JOURNAL OF BACTERIOLOGY, July 1988, p. 2947–2953
0021-9193/88/072947-07$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 170, No. 7

Malate Dehydrogenase from the Thermophilic Green Bacterium *Chloroflexus aurantiacus*: Purification, Molecular Weight, Amino Acid Composition, and Partial Amino Acid Sequence

ANNA KRISTIN ROLSTAD, ELLEN HOWLAND,† AND REIDUN SIREVÅG*

Department of Biology, Division of Molecular Cell Biology, University of Oslo, Blindern, Oslo 3, Norway

Received 6 January 1988/Accepted 24 March 1988

Malate dehydrogenase (MDH; EC 1.1.1.37) from the thermophilic green nonsulfur bacterium *Chloroflexus aurantiacus* was purified by a two-step procedure involving affinity chromatography and gel filtration. The enzyme consists of identical subunits which had molecular weights of approximately 35,000. In its active form at 55°C, it formed tetramers. At lower temperatures, inactive dimers and trimers existed. Antibodies against the purified enzyme were produced, and immunotitration and enzyme-linked immunosorbent assays showed that there was an immunochemical homology between the MDH from *C. aurantiacus* and MDHs from several other bacteria. The amino acid composition of *C. aurantiacus* MDH was similar to those of other MDHs. The N-terminal amino acid sequence was enriched with hydrophobic amino acids, which showed a high degree of functional similarity to amino acids at the N-terminal ends of both *Escherichia coli* and *Thermus flavus* MDHs. The activity of the native enzyme was inhibited by high concentrations of substrate and had temperature and pH optima consistent with the optimal growth conditions for the organism.

The thermophilic, phototrophic green bacterium *Chloroflexus aurantiacus* is found in nature in hot springs and is closely associated with blue-green bacteria. It is capable of living photoautotrophically, photoheterotrophically, or chemoheterotrophically and has an optimum temperature for growth of 55°C. The organism is able to use a variety of carbon sources during photoheterotrophic growth; organic acids like acetate, malate, and lactate are utilized efficiently (15). We have previously shown (13) that such compounds are metabolized in *C. aurantiacus* via the tricarboxylic acid cycle, the glyoxylate cycle, or both, in both cases of which the reaction that is catalyzed by malate dehydrogenase (MDH) is functioning.

In order to obtain information about the molecular mechanism of thermophilic proteins in general and to do comparative and phylogenetic studies within the group of green phototrophic bacteria, we purified and characterized the MDH (EC 1.1.1.37) from *C. aurantiacus*. This enzyme has previously been purified and characterized from several bacterial sources, including both mesophilic and thermophilic sources. In all cases, the enzyme appears to consist of identical subunits, each of which has a molecular weight (MW) of approximately 35,000. The various native enzymes, however, exist either as dimers or tetramers. Recently, Tayeh and Madigan (24) studied MDHs from several purple bacteria and found that, although most of the species tested had the tetrameric form, the dimeric form was also represented within the group.

One approach to understanding the nature of thermal stability in proteins is to compare a protein from a thermophilic organism with the corresponding protein from a related mesophilic organism or from a mesophilic strain of the same organism. Such comparative studies can also give information about the evolutionary relationship between species.

* Corresponding author.
† Present address: Biology Department, Rensselaer Polytechnic Institute, Troy, NY 12181.

C. aurantiacus has several features in common with the other member of the green bacteria, *Chlorobium limicola* forma sp. *thiosulfatophilum*, but their phylogenetic relationship remains obscure. The two organisms have the same type of bacteriochlorophyll, and their antenna chlorophylls are organized in special structures, chlorosomes. In contrast to *C. aurantiacus*, *Chlorobium limicola* forma sp. *thiosulfatophilum* is a photoautotrophic obligate anaerobic organism which uses the reductive tricarboxylic acid cycle as a mechanism to fix CO_2. The mechanism of CO_2 fixation in *C. aurantiacus* is not known (8). Results from 16S rRNA analyses indicate that these organisms are phylogenetically only very distantly related to each other and to the purple bacteria (7). Apparently, *C. aurantiacus* diverged very early from most other photosynthetic procaryotes, and therefore, knowledge about this organism might be important in tracing the evolution of the phototrophic bacteria.

