimal pH: OAA reduction. The optimal pH for both isoenzymes was pH 7.6 (Fig. 5). Although both forms showed about a 50%

FIG. 2. Gradient elution profile of $(\text{NH}_4)_2\text{SO}_4$ -fractionated M-MDH from SE-cellulose. The 70 to 90% $(\text{NH}_4)_2\text{SO}_4$ fraction of M-MDH was applied to a column, 2 by 30 cm, of SE-cellulose. The sample contained 227.5 mg of protein in 65 ml of 0.03 M potassium phosphate buffer, pH 6.0, containing 0.001 M ME, and was applied on 17.8 g of cellulose (0.17 meq/g). The column was washed with 267 ml of the same buffer. Then 380 ml of a linear gradient extending from 0.03 to 0.08 M potassium phosphate, pH 6.0, was used to elute the M-MDH. This gradient volume was followed by 55 ml of 0.08 M potassium phosphate and 0.001 M ME, pH 6.0. Each fraction contained 4.4 ml per tube. Symbols: $\circ$, absorbancy at 280 nm; $\bullet$, percent maximal MDH activity; dashed line, potassium phosphate gradient.

FIG. 3. DEAE-cellulose chromatography of the 58 to 69% $(\text{NH}_4)_2\text{SO}_4$ fraction of S-MDH. The elution profile represents that obtained from 612 mg of protein in 38 ml of 0.002 M potassium phosphate buffer, pH 7.5, containing 0.001 M ME, loaded on 6 g

reduction in activity at pH 5.5, there was an 11% difference at pH 8.5, with the M-MDH being more sensitive.

Optimal pH: malate oxidation. The optimal for both M-MDH and S-MDH was pH 10.0. Figure 5 shows essentially no difference between the two forms.

Substrate concentration effects and apparent K_m determinations: OAA reduction. For S-MDH, the maximal reaction rates were achieved at OAA concentrations from about 0.25 to 0.35 mM. The maximal rates were obtained at about 0.35 to 0.7 mM OAA for M-MDH (Fig. 6). Beyond 0.7 mM OAA, both isoenzymes were increasingly inhibited. At 40 mM substrate, the M-MDH was inhibited 51% and the S-MDH was inhibited 73%.

Assays in Tris buffer revealed identical inhibition patterns as in phosphate buffer.

The apparent K_m for S-MDH was 6.0×10^{-5} M and the K_m for M-MDH was 7.4×10^{-5} M for OAA reduction.

Kinetic behavior with malate: L-malate concentration effects, apparent K_m determi-

of DEAE-cellulose (0.87 meq/g) in a column, 1 by 10 cm. The protein was eluted with 300 ml of the same buffer. Each fraction contained 3 ml. Symbols: $\circ$, absorbancy at 280 nm; $\bullet$, percent maximal MDH activity.

FIG. 4. SE-cellulose chromatography of the DEAE-cellulose-purified S-MDH. The elution profile represents the chromatography of 183 mg of protein contained in 26 ml of 0.008 M citrate-phosphate buffer, pH 5.5, and 0.001 M ME applied on 14.4 g of SE-cellulose in a column, 2 by 30 cm. The column was washed with 185 ml of the same buffer, and S-MDH was eluted with 345 ml of a linear gradient from 0.008 to 0.05 M citrate-phosphate buffer, pH 5.5, containing 0.001 M ME. Each fraction contained 3 ml. Symbols: O, absorbancy at 280 nm; ●, percent maximal MDH activity; dashed line, citrate-phosphate gradient.

FIG. 5. Optimal pH determination. (A) Oxalacetate (OAA) reduction. The reaction mixture consisted of 0.39 μmol of NADH_2 , 0.9 μmol of OAA, enzyme in 0.1 ml, and sufficient 0.1 M potassium phosphate buffer to produce a total volume of 3 ml. (B) Malate oxidation. The reaction mixture consisted of 1.23 μmol of NAD, 19.5 μmol of L-malate, enzyme in 0.1 ml, and sufficient 0.075 M sodium glycinate buffer to equal a total volume of 3 ml. The reactions were monitored in a spectrophotometer at 340 nm; the temperature was 25 C. Symbols: ●, M-MDH activity; O, S-MDH activity.

nations, and behavior with D-malate. The L-malate concentrations producing maximal

MDH activity were 5 to 7 mM for S-MDH and 6 to 9 mM for M-MDH (Fig. 7). Both isoenzymes were increasingly inhibited by the substrate beyond 9 mM; at 200 mM, S-MDH was inhibited 47% and M-MDH was inhibited 34%.

