Registry No. MeOH, 67-56-1; EtOH, 64-17-5; $CH_3(CH_2)_4OH$, 71-41-0; $CH_3(CH_2)_7OH$, 111-87-5; *i*-PrOH, 67-63-0; $PhCH_2OH$, 100-51-6; 12-HDDA, 505-95-3; 16-HHDA, 506-13-8; alcohol dehydrogenase, 9031-72-5.

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Malate Dehydrogenase from *Rhizobium japonicum* 3I1b-143 Bacteroids and *Glycine max* Root-Nodule Mitochondria†

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ABSTRACT: The enzyme malate dehydrogenase (EC 1.1.1.37) has been purified (85-fold) from mitochondria of *Glycine max* (Williams 79 cultivar) root nodules and from *Rhizobium japonicum* 3I1B-143 bacteroids (400-fold). The mitochondrial malate dehydrogenase (m-MDH) has a native molecular weight of 69 000. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of the protein resulted in two protein bands of equal intensity with molecular weights of 39 000 and 37 000, respectively. The pH optimum of m-MDH is 6.5–7.3 for the conversion of oxaloacetate (OAA) to L-malate and 8.2–8.6 for the conversion of L-malate to OAA. Reduced nicotinamide adenine dinucleotide (NADH), but not OAA, protected m-MDH against heat inactivation at 55 °C. The bacteroid malate dehydrogenase (b-MDH) has a native molecular weight of 139 000 as analyzed by native polyacrylamide gel electrophoresis. SDS–polyacrylamide gel electrophoresis of b-MDH resulted in one protein band with a molecular weight corresponding to 36 000. The pH optimum for conversion of OAA to L-malate is 8.0–8.5, while the pH optimum in the reverse direction is 8.6–9.0. NADH, but not OAA, provided almost complete protection against heat inactivation at 55 °C. Arrhenius plots of the two enzymes gave Q_{10} values of 2.34 for the b-MDH and 1.62 for the m-MDH. The Michaelis constants of both malate dehydrogenases were determined and found to be similar to those of malate dehydrogenases from other sources. Kinetic analysis of b-MDH indicated an ordered bi-bi mechanism with NAD^+ adding first and NADH leaving last.

The metabolism of organic acids fulfills a principal role in the symbiotic nitrogen-fixation process (Bach et al., 1958; Bergersen & Turner, 1967; Finan et al., 1983; Glenn et al., 1980; Reibach & Streeter, 1984; Ronson et al., 1981). In the symbiotic relationship between leguminous plants and the *Rhizobium* sp., for example, soybeans and *Rhizobium japonicum*, the actual site of nitrogen fixation occurs within the bacteroid, the phenotypically distinct state of the bacteria found within the plant root-nodule tissue. The plant provides the energy for the nitrogen-fixation process by supplying the bacteroids with photosynthetically derived carbon compounds.

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Organic acids (cited in this paper as the ester form) are believed to be the principal compounds that the bacteroids require. Exogenous organic acids, particularly succinate, fumarate, and malate, promote the greatest rates of nitrogen fixation in isolated suspensions of soybean root-nodule bacteroids (Bergersen & Turner, 1967; Bach et al., 1958). In the soybean nodule, the major organic acids are (μmol/g fresh weight) malonate (5.40 ± 0.4), malate (4.12 ± 0.21), fumarate (1.27 ± 0.21), and succinate (0.76 ± 0.10) [D. G. Blevins, unpublished results; see also Stumpf & Burris (1981)]. Reibach & Streeter (1983) isolated radiolabeled carbon metabolites of nodules via translocation of photosynthate synthesized from $^{14}CO_2$. The major organic acid was malate, comprising 45% of the total radioactivity of the organic acid fraction. Lawrie & Wheeler (1975) observed high levels of radiolabeled $^{14}CO_2$ incorporation into malate in *Vicia faba* nodules. In *Pisum sativum* nodules, De Vries et al. (1980) have estimated the malate concentration to be 3.4 mM. Reibach & Streeter (1984) reported that malate, succinate, and ketoglutarate are actively transported into *R. japonicum* bacteroids whereas glucose and glucose 6-phosphate are not.

To determine the role of organic acids in symbiotic functioning, characterization of both metabolite concentrations and enzyme activities is required. The carbon metabolic pathways of the host plant and of the bacteroid are still obscure. Although a fully functional citric acid cycle has been implied to operate in the bacteroid (Stovall & Cole, 1978), few of the enzymes have been measured and characterized.

There are few reports describing malate dehydrogenase from either the bacteroid or the plant root nodule. Malate dehydrogenase catalyzes the reversible reaction malate + NAD^+ $\rightleftarrows$ oxaloacetate + NADH. Malate dehydrogenase has been measured in bacteroid extracts from *Phaseolus vulgaris* (Grimes & Fottrell, 1966), *P. sativum* (Kurz & La Rue, 1977; De Vries et al., 1980), and *Glycine max* (Karr et al., 1984). Kurz & La Rue (1977) reported that pea bacteroid malate dehydrogenase activity decreased with plant age whereas in soybean bacteroids the activity remained constant (Karr et al., 1984). De Vries et al. (1980) examined the effect of O_2 stress on the specific activity of malate dehydrogenase in roots of infected plants and uninfected plants. The specific activity of cytosolic malate dehydrogenase drops 25% in the uninfected plant when O_2 is limited compared to a 1% decrease of the cytosolic malate dehydrogenase in infected plant roots.