MATERIALS AND METHODS

Organisms and culture conditions. *C. aurantiacus* J-10-fl was grown in the complex Roux medium described by Pierson and Castenholz (17), except that 10 mM Tris hydrochloride (pH 8.0) replaced the glycyl-glycine buffer. When the effect of malate in the growth medium on MDH activity was tested, the cells were grown in the defined medium described by Madigan et al. (15) as modified by Løken and Sirevåg (13). The cells were grown in 5- or 10-liter bottles completely filled with medium and equipped with screw caps or rubber stoppers. The bottles were incubated in a water bath at 55°C and illuminated by two 20-W fluorescent tubes.

Rhodospirillum rubrum was grown on malate medium, and *Chlorobium limicola* forma sp. *thiosulfatophilum* was grown on thiosulfate medium (20) in the light at 30°C. *Escherichia coli* and *Bacillus subtilis* were grown in 1% tryptone–0.5% yeast extract–1% NaCl at 37°C.

Cell extracts. Cells of *C. aurantiacus* were harvested at room temperature in a Cepa continuous centrifuge, washed twice with 20 mM phosphate buffer (pH 7.5), and stored as cell paste at −20°C. For analysis, the thawed cells were

suspended in the same buffer to a concentration of 1 g (wet weight) of cells per ml, broken by ultrasonic treatment at 10 Hz for several 15-s intervals, and centrifuged at 200,000 × *g* for 90 min at 10°C. The supernatant was the crude extract.

Cells of the other organisms were collected by centrifugation, washed 3 times in 20 mM phosphate buffer (pH 7.5), suspended in the same buffer to 1 g (wet weight) of cells per ml, and broken by ultrasonic treatment. The broken cells were centrifuged at 30,000 × *g* for 90 min at 4°C. The crude extracts were adjusted to a protein concentration of 15 to 20 mg/ml in the same phosphate buffer.

Enzyme assays. MDH was assayed at 55°C by following the rate of change of the A_{340} caused by the oxidation of NADH or the reduction of NAD. The standard assay mixture contained 50 mM phosphate buffer (pH 7.5), 0.15 mM NADH, 0.2 mM oxalacetate, and enzyme in a total of 1 ml. The oxidation of malate was measured in the presence of 75 mM phosphate buffer (pH 7.5), 1 mM NAD, 25 mM L-malate, and enzyme. One unit of MDH activity is defined as the amount of enzyme that catalyzes the oxidation-reduction of 1 μmol of NADH or NAD^+ per min.

Lactate dehydrogenase (LDH; EC 1.1.1.27) was assayed in the same manner as MDH, except that 0.2 mM pyruvate was used instead of oxalacetate.

Protein determination. Protein was determined by the method of Lowry et al. (14), with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) used as a standard.

Affinity chromatography. The protein concentration in the crude extract was adjusted to 20 mg/ml in 20 mM phosphate buffer (pH 7.5) before the extract was heated to 55°C and applied directly to an affinity column (1.6 by 20 cm; Blue Sepharose CL-6B; Pharmacia, Uppsala, Sweden) that was equilibrated with the same buffer at 55°C. MDH was eluted from the column with 0.15 mM NADH. The fractions containing the highest activities of MDH were pooled, and $(NH_4)_2SO_4$ was added to 70% saturation. After centrifugation, the pellet was dissolved in 20 mM phosphate buffer (pH 7.5) and dialyzed against 200 mM NaCl in the same buffer.