The specific activity as measured by malate oxidation at optimal conditions was 92 for S-MDH and 126 for M-MDH.

The apparent K_m for M-MDH and S-MDH was 9.8×10^{-4} M and 2.6×10^{-4} M L-malate, respectively.

Substitution of D-malate for L-malate under the conditions for optimal L-malate oxidation produced no reduction of NAD, even at 100-fold increases in enzyme concentration.

Michaelis constant determinations of the coenzyme. The apparent K_m value of NADH_2 for the isoenzymes was 2×10^{-4} M for M-MDH, and that for S-MDH was 4.8×10^{-5} M.

The concentration of 0.13 mM NADH_2 used in previous assays was not inhibitory to either isoenzyme. Reduced NADP (NADPH_2) at a similar concentration in the OAA reduction assay would not substitute for NADH_2 .

The apparent K_m values for NAD for both isoenzymes were 7.5×10^{-4} M for S-MDH and 1.9×10^{-4} M for M-MDH.

The concentration 0.41 mM NAD was not inhibitory to either isoenzyme. NADP would not replace NAD in the malate oxidation assay.

FIG. 6. Effect of various oxaloacetate (OAA) concentrations on the activity of the MDH isoenzymes. All reactions were followed in a spectrophotometer at 340 nm and 25 C. Each assay mixture contained 0.39 μmol of NADH_2 , enzyme in 0.1 ml, OAA adjusted to pH 7.6, and sufficient 0.1 M potassium phosphate buffer, pH 7.6, to produce a total volume of 3 ml. Enzyme velocities are expressed as percent maximal MDH activity with the maximal rate for each isoenzyme as 100%. Symbols are the same as in Fig. 5.

FIG. 7. Effect of various L-malate concentrations on the activity of the MDH isoenzymes. All reactions were followed in a spectrophotometer at 340 nm and 25 C. Each assay mixture contained 1.23 μmol of NAD, 0.1 ml of enzyme, L-malate adjusted to pH 10.0, and sufficient 0.075 M sodium glycinate buffer, pH 10.0, to equal a total volume of 3 ml. Rate changes with concentration were expressed as percent maximal MDH activity with the maximal rate for each isoenzyme as 100%. Symbols are the same as in Fig. 5.

Relative reactive rates of the MDH isoenzymes with analogues of NAD. Table 2 shows a comparison of isoenzyme reaction rates with NAD and four analogues of NAD at two concentrations of L-malate.

None of the analogues was reduced better than NAD at the high substrate concentration, but APHD and APAD stimulated both isoenzymes at the low substrate concentration. M-MDH was stimulated more than S-MDH by APHD at 1 mM malate, but the isoenzymes

were affected almost equally by APAD. At 6.5 mM malate, M-MDH was less active with APAD than S-MDH. Although TNAD was not used as well as NAD, M-MDH was less active with the analogue than S-MDH at both substrate concentrations. The relative reactivities with analogues therefore served to distinguish between the isoenzymes. With PAAD, the isoenzymes exhibited only 3 to 6% of the NAD reactivity.

Thermal characteristics of the isoenzymes

TABLE 2. *Relative reaction rates of MDH isoenzymes with NAD analogues*

Analogue	Malate (mM)	S-MDH ^a	M-MDH ^a	S-MDH/M-MDH ^b
NAD	1.0	1.00	1.00	1.00
	6.5	2.88	2.25	1.28
APHD	1.0	1.73	2.09	0.83
	6.5	2.46	2.00	1.23
APAD	1.0	2.07	1.94	1.07
	6.5	2.62	1.74	1.51
PAAD	1.0	0.03	0.06	0.50
	6.5	0.17	0.13	1.24
TNAD	1.0	0.24	0.16	1.50
	6.5	1.18	0.61	1.93

^a Ratios of reaction rates relative to rate with NAD at 1.0 mM malate. Reaction rates are the micromoles of coenzyme or analogue reduced per minute.