This report describes the purification of the bacteroid malate dehydrogenase (b-MDH)[1] and the plant root-nodule mitochondrial malate dehydrogenase (m-MDH). We have found that plant enzymes [and lipids; see Miller & Tremblay (1983)] adhere to the bacteroid exopolysaccharides during isolation and subsequently contaminate bacteroid crude extracts. The comparative nature of this report arises from the need to distinguish b-MDH from m-MDH. This paper describes the physical–chemical characterization of these two enzymes.

Experimental Procedures

Source of Materials. Soybean seeds (Williams 79 cultivar) were inoculated with *R. japonicum*, strain 3I1b-143, which was supplied by Dr. Harold Evans, Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, OR.

Buffers and chemicals were purchased from Sigma Chemical Chemical Co. unless stated otherwise. Polyclar AT [poly(vinylpyrrolidone), PVP] was purchased from GAF Corp., New York. Before use, PVP was washed with 10% hydrochloric acid, neutralized, washed with distilled water, and dried.

Electrophroresis chemicals, hydroxylapatite, and DEAE-Affi-Gel Blue were purchased from Bio-Rad Laboratories. Amicon Matrex affinity gels were purchased from Amicon Corp., Danvers, MA. SDS molecular weight standards and native molecular weight standards were purchased from Sigma and Bio-Rad.

Nodules were grown from soybean seeds coated with a Bactosoil-based inoculum of *R. japonicum* 3I1b-143 and planted in 8-in. pots containing Perlite as the growth support. Plants were supplied daily with a nitrogen-free nutrient solution (Ahmed & Evans, 1960) for 5 days and flushed with tap water on the sixth and seventh days. Nodules were harvested from 6–8 week old greenhouse-grown plants and then frozen at −80 °C until used.

Isolation of Bacteroids and Purification of Malate Dehydrogenase. Nodules were disrupted (Karr et al., 1984) with a Waring blender in a solution of 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM potassium phosphate buffer, pH 7.2 (MEP), containing 17% sucrose (w/v) (MEPS), and in PVP (1 g of nodule, $^1/_3$ g of PVP, and 10 mL of MEPS). A typical preparation began with 20 g of nodules. The preparation was filtered through four layers of cheesecloth and centrifuged at 400*g* for 10 min at 5 °C. The pellet was discarded, and the supernatant was centrifuged at 8000*g* for 20 min at 5 °C.

The pellet was dispersed into the MEPS buffer (1 mL/g original weight of nodules) and was layered onto a gradient consisting of 30% (10 mL), 40% (5 mL), and 57% (6 mL) (w/w) sucrose in MEP. The gradient tubes were centrifuged in an SW-28 rotor at 72000*g* for 35 min at 5 °C in a Beckman L8-55 ultracentrifuge. The bacteroid layer at the 40%/57% sucrose interface was collected, and after dilution with 2 volumes of MEP, it was applied to a second gradient composed of 30% (15 mL) and 40% (10 mL) (w/w) sucrose. The tubes were centrifuged as described above. The pellet was suspended (1 mL/g original weight of nodules) in 25 mM phosphate–MED (5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT) buffer and broken in a prechilled French pressure cell at 16 000 psi. The ruptured bacteroid suspension was centrifuged in an SS-34 rotor at 33300*g* for 30 min at 5 °C with a Sorvall RC-5B centrifuge. The resulting supernatant, referred to as the crude extract, was concentrated with $(NH_4)_2SO_4$ (25% saturation), pH 7.2, for 30 min at 4 °C and centrifuged as described above. The $(NH_4)_2SO_4$ (25% saturation) supernatant was separated from the pellet, and the $(NH_4)_2SO_4$ concentration was adjusted to 90% saturation, pH 7.2, and centrifuged as described earlier. The pellet was suspended in 25 mM phosphate–MED buffer and fractionated on a DEAE-Affi-Gel Blue column (2.5 cm × 22 cm) by using a linear 0–700 mM NaCl gradient in 25 mM phosphate–MED buffer. The active fractions from the DEAE-Affi-Gel Blue column were combined and desalted by repeated concentration/dilution cycles with an Amicon ultrafiltration protein concentrator (PM-10 membrane). The desalting buffer was 5 mM phosphate–MED buffer.

The concentrate was applied to a hydroxylapatite column (2.5 cm × 5 cm) in 5 mM phosphate–MED buffer, pH 7.2, and protein was eluted from the column by using a linear 5–500 mM phosphate buffer gradient. The fractions con-

[1] Abbreviations: BSA, bovine serum albumin; b-MDH, bacteroid malate dehydrogenase; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CoA, coenzyme A; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HAP, hydroxylapatite; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; m-MDH, mitochondrial malate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; pI, isoelectric point; PMSF, phenylmethanesulfonyl fluoride; PVP, poly(vinylpyrrolidone); OAA, oxaloacetate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

taining bacteroid malate dehydrogenase activity were collected, concentrated to approximately 10 mL with the protein concentrator, and desalted against 25 mM phosphate–MED buffer. The desalted enzyme preparation was applied consecutively to the Amicon Matrex affinity gels (1 cm × 3 cm) Red A, Orange A, and Green A. In each case the enzyme was eluted with 25 mM phosphate–MED buffer containing 1.4 M NaCl and desalted between each column run as described above.

Standard Assay for Malate Dehydrogenase. Enzyme assays were monitored at 340 nm in a 3-mL quartz cuvette with a Gilford Model 250 spectrophotometer. Enzyme activity was measured at room temperature (22–25 °C) unless stated otherwise. The final enzyme reaction mixture for the contained 0.2 mM NADH, 0.5 mM oxaloacetate, and 94 mM HEPES buffer, either at pH 7.4 (m-MDH) or at pH 8.3 (b-MDH) (*Biochemical Information*, 1973). The NADH and oxaloacetate solutions were prepared daily and neutralized with 100 mM HEPES buffer.