Gel filtration. Gel filtration experiments were performed at room temperature. For the purification step, a column (1.4 by 60 cm; Sepharose CL-6B; Pharmacia) that was equilibrated with 200 mM NaCl in 20 mM phosphate buffer (pH 7.5) containing 0.02% NaN_3 was used. Fractions containing the highest activity of MDH were pooled, treated with $(NH_4)_2SO_4$ as described above, and stored at 4°C. For MW determination of the native MDH, a column (1.6 by 100 cm; Sephadex G-100; Pharmacia) that was equilibrated with 65 mM Tris hydrochloride (pH 6.8) containing 100 mM NaCl and 0.02% NaN_3 was used. One milligram each of purified MDH and MW standards (L-amino acid oxidase [MW, 132,000]; lipoxidase [MW, 97,400]; bovine serum albumin [MW, 66,000]; egg albumin [MW, 45,000]; carbonic anhydrase [MW, 29,000]) was applied to the column in separate steps and eluted with the same buffer. The elution volume of MDH was determined by measuring enzyme activity, whereas the elution volumes of the protein markers were determined by measuring protein (optical density at 280 nm).

PAGE. For determination of subunit MW, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (11) on a 0.75-mm 8 to 18% linear gradient containing 0.1% SDS. SDS-PAGE low-MW standards (Bio-Rad Laboratories, Richmond, Calif.) were used. Samples were boiled for 5 min in an equal volume of 2× sample buffer (11), and electrophoresis was carried out at a constant current of 30 mA at 25°C.

Isoelectric focusing was performed on 0.2-mm-thick horizontal slab gels containing 4% polyacrylamide and ampholytes (Bio-Rad or LKB Instruments, Inc., Rockville, Md.) at pH ranges of 3 to 10 and 3.5 to 5. Proteins in the gels were stained with Coomassie brilliant blue (6).

For the MW determination of native MDH, electrophoresis under nondenaturating conditions was performed on a 4 to 30% polyacrylamide (Pharmacia) gradient gel. Before loading, the sample was dialyzed against 90 mM Tris hydrochloride–80 mM H_3BO_3–2.5 mM sodium EDTA (pH 8.4) and was then dissolved in the same buffer containing 10% glycerol and bromophenol blue. The MW markers bovine serum albumin (MW, 66,000), lipoxidase (MW, 97,400), L-amino acid oxidase (MW, 132,000), and catalase (MW, 232,000) were dissolved in the same way. After the gel was preelectrophoresed for 20 min at 70 V, the samples were loaded and run at 70 V for 20 min and then were run for 16 h at 150 V. After electrophoresis, the gel was fixed in a solution of 10% 5-sulfosalicylic acid for 30 min and stained with Coomassie brilliant blue R-250.

Activity staining with Nitro Blue Tetrazolium was performed by the method of Dietz and Lubrano (5). The gels to be tested were incubated for 1 h in the dark at 55°C in a solution containing 0.5 mM NAD, 20 mM L-malate or L-lactate, 0.03 mg of phenazine methosulfate per ml, 50 mM Tris hydrochloride (pH 8.2), and 0.1 mg of Nitro Blue Tetrazolium chloride per ml.

The MW of cross-linked MDH was estimated by the method of Lam (12), with the following modifications. A total of 20 μl of purified MDH (1.2 mg of protein per ml) was applied on a dialyzing membrane (Sartorius) which had a nominal cutoff at an MW of 10,000. The samples were dialyzed against 0.1 M phosphate buffer (pH 7.5) and then heated to either 22 or 55°C before glutaraldehyde was added to a final concentration of 0.1%. After incubation for 5 min at the desired temperature, Tris hydrochloride (pH 7.5) was added to a final concentration of 50 mM to stop the reaction. The glutaraldehyde samples and low-MW standards (Bio-Rad) were diluted in an equal volume of sample buffer (11) and boiled for 5 min before electrophoresis was performed on an 8 to 25% or 10 to 15% gel gradient (Phast Gel Gradient; Pharmacia) in 112 mM Tris acetate buffer (pH 6.4). The gels were run at 10 mA for 16 V · h at 15°C and stained (Phast Gel Blue R; Pharmacia) for 8 min at 50°C.