^b Comparison of S-MDH ratios relative to M-MDH ratios.

of MDH. The heat stability of the isoenzymes was analyzed by two methods. The first method exposed the isoenzymes to various temperatures for 15 min (Fig. 8A). The maximal activity, as compared with the unheated controls, was reduced to 50% at 51 C for the M-MDH and at 55 C for S-MDH.

Heat stability was also compared at 50 C over a period of 60 min (Fig. 8B). The maximal activity, as compared with unheated samples of each isoenzyme, was reduced 80% after 60-min exposure to 50 C for the M-MDH. At the same time period of 60 min, only 34% of the activity was lost for the S-MDH.

DISCUSSION

This report establishes that the two electrophoretic forms of MDH from *P. polycephalum* are isoenzymes which are distinguishable by their cellular compartmentalization and their physicochemical and catalytic properties.

Although there is distinct evolutionary divergences separating the Myxomycetes from the other organisms studied in depth, it is intriguing that the purified *Physarum* MDH isoenzymes have many properties in common with more complex systems such as vertebrates.

The electrophoretic mobility of M-MDH being more cathodal and S-MDH more anodal is consistent with vertebral tissues (27), *Saccharomyces* (2), *Drosophila* (26), and cactus (29). However, electrophoretic mobility per se is not a clear indicator of identification, as can be seen from many examples where the relative mobility is reversed, such as in certain fish (3, 43), snails (28), and sea urchins (32).

The properties of the *Physarum* extracts are

different enough from those of other organisms to necessitate the development of this specific purification scheme. Also, the behavior of isoenzymes during purification is another way of distinguishing between them, and constitutes a segment of the isoenzyme characterization (17). The *Physarum* isoenzymes exhibited dissimilar properties when subjected to isoelectric precipitation, acetone and ammonium sulfate fractionation, and chromatography on ion-exchange celluloses. The purified isoenzymes, when examined for catalytic activity, proved to be quite similar to vertebral systems. In *P. polycephalum*, the optimal pH for OAA reduction is 7.6 for both isoenzymes, as contrasted with 7.8 for M-MDH and 7.6 for S-MDH in vertebrates (8, 18). The reactivities of *Physarum* MDH at higher pH values served to distinguish between the two isoenzymes. The optimal pH of 10.0 for malate oxidation is the same as for vertebrates (20). The pH optima of *Drosophila* (26), *Neurospora* (30), *Phycomyces* (42), and *Saccharomyces* (2) are significantly different from those of *Physarum*.

The optimal OAA concentrations, 0.25 mM for S-MDH and 0.35 mM for M-MDH, are quite close to the 0.33 mM values for both S- and M-MDH for vertebral systems (18). The optimal malate concentrations are also equivalent (20).

The substrate and coenzyme apparent K_m values tend to agree more with those reported for vertebrates (8, 11, 15, 18, 36, 38) and higher plants (1, 12, 37) than those of *Drosophila* (26), *Chlorella* (41), sea urchin (32), or fungi (2, 30, 42). The discrepancies noted between the vertebrate values and those of *Physarum* are with coenzymes rather than substrates.

The stereospecificity of the isoenzymes for substrate is noted by the absence of activity with D-malate. Also, the L-malate inhibition pattern, whereby S-MDH is inhibited more than M-MDH, is in agreement with vertebrate systems (18, 20).