Determination of Optimum pH. The pH optimum of the reaction in the direction of malate formation was determined by using the standard assay procedure for malate dehydrogenase above but substituting a mixture of mono-, di-, and tripotassium phosphate salts for the HEPES buffer. Assays were performed in quadruplicate, and the pH was measured at the end of the assay. The pH optimum of the reaction in the direction of oxaloacetate formation was determined under the following assay conditions (final concentrations): 96 mM HEPES or CHES buffer, 0.17 mM NAD^+, 1.7 mM L-malate, 0.004 unit of citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.7, type II from pigeon breast muscle), and 0.024 mM acetyl-CoA. An increase in the L-malate concentration or the NAD^+ concentration did not increase NADH formation. The background rate was determined by mixing all reagents minus the malate dehydrogenase enzyme and monitoring for 2 min. The reaction was initiated by enzyme addition to the cuvette and monitored for 3–5 min. For kinetic analyses, initial rates of NADH oxidation or NAD^+ reduction were determined in quadruplicate, and the data were treated by linear regression analysis utilizing the computer programs of Cleland (1967).

Arrhenius Plot and Heat Stabilization. Temperature control was achieved by using a Haake circulating water bath attached to the Gilford cuvette chamber, which permits rapid circulation of water and temperature control (±0.1 °C). Cuvettes contained the standard assay mixture for malate dehydrogenase at its respective pH (pH 8.3 for b-MDH and pH 7.4 for m-MDH). The assay mixture was preincubated in the cuvette chamber for 5 min and monitored for the background rate. Addition of enzyme was used to start the reaction. Every temperature point was repeated in quadruplicate, and the temperature was measured directly at the end of each assay.

Heat Stabilization. Aliquots of enzyme solutions were incubated in a NAPCO Model 220 water bath (±1.0 °C). Enzyme was diluted 1:1 with 100 mM HEPES buffer (pH 7.4 for m-MDH and pH 8.3 for b-MDH) containing one of the following: 12 mM NADH or 15 mM OAA. For controls, 100 mM HEPES buffer, pH 7.9, was added. After dilution, the enzyme was placed in the water bath at 55 ± 1.0 °C. Aliquots were taken from the enzyme solution at indicated intervals and stored on ice until the activity could be determined by using the standard assay mixture. Each time point was repeated in triplicate. The pHs of the heated enzyme solutions were measured, after they were cooled to room temperature, and were found not to have changed significantly.

Enzyme Inhibition. Equal amounts of enzyme were mixed with inhibitors (1 mM final concentration for each inhibitor) at room temperature (22–25 °C) and assayed for activity with the standard assay mixture at the indicated times.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) or by the method of Bradford with BSA as the protein standard. The Bradford (1976) method was used in the final purification steps as the Lowry et al. (1951) protein determination method was insensitive at these protein concentrations (≪0.5 mg).

Electrophoretic Methods. Polyacrylamide vertical slab-gel (1.5 mm × 160 mm × 180 mm) electrophoresis utilizing a discontinuous buffer system was performed according to the method of Laemmli (1970). The upper and lower buffer reservoirs contained Tris-HCl (0.025 M) and glycine (0.192 M) with/without 0.1% SDS (w/v), pH 8.3. The chamber was cooled by circulating tap water. The separation gel, in 0.38 M Tris-HCl, pH 8.8, consisted of 28 mL of 12% acrylamide/bis(acrylamide) (30.0 g of acrylamide/0.8 g of *N*,*N*-methylenebis(acrylamide) per 100 mL of H_2O) (with/without 0.4% SDS). The stacking gel, in 0.5 M Tris-HCl, pH 6.8, consisted of 4.5 mL of 6% acrylamide solution (with/without 0.4% SDS w/v). Riboflavin (native gels only) was used as the radical-forming agent because residual ammonium persulfate reacted with the bacteroid malate dehydrogenase, causing multiple activity bands on native gels stained for activity and miscalculation in native molecular weight determinations (data not shown). Activity stain solution consisted of 100 mL of 0.2 M DL-malate, 0.003 M nitro blue tetrazolium, 0.004 M NAD^+, and 0.0007 M phenazine methosulfate, pH 8.3 (Gabriel, 1971). Gels were incubated in the activity stain solution for approximately 15 min at room temeprature or until violet bands appeared. Silver stain was utilized for protein detection in acrylamide slab gels; Bio-Rad silver stain reagents and protocol were followed except for extending the developer step to 30 min. Low protein content of the samples required a longer developing time and resulted in both darker background and more intense staining of the protein standards. SDS low molecular weight standards from Sigma were used to calculate the subunit molecular weights of the two enzymes; a standard curve was used. Native molecular weight determinations (*Sigma Technical Bulletin*, 1983) were made in polyacrylamide slab gels (1.5 mm × 160 mm × 180 mm) over the following range of acrylamide concentrations: 7.5, 8.5, 9.5, 10.5, 11.5, and 12.4%. α-Lactalbumin was not used as a molecular weight standard because of inaccurate protein migration at the lower gel concentrations. Native gels were first stained for activity and then for protein.