Sedimentation equilibrium analysis. The MW of the native enzyme was estimated by sedimentation equilibrium analysis in an analytical ultracentrifuge (model E; Beckman Instruments, Inc., Fullerton, Calif.). The purified MDH (0.5 mg/ml) in 90 mM Tris hydrochloride–80 mM H_3BO_4–2.5 mM sodium EDTA (pH 8.4) was centrifuged at 15,000 rpm for 24 h at 12°C.

Amino acid composition. The sample was hydrolyzed in 6 M HCl under vacuum and analyzed in an automatic amino acid analyzer (LC-5000; Biotronic). Half-cystine residues were detected in trace amounts only and are not listed. The content of tryptophan residues was not determined.

N-terminal amino acid sequence. N-terminal amino acid analysis was performed on an automatic sequence analyzer (JEOL-K47). Analysis of the phenylthiohydantoin residues was performed as described by Sletten et al. (21).

Immunochemical experiments. Antiserum was produced by injecting rabbits with purified MDH. For the first injection, 100 μg of MDH in 0.5 ml of 150 mM NaCl was mixed with an equal volume of Freund complete adjuvant and injected subcutaneously at 8 to 10 sites on the back of the animal. For subsequent injections 3 weeks later, Freund incomplete adjuvant was used. Blood containing antibodies

TABLE 1. Purification of MDH from *C. aurantiacus*

Purification step	Enzyme activity (U)[a]	Protein (mg)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	7,044	587	12	1	100
Affinity chromatography	4,321	7.5	580	48	61
Gel filtration	2,502	2.4	1,040	86	36

[a] Units are micromoles per minute.

against MDH was collected from the vein at the edge of the ear of the rabbit, and the serum was prepared by standard methods. To rule out nonspecific reactions in the immunochemical experiments, controls with preimmune serum were always used.

Cross-reaction of the antibodies production with MDHs from other organisms was tested by double-diffusion tests in 1% agarose gels containing 80 mM barbitone buffer (pH 8.2). The antiserum was placed in the center well, and the extracts to be tested and the purified *Chloroflexus* MDH were placed in the peripheral wells. The gels were incubated in a humidity chamber at 37°C overnight.

The immunoglobulin G fraction containing antibodies against *Chloroflexus* MDH was isolated as follows. The serum was diluted with 150 mM NaCl and precipitated with $(NH_4)_2SO_4$, and the precipitate was washed and dialyzed as described by Hudson and Hay (9). The activity of MDH in the immunoglobulin fraction was measured at 30°C, and in the immunotitration experiments, corrections were made for the values that were obtained.

For the enzyme-linked immunosorbent assay (ELISA), a conjugate of peroxidase and the immunoglobulin fraction containing antibodies against *Chloroflexus* MDH was made by the method of Avrameos et al. (2).

A total of 0.1 ml of purified *Chloroflexus* MDH (2 μg/ml) or 0.1 ml of crude extracts of *E. coli* and *B. subtilis* (protein concentration, 0.5 mg/ml) was added to the wells of microtiter plates (Nunc, Roskilde, Denmark), which were incubated at 5°C overnight. The coated plates were washed 3 times with 10 mM phosphate (pH 7.2)–150 mM NaCl (phosphate-buffered saline) containing 0.1% Tween 20. Free sites on the plates were blocked by reaction with 0.1% bovine serum albumin for 1 h at 37°C, followed by a further wash with phosphate-buffered saline–Tween 20. Enzyme conjugates, at dilutions from 1:10 to 1:1,000, were added to the wells (0.1 ml per well) and incubated for 3 to 4 h at 37°C. This was followed by washing three times with phosphate-buffered saline–Tween 20, and then 0.1 ml of substrate (0.18 mg of azino-benzthiazolin sulfonate per ml and 0.8 μl of 3% H_2O_2 per ml) was added. Color development was monitored after incubation at 37°C by measuring the A_{405} on a spectrophotometer (Titertek Uniskan; Flow Laboratories, Inc., McLean, Va.).