The substrate inhibition pattern for OAA is unusual. From a number of sources, the effect of excess OAA on the S-MDH is negligible, whereas the M-MDH is inhibited (2, 3, 18, 20, 28). Ternary complexes formed from enzyme, substrate, and coenzyme, either viewed as abortive (35) or of reduced catalytic function (15), are suggested as the cause of this inhibition phenomenon. In *P. polycephalum*, both isoenzymes are inhibited, with the S-MDH being inhibited much more strongly than M-MDH. A similar instance has been reported for chicken heart (18) in the early stages of inhibition, but the M-MDH was inhibited much more strongly

FIG. 8. Comparison of the thermal stability of M-MDH and S-MDH. (A) Samples of 1 ml of each isoenzyme containing 1 enzyme unit (as measured by OAA reduction) previously warmed to 25 C for 15 min were plunged into preset water baths at temperatures between 25 and 70 C and left for 15 min. At the end of the time period, the samples were immediately plunged into ice. (B) Samples of 1 ml of each isoenzyme containing 1 enzyme unit (as measured by OAA reduction) were warmed to 25 C for 15 min and were immediately plunged into a preset water bath at 50 C. Samples of each isoenzyme at 50 C were removed every 5 min for 30 min, then every 10 min for an additional 30 min, and cooled immediately. Warmed (25 C) samples not placed in the baths were placed in ice and served as the control for 100% activity. Each sample was analyzed for enzyme activity (OAA reduction) by use of previously determined optimal assay conditions. Symbols are the same as in Fig. 5.

than S-MDH as the OAA concentration increased. In other organisms (7, 39), phosphate ions are known to prevent the inhibition of M-MDH by OAA. However, we found identical inhibition patterns in Tris buffer as well as phosphate buffer. The OAA inhibition pattern therefore constitutes a definite difference between *P. polycephalum* and higher organisms. However, the M-MDH and S-MDH are distinguishable on the basis of their substrate inhibition patterns.

Like other systems, neither isoenzyme could utilize either NADP or NADPH₂, but both could utilize several of the analogues of NAD. Both isoenzymes readily used APHD and APAD in place of NAD. Also, the relative reactivities

with the analogues served to distinguish between the isoenzymes. In general, the reactivities of the isoenzymes with the various analogues tend to agree with results on vertebrate (44), plant (12, 29, 37), and fungal (2, 4, 19, 42) systems.

That S-MDH is more heat-stable than M-MDH agrees with the results on MDH from chicken (18), tuna (20), spinach (37), yeast (2), and *Ascaris* (45). However, the opposite condition has been reported for *Drosophila* (26) and cactus (29).

Although it is generally accepted that M-MDH participates in the tricarboxylic acid cycle (29), the role for S-MDH is unclear. The usefulness of having two localized isoenzymes

becomes very important when one considers that: in aerobic organisms NADH_2 produced by the Embden-Meyerhof pathway (EMP) is primarily oxidized by means other than lactate dehydrogenase, but NADH_2 cannot permeate mitochondria (34); a likely major control on the EMP is the NAD to NADH_2 ratio (5); and mitochondrially formed acetyl-coenzyme A (CoA) must be transferred to the cytoplasm (e.g., for lipid synthesis) (24). In vertebrates, the electrons of NADH_2 are thought to enter the mitochondria, and acetyl-CoA exit, by shuttle mechanisms.

According to the model of Lowenstein (24), citrate formed intramitochondrially is shuttled extramitochondrially where it is cleaved to OAA and acetyl-CoA. The OAA formed is then reduced to malate, and malate reenters the mitochondrion where it is converted to OAA and again condenses with acetyl-CoA to form citrate.

However, the malate-aspartate shuttle (22) is thought to be the principal means for removal of reducing equivalents from the cytoplasm to the mitochondria. Intramitochondrially, malate is oxidized to OAA by the tricarboxylic acid cycle with the concomitant reduction of NAD . The OAA so formed is then transaminated with glutamate to form aspartate and α -ketoglutarate. After the latter two compounds exit the mitochondria, they are transaminated back to OAA and glutamate. Glutamate reenters the mitochondrion to complete its part of the cycle. OAA is reduced to malate and thus transfers the electrons from extramitochondrial NADH_2 to malate, which reenters the mitochondrion. This model is supported by the finding that energy-rich mitochondria export malate nonenergetically (as a possible source of cytoplasmic NADH_2) and import malate in the energy-poor state (25).

P. polycephalum is an obligate aerobe and malate is known to permeate its mitochondria (6), but whether these shuttle systems apply to *P. polycephalum* is unknown.

Future studies will entail the detailed physicochemical properties of the MDH proteins, and analyses of the regulatory properties and the nature of the catalytic center.

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