Analytical isoelectrofocusing was performed by the LKB electrofocusing procedure and with LKB ampholine PAG plates (pH 3.5–9.5). The sample consisted of crude soluble protein (5 mg/mL) from bacteroids in a 1% glycine and ampholyte (pH 3.5–9.5) buffer. The sample was applied to the gel by using filter paper (Whatman No. 3, 6 mm × 6 mm) that had been previously soaked in the sample and then placed on the gel surface. The electrical power supply output was set on the constant current mode; the initial settings were adjusted to 5 W and 250 V. The proteins were subjected to electrophoresis for approximately $1^1/_2$ h. The gel was immediately stained for malate dehydrogenase activity. The proteins were fixed onto the gel with a trichloroacetic acid/sulfosalicylic acid solution. The gel was stained for the isoelectric point (pI) standard protein markers with Coomassie blue protein stain. The pI was determined by comparison to

Table I: Comparison of b-MDH and m-MDH Purification Procedures[a]

	total vol (mL)	total protein (mg)	total act. (μmol/min)	sp act. (μmol min^{-1} mg^{-1})	% recovery	x-fold purification
bacteroid						
bacteroid crude extract	29.9	400	1256	3.14	100	
25–90% $(NH_4)_2SO_4$ supernatant	34	208	1193	5.74	95	2
DEAE-Affi-Gel Blue	50	11.7	497	42.5	42	7
hydroxylapatite	26	2.16	538	249	43	79
Amicon Matrex gels	17	0.26	330	1270	26	404
mitochondria						
mitochondrial crude extract	20.5	23.5	462	19.7	100	
25–90% $(NH_4)_2SO_4$ supernatant	24	5.04	412	81.8	89	4
DEAE-Affi-Gel Blue	20	0.40	221	552.5	48	28
hydroxylapatite	4	0.09	102	1133	22	58
Amicon Matrex gels	10.5	0.02	3350	1675	7	85

[a] Purification of b-MDH began with 20 g of 4–6 week old greenhouse-grown soybean nodules. Purification of m-MDH began with 40 g of 4–6 week old greenhouse-grown soybean nodules. For details of purification procedures, see Materials and Methods.

the pI standard protein markers (FMC Corp., Marine Colloid Division, Rockland, ME).

Results

The purification data of bacteroid malate dehydrogenase (b-MDH) and of soybean root-nodule mitochondria malate dehydrogenase (m-MDH) are shown in Table I. The specific activity of b-MDH in crude extracts is 3 μmol min^{-1} (mg of protein)$^{-1}$ whereas m-MDH, typically, has a higher specific activity of 20 μmol min^{-1} (mg of protein)$^{-1}$. The specific activity of b-MDH is the highest found in this laboratory for any of the enzymes examined in bacteroid crude extracts (Karr et al., 1984) and is similar to the values reported from other sources (Kurz & La Rue, 1977; De Vries et al., 1980). The protein has been purified approximately 400-fold to a specific activity of 1200 μmol min^{-1} (mg of protein)$^{-1}$. Mitochondrial malate dehydrogenase has been purified approximately 85-fold to a specific activity of 1700 μmol min^{-1} (mg of protein)$^{-1}$.

Native and SDS discontinuous polyacrylamide gel electrophoreses of crude extracts or partially purified extracts after ammonium sulfate precipitation and DEAE-Affi-Gel Blue chromatography show indistinct protein bands (data not shown). Once the protein extract is eluted from a hydroxylapatite column, individual protein bands become sharply focused. Bacteroid protein extracts contain 0.1–1.0 mg of polysaccharides (mg of protein)$^{-1}$ (Smith and Emerich, unpublished results). Exopolysaccharides are highly charged and thus can adhere to proteins and interfere with their purification (Emerich & Burris, 1978). The hydroxylapatite step has been demonstrated to remove exopolysaccharides from *Bacillus polymyxa* extracts (Emerich & Burris, 1978) and also from *R. japonicum* bacteroid extracts (Smith and Emerich, unpublished results). Eluting the column with low concentrations of phosphate buffer (1–5 mM) removes the exopolysaccharides, but the proteins remain on the column. Hydroxylapatite chromatography of partially purified b-MDH obtained from bacteroids isolated by the method of Emerich et al. (1979) is shown in Figure 1. The hydroxylapatite column was eluted with a 5–500 mM potassium phosphate gradient containing 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT at pH 7.0. The first peak of activity was identified by native gel electrophoresis as being m-MDH (present as a contaminant due to incomplete separation of bacteroids from plant components) whereas the second activity peak corresponded to b-MDH. Figure 2 shows a native discontinuous polyacrylamide gel of an 8000*g* bacteroid pellet prior to sucrose density gradient centrifugation. The gel was stained by using a malate dehydrogenase activity stain (Gabriel, 1971). The presence of m-MDH exemplifies the extent of contamination

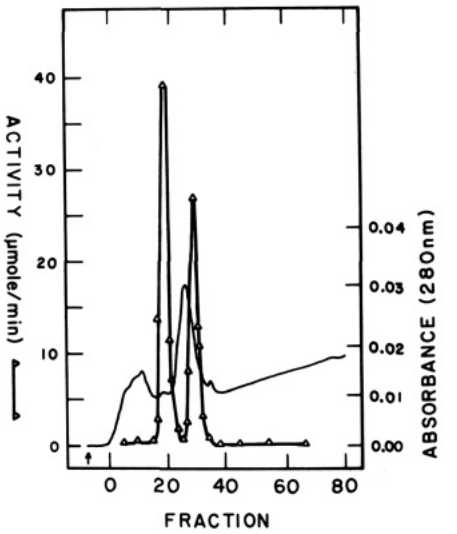

FIGURE 1: Elution profile of partially purified b-MDH from a hydroxylapatite (HAP) column. The crude extract (containing both b-MDH and m-MDH) was applied to a HAP column (1.0 cm × 5.0 cm) in 5 mM phosphate–MED buffer, pH 6.8. The column was washed with 5 column volumes of buffer, and a gradient (5–500 mM phosphate, 400-mL total volume) was started at the arrow. Fractions contained 2.5 mL per tube. The first activity peak (fractions 16–21) was determined to be m-MDH by activity stain on native discontinuous polyacrylamide gels (10% acrylamide), and the second activity peak was b-MDH (fractions 27–31).