RESULTS

Purification of MDH. The specific activity of MDH in various extracts of *C. aurantiacus* grown under photoheterotrophic conditions on the complex Roux medium was in the range of 7 to 60 U/mg of protein. Similar specific activities were obtained in growth experiments when malate was the sole carbon source.

The thermophilic MDH from *C. aurantiacus* was purified in a two-step procedure by affinity chromatography and gel filtration. It should be noted that in order for the enzyme to become efficiently bound to and eluted from the affinity column, temperatures at around 55°C are required.

When the fractions from the affinity column which contained the highest activity of MDH were pooled and subjected to gel electrophoresis, five different proteins were visible after staining. In addition to MDH activity, some of the fractions contained LDH activity.

In the gel filtration step, MDH was eluted as a single peak, which, on electrophoresis in the native state in polyacrylamide gels, revealed only a single band. This was also the case after isoelectric focusing, which revealed a single band with an isoelectric point of 4.2. Activity staining confirmed the identity of MDH and showed that no LDH was present in either case. With regard to gel filtration, it was observed that the matrix of a freshly prepared Sepharose CL-6B column bound LDH.

The results of the purification procedure are summarized in Table 1. A further indication that the enzyme was at a high degree of purity came from the examination of its amino-terminal group, which revealed only single amino acids in the various positions. The specific activity of the purified MDH from *C. aurantiacus* was in the same range as that reported for MDHs from other bacteria (10, 22, 24).

Subunit structure. The MW of the subunits of MDH was determined from its electrophoretic mobility on SDS-PAGE, in which the protein migrated as a single band (Fig. 1). This indicates that MDH consists of only one kind of subunit. By comparison with the electrophoretic mobility of known markers, the MW was estimated to be approximately 35,000, which corresponds to the MW reported for MDHs with identical subunits from animal, plant, and bacterial sources (22, 24).

MW and quaternary structure. The MW of purified native *Chloroflexus* MDH was determined by various methods. When the MW was determined by sedimentation equilibrium centrifugation, a value of 103,000 was obtained. Results derived from gel filtration indicated an MW of 110,000, whereas analysis by nondenaturating PAGE showed one band corresponding to an MW of 120,000. When SDS-PAGE was performed after the enzyme was cross-linked with

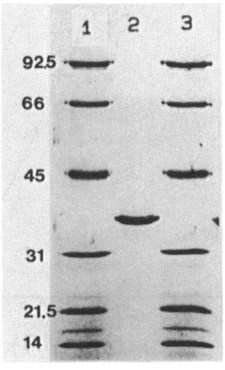

FIG. 1. SDS-PAGE of purified MDH from *C. aurantiacus*. Lanes 1 and 3, MW standards (indicated in thousands); lane 2, purified MDH.

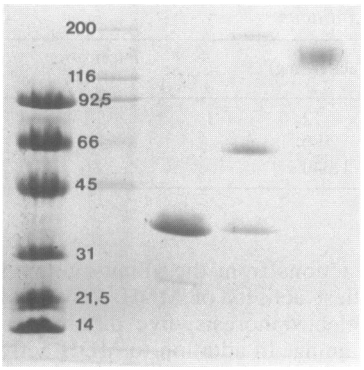

FIG. 2. SDS-PAGE of purified MDH from *C. aurantiacus* cross-linked with glutaraldehyde at 22 and 55°C (lanes are indicated from the left). Lane 1, Low-MW standards (indicated in thousands); lane 2, high-MW standards (indicated in thousands); lane 3, MDH subunits; lane 4, MDH cross-linked with glutaraldehyde at 22°C; lane 5, MDH cross-linked with glutaraldehyde at 55°C.

glutaraldehyde at 55°C, one major band corresponding to an MW of 145,000 could be seen on the gel (Fig. 2, lane 5). When the cross-linking was done at 22°C, two additional bands were present on the gel (Fig. 2, lane 4).