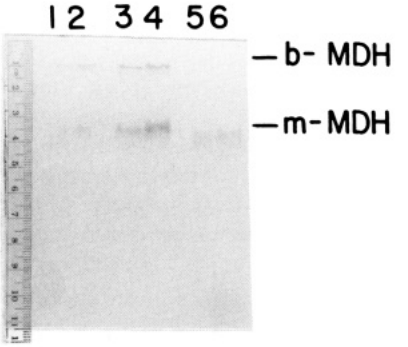

FIGURE 2: Activity-stained native polyacrylamide gel. Lanes 1–4 contain a clarified crude protein extract (membranes precipitated) from an 8000*g* bacteroid pellet. *R. japonicum* malate dehydrogenase corresponds to the upper activity band in lanes 1–4. The 10% polyacrylamide gel was stained by the method of Gabriel (1971). Lanes 5 and 6 contain malate dehydrogenase from sucrose density gradient purified plant mitochondria.

by plant enzymes. Furthermore, a single gradient step, either linear or discontinous, is not sufficient to remove contaminating material to acceptable levels (data not shown). The isolation

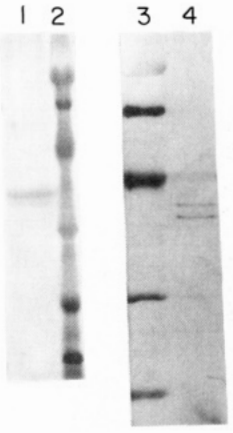

FIGURE 3: Two silver-stained SDS discontinuous polyacrylamide gels (12% acrylamide) of b-MDH and m-MDH after purification. Lane 1 contains b-MDH (approximately 100 ng of protein). Lane 4 shows the two protein bands of m-MDH (approximately 10 ng of protein). Lanes 2 and 3 contain molecular weight standards from Bio-Rad.

method described under Experimental Procedures removes all measurable m-MDH from bacteroid extracts.

Figure 3 shows a 12% SDS discontinuous polyacrylamide gel of the two enzymes after purification. b-MDH (lane 1, approximately 100 ng of protein) appears to be >90% pure as judged by SDS gel electrophoresis. The darkened background and overstaining of the molecular weight standard proteins (lanes 2 and 3) are from prolonged development (>30 min). Lane 4 shows the two subunits of m-MDH (<20 ng of protein). The two protein bands from the m-MDH have not been shown conclusively to be subunits of the enzyme, but throughout the purification each band copurifies and stains with equal intensity, suggesting that m-MDH may be composed of protein subunits that differ in their molecular weights. Estimation of the subunit molecular weight from known molecular weight standards ($r^2 = 0.96$) gave values for b-MDH of 36 000 ± 2000 and for m-MDH of 39 000 ± 1000 and 37 000 ± 1000, respectively. Native molecular weights have been estimated to be 138 600 ± 8200 for b-MDH and 69 400 ± 2500 for m-MDH. The isoelectric points of b-MDH and m-MDH were estimated to be 7.5–7.7 and 5.8–5.9, respectively.

Purified b-MDH (15 μg/mL) is stable for more than 1 month in 25 mM phosphate buffer containing 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT, pH 7.4 at 4 °C, retaining more than 80% of its activity. Mitochondrial malate dehydrogenase is very unstable. Under these same conditions, less than 20% of its activity remains. Mitochondrial malate dehydrogenase is stored at 4 °C in 25 mM phosphate–MED buffer.

When subjected to 55 °C for 20 min, both enzymes lose appreciable activity (Table II). The presence of NADH stabilizes both enzymes equally. Eighty to ninety percent of the activity of both enzymes is retained in the presence of NADH. NADH appears to activate m-MDH in the zero-time control in Table II, but in other experiments this was not observed. In the presence of oxaloacetate, b-MDH loses nearly 95% of its original activity whereas m-MDH loses 10–40% of its original activity. Although b-MDH is significantly more sensitive to oxaloacetate at 55 °C, both enzymes display decreased activity in the presence of oxaloacetate in the zero-time control assays.

Arrhenius plots have been used to distinguish between bacterial enzymes of different species (Meynell & Meynell, 1975). Figure 4 shows that b-MDH ($Q_{10} = 2.17 \pm 0.17$) and m-MDH ($Q_{10} = 1.56 \pm 0.05$) have distinctly different Q_{10}

Table II: Activity Remaining after Heat Treatment at 55 °C[a]

enzyme	addition[c]	μmol/min (% initial act.) at time of treatment[b] 0 min	20 min
b-MDH	control	12.01 ± 0.20 (100)	3.18 ± 0.41 (26)
b-MDH	NADH	12.01 ± 0.25 (100)	10.41 ± 0.39 (87)
b-MDH	OAA	7.67 ± 0.15 (100)	0.39 ± 0.08 (5)
m-MDH	control	1.39 ± 0.07 (100)	0.50 ± 0.07 (36)
m-MDH	NADH	1.83 ± 0.06 (100)	1.48 ± 0.04 (81)
m-MDH	OAA	0.60 ± 0.01 (100)	0.34 ± 0.01 (57)

[a] Assays were performed as described under Experimental Procedures. [b] After heat treatment for the specified time, samples were cooled in an ice bath until assays could be performed. [c] Concentrations of NADH and OAA during heat treatment were 12 and 15 mM, respectively. An equal volume of 100 mM HEPES buffer, pH 7.9, was added to the controls.

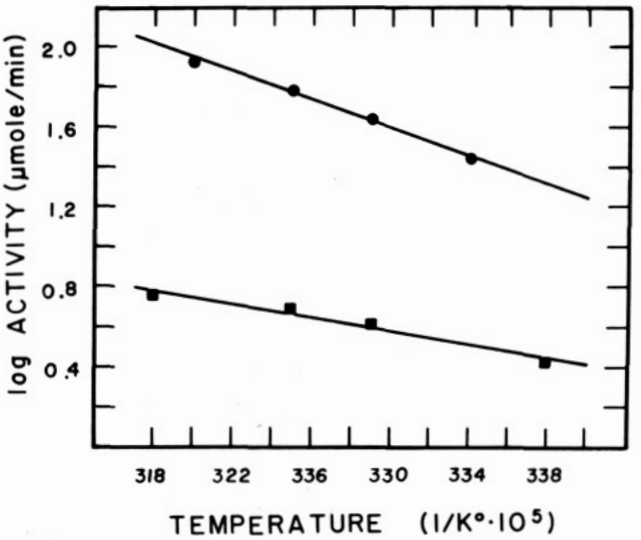

FIGURE 4: Arrhenius plot of b-MDH and m-MDH. The Q_{10} coefficient (change in activity per 10 K change in temperature) for b-MDH (●) is 2.17 ± 0.17 and for m-MDH (■) is 1.56 ± 0.05. Enzyme activity was measured by following the formation of malate.

coefficients, which would be expected since these enzymes are from a procaryote and an eucaryote, respectively. The energy of activation was estimated to be 15.8 kcal/mol for b-MDH and 7.7 kcal/mol for m-MDH.

The pH profiles of the two enzymes in the direction of malate formation are shown in Figure 5A. The optimum pH range for the b-MDH is between 8.0 and 8.5 whereas m-MDH has an optimum pH range between 6.0 and 7.6. The pH optimum in the direction of oxaloacetate formation for both enzymes is shown in Figure 5B. Bacteroid malate dehydrogenase has an optimum pH range between 8.6 and 9.0, and m-MDH has an optimum pH range between 8.3 and 8.6.

The effects of several active-site inhibitors on MDH from both sources were determined (Figure 6). Iodoacetamide had little effect on b-MDH whereas it caused a progressive inhibition of m-MDH to the extent of 85% after 70 min of incubation (Figure 6). The pseudo-first-order rate constant for iodoacetamide inhibition of m-MDH was 0.0260 min^{-1}. Phenylmethanesulfonyl fluoride (PMSF) also displayed a differential inhibition between the two enzymes. Bacteriod malate dehydrogenase was not inhibited by PMSF, but the m-MDH was inhibited with a pseudo-first-order rate constant of 0.0150 min^{-1} for the second phase of inhibition. The rapid first phase of inhibition was found to be due to the ethanol used to solubilize the PMSF (data not shown). Pyrocarbonate (1 mM) rapidly and completely inhibited both enzymes (data not shown). This inhibitor reduced the activity of b-MDH

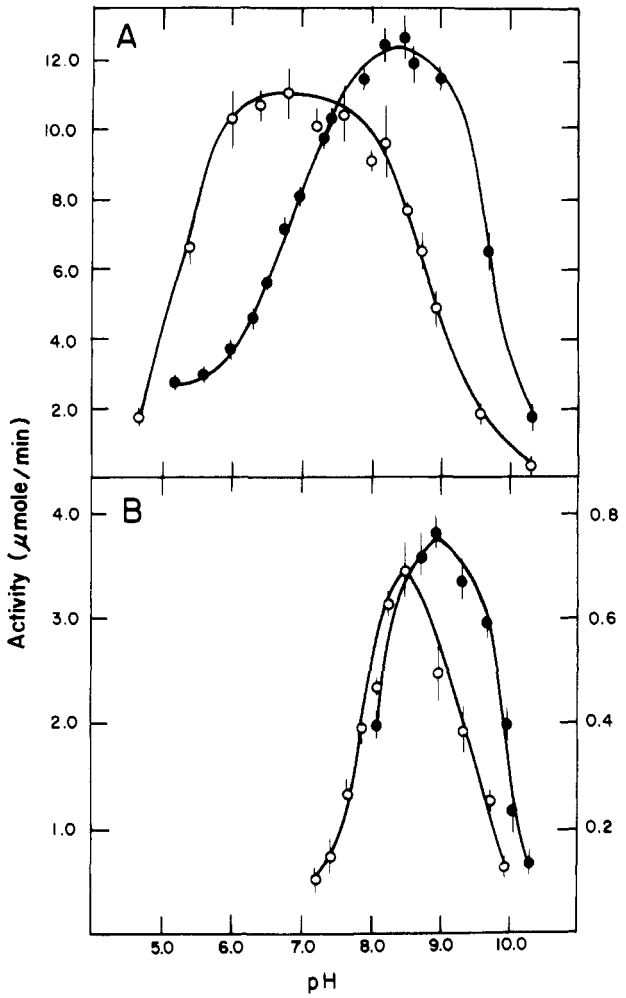

FIGURE 5: Effect of pH on purified b-MDH (●) and m-MDH (○). Panel A shows the effect of pH on oxaloacetate reduction by b-MDH and m-MDH. Panel B shows the effect of pH on malate oxidation of purified b-MDH and m-MDH. The pH was determined after measurement of the enzyme activity. See Experimental Procedures for assay conditions.