None of the values obtained by the various methods indicated that the native enzyme was a dimer, but according to the data, the quaternary structure could have been trimeric as well as tetrameric.

Catalytic properties. The K_m value for oxalacetate reduction was 2.3×10^{-5} M, as determined by double-reciprocal plots. The threshold value for inhibition by high concentrations of oxalacetate was 200 μM, which is similar to that found for other MDHs. The temperature and pH optima of the purified enzyme are shown in Fig. 3 and 4, respectively. For both temperature and pH, the optimum values obtained were the same as those for growth of the organism. When the specific activity was determined at 30°C, it was found to be significantly lower than that obtained at 55°C.

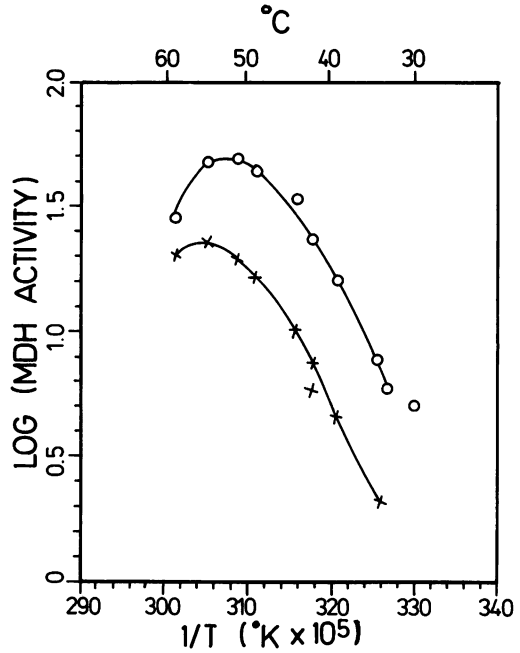

FIG. 3. Effect of temperature (T) on the initial rate of oxalacetate reduction (○) and malate oxidation (×) by purified MDH from *C. aurantiacus*.

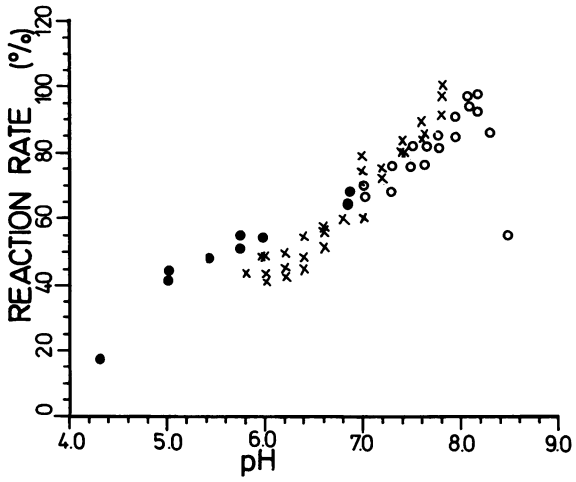

FIG. 4. Effect of pH on the initial rate of oxalacetate reduction by purified MDH from *C. aurantiacus*. Symbols: ●, citrate buffer; ×, phosphate buffer; ○, Tris hydrochloride buffer.

Amino acid composition. The amino acid composition of the MDH from *C. aurantiacus* is presented in Table 2. Half-cystine was detected only in trace amounts in *C. aurantiacus* and is not listed. Tryptophan residues were not determined. The reported content of these amino acid residues in other MDHs is also low (3). However, as can be seen from the amino acid sequence in Fig. 5, a tryptophan residue was found near the N-terminal end. Comparison of the amino acid composition of the MDH from *C. aurantiacus* with those of the MDHs from *E. coli* (3) and the extremely thermophilic organism *Thermus flavus* (18) revealed only small differences.