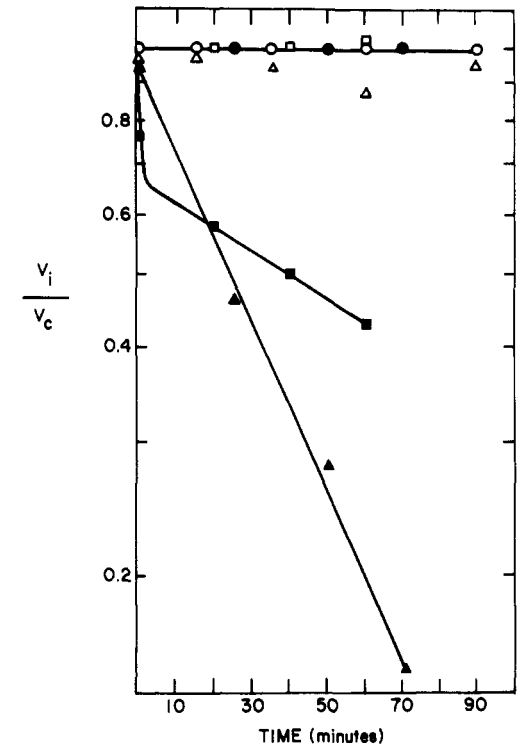

FIGURE 6: Reaction of active-site reagents with b-MDH and m-MDH. Assay conditions are described under Experimental Procedures. b-MDH, open symbols; m-MDH, closed symbols. All inhibitors were present at 1 mM concentrations: iodoacetamide (△, ▲), phenylmethanesulfonyl fluoride (□, ■), and no inhibitor controls (○, ●).

Table III: Kinetic Constants for b-MDH and m-MDH[a]

	substrate (μM)			
enzyme	malate	NAD^+	oxaloacetate	NADH
b-MDH	730	690	40	20
m-MDH	10020	1270	70	44

[a] Assays were performed as described under Experimental Procedures. Concentrations of the variable substrate ranged from approximately 0.2 to 5 K_m. The concentration of the fixed substrate was approximately 10 K_m.

by 90% and m-MDH by 85% in the zero-time assays, with complete inactivation within 5 min for both enzymes. Pyridoxal 5′-phosphate caused 30–40% inhibition of both enzymes after 60 min with pseudo-first-order rate constants for inactivation of b-MDH and m-MDH of 0.00768 min^{-1} and 0.0161 min^{-1}, respectively (data not shown).

The kinetic mechanism of both b-MDH and m-MDH is similar to that previously reported for malate dehydrogenase from other sources (Dixon & Webb, 1979; Silverstein & Subele, 1969). The initial velocity patterns of both b-MDH and m-MDH indicated a sequential mechanism (data not shown). The product inhibition patterns showed NADH vs. NAD^+ to be competitive and NADH vs. malate, NADH vs. oxaloacetate, NAD^+ vs. oxaloacetate, and malate vs. oxaloacetate to be noncompetitive (data not shown). These kinetic patterns are consistent with an ordered bi–bi mechanism with NADH binding first, followed by the binding of oxaloacetate; malate is released as the first product, followed by NAD^+. The various kinetic constants are shown in Table III.

Discussion

The activities of a variety of enzymes from *Rhizobium* bacteroids have been reported by many laboratories. However, few investigators have taken precautions to ensure that bacteroid preparations are entirely freed of contaminating plant enzymes. Recently, Miller & Tremblay (1983) characterized the lipid composition of *Rhizobium meliloti* membranes and have reported the presence of plant-type lipids in their bacteroid preparations. These authors attribute these results to the presence of contaminating plant membranes in their bacteroid membrane preparations. Our experience with m-MDH and b-MDH has indicated that contamination of bacteroid extracts with plant proteins is unavoidable when most commonly used isolation methods are employed. Alternate procedures must be adopted to remove plant material adhering to the bacteroid exopolysaccharide. The two-step sucrose gradient procedure used in this report greatly reduces this contamination. The high activity of mitochondrial malate dehydrogenase makes it a suitable candidate for monitoring plant enzyme contamination. Thus we have sought to determine the characteristic biochemical differences between m-MDH and b-MDH that are to be used as an index of contamination.

The mitochondrial and bacteroid malate dehydrogenases have been purified 85- and 400-fold, respectively, and each can be readily distinguished on activity-stained native polyacrylamide gels due to differences in migration. The native molecular weights of the bacteroid and mitochondrial enzymes are 138 600 and 69 400, respectively. The molecular weights of many bacterial MDH's are approximately 70 000. However, certain species of the order Eubacteriales have molecular

weights around 120 000 (Murphy et al., 1967a). The only representative of the family Rhizobiaceae included in the survey was *Chromobacterium violaceum*, which contained a low molecular weight MDH (Murphy et al., 1967a). In contrast, the molecular weight of MDH from *R. japonicum* reported here is 140 000. The families Corynebacteriaceae and Brevibacteriaceae each contain representatives that possess either the low or the high molecular weight forms. Thus the finding that the family Rhizobiaceae has species that represent both forms is not unprecedented. The subunit molecular weights of the MDH's from *Escherichia coli* and *Bacillus subtilis* range between 33 000 and 35 000 (Banaszak & Bradshaw, 1975).

The molecular weight estimations of mammalian m-MDH's are approximately 70 000 (Banaszak & Bradshaw, 1975). There are few reports describing the molecular weights of plant m-MDH's, but they generally are similar to those of mammalian sources (Murphy et al., 1967a; Yang & Scandalious, 1974). Mammalian MDH's are composed of two identical subunits of approximately 33 000–35 000 molecular weight (Banaszak & Bradshaw, 1975). There are two reports describing dissimilar subunits of m-MDH (Mann & Vestling, 1970; Blackwood & Miflin, 1976). Blackwood & Miflin (1976) proposed dissimilar subunits for the MDH's from both maize and barley. Thus, the presence of dissimilar subunits of m-MDH may not be uncommon in the plant kingdom. The soybean root-nodule m-MDH appeared as a single band on native polyacrylamide and isoelectrofocusing gels. SDS–polyacrylamide gel electrophoresis of active fractions of m-MDH, obtained at successive stages of purification, always contained two bands. These two bands copurified and remained in an approximate ratio of 1:1. These results suggest that soybean root-nodule m-MDH is composed of two dissimilar subunits; however, a more rigorous examination of the subunit composition is necessary.

The effects of active-site reagents on MDH from soybean root-nodule mitochondria and bacteroids may be useful criteria for differentiating between these two enzymes (Figure 6). Both enzymes are inhibited to the same extent by pyridoxal 5′-phosphate (30–40%) and pyrocarbonate (100%) but are differentially affected by iodoacetamide and PMSF (Figure 6). Inhibition by pyridoxal 5′-phosphate has been reported for mammalian m-MDH (Roy & Coleman, 1979; Wimmer et al., 1975). NAD^+ and NADH, but not malate, protect against this inhibition. This suggests the presence of a lysine residue near or at the nucleotide binding site (Roy & Coleman, 1979; Wimmer et al., 1975). Both b-MDH and soybean root-nodule m-MDH are affected similarly by pyridoxal 5′-phosphate, resulting in a 35% loss of activity after 60 min. The bacteroid enzyme is unaffected by the presence of either iodoacetamide or PMSF, but the mitochondrial enzyme is strongly inhibited by both of these compounds. The inhibition by pyrocarbonate and iodoacetamide suggests the presence of a histidine residue at the active site of m-MDH and agrees with the results reported for mammalian m-MDH (Jurgensen & Harrison, 1982; Gregory, 1975). Soybean root-nodule m-MDH is also inhibited by PMSF, an inhibitor of serine proteases. The b-MDH is not inhibited by either iodoacetamide or PMSF to any appreciable extent (95% of original activity after 90-min exposure). Since the effects of active-site reagents on bacterial malate dehydrogenases have not been investigated, it is not known if these results represent characteristic differences between bacterial and mitochondrial MDH's.

The pH profiles of both enzyme activities in the direction of oxaloacetate formation are similar, but the pH profiles for the reverse direction are uniquely different between pH 6.0 and pH 8.0 (Figure 5). These results together with the active-site residue inhibitor results suggest the low pH inflection is due to the protonation/deprotonation of a histidine residue whereas the high pH inflection is due to lysine (or serine or cysteine).

The heat stability of b-MDH and m-MDH is similar to the heat stabilities displayed by other bacterial and mitochondrial enzymes. The MDH from *E. coli* or *B. subtilis* was stable at 50 °C for 20 min but lost more than 95% when subjected to 55 °C for the same period of time (Murphy et al., 1967b). The thermophile *Bacillus stearothermophilus* was stable up to 75 °C but lost all activity when heated to 80 °C for 20 min (Murphy et al., 1967b). The soybean root-nodule m-MDH had the same heat stability as three of the five maize isozymes (Yang & Scandalios, 1974) but was considerably more stable than pea m-MDH (Zschoche & Ting, 1973). The maize m-MDH isozymes showed differences in stability at 53 °C ranging between 35% and 60% retention of activity (Yang & Scandalios, 1974). The m-MDH from *P. sativum* leaves and roots lost 60% and 75% of the activity, respectively, after heating at 55 °C for 10 min. Both b-MDH and m-MDH are stabilized against heat inactivation by the presence of NADH, but in the presence of oxaloacetate the bacteroid enzyme is considerably more sensitive to elevated temperature than m-MDH (Table II). Oxaloacetate has been reported to inhibit many MDH's, but the nature of this inhibition is not known (Yang & Scandalios, 1974; Davis & Merrett, 1973).

Each enzyme displays a different heat of activation, a characteristic that is often used to distinguish between species (Figure 4). The energy of activation of the b-MDH is twice as great as that of the soybean root-nodule m-MDH. Both enzymes display the same sequential bi-bi kinetic mechanism typical of malate dehydrogenases from other sources. The Michaelis constants for the substrates are similar to those reported for other malate dehydrogenases (Zschoche & Ting, 1973; Yang & Scandalios, 1974; Blackwood & Miflin, 1976; Davis & Merrett, 1973).

In summary, the malate dehydrogenases from soybean root-nodule bacteroids and mitochondria have been highly purified. Both enzymes have been characterized and have been found to differ significantly in several properties that will serve as markers to discriminate between the sources of the enzyme. Characterization of malate dehydrogenase from both symbionts will aid in determining the role organic acids play in the nitrogen fixation process.

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Registry No. MDH, 9001-64-3; NAD^+, 53-84-9; NADH, 58-68-4; OAA, 328-42-7; L-malate, 97-67-6.

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