Amino acid sequence. The amino acid sequence of the first 35 residues from the N-terminal end of the MDH from *C. aurantiacus* is shown in Fig. 5. Compared with the overall amino acid composition (Table 2), the N-terminal end of the molecule is enriched with the hydrophobic amino acids alanine, isoleucine, valine, and leucine.

TABLE 2. Amino acid composition of MDH from *C. aurantiacus*

Amino acid	mol%[a]
Asp	9.2
Thr	5.2
Ser	4.9
Glu	10.2
Pro	5.1
Gly	9.7
Ala	13.2
Val	8.4
Met	2.9
Ile	5.5
Leu	9.5
Tyr	3.2
Phe	2.4
His	1.2
Lys	5.8
Arg	3.6

[a] Values of half-cystine residues are not listed. Tryptophan residues were not determined.

1 5 10 15
Met-(Arg)-Lys-Lys-Ile-Ser-Ile-Ile-Gly-Ala-Gly-Phe-Val-Gly-Ser-Thr-Thr-
20 25 30 35
Ala-His-Trp-Leu-Ala-Ala-Lys-Glu-Leu-Gly-Asp-Ile-Val-Leu-Leu-Asp-Ile-Val-

FIG. 5. Amino acid sequence of the N-terminal end of the MDH from *C. aurantiacus*.

Immunochemical analysis. The immunoglobulin fraction from the antiserum against the MDH from *C. aurantiacus* inactivated the enzyme 100% (Fig. 6). When antiserum against the MDH from *C. aurantiacus* was tested against extracts from other phototrophic bacteria and *E. coli* in double-immunodiffusion tests, no immunochemical reaction was observed. However, when the more sensitive ELISA was employed, a positive reaction occurred with extracts from both *E. coli* and *B. subtilis*. The activity of MDH in extracts from both *E. coli* and *Chlorobium limicola* forma sp. *thiosulfatophilum* was inhibited 90%, and that in extracts from *B. subtilis* was inhibited 80%, when structural homology was tested in immunotitration experiments. The results are summarized in Table 3.

TABLE 3. Structural homology between MDH from *C. aurantiacus* and MDH from other bacteria tested by ELISA and immunotitration

Protein source	ELISA		Immunotitration inactivation (%)[a]
	Protein (μg)	Reaction	
Purified MDH from *C. aurantiacus*	0.2	+	100
Extract from *E. coli*	50.0	+	90
Extract from *B. subtilis*	50.0	+	80
Extract from *Chlorobium limicola* forma sp. *thiosulfatophilum*	40.0	ND[b]	90

[a] Samples contained the following in 20 mM phosphate buffer (pH 7.5): 0.15 U of MDH per ml, 0.15 mM NADH, 2.5 to 60 μl of the immunoglobulin fraction (35 mg of protein per ml), and 0.3 mM oxalacetate in a total volume of 1 ml. The activity of MDH from *C. aurantiacus* was measured at 30 and 55°C. For the other organisms, the activity was measured at 30°C.
[b] ND, Not determined.

DISCUSSION

When *C. aurantiacus* grows under phototrophic conditions, light is the main energy source, and the role of the tricarboxylic acid cycle is mainly to provide precursors of amino acids for biosynthesis. The specific activity of MDH observed in the cell extracts of *C. aurantiacus* varied between 7 and 60 U/mg in these experiments and was high enough to account for the observed generation time of 10 to 12 h. The enzyme activities were within the same range as those reported for other organisms, including phototrophs and chemotrophs (10, 22, 24). When the organism was grown with malate as the sole organic carbon source, the specific activity was approximately the same as that obtained on the complex medium, indicating that MDH is a constitutive enzyme and is not subject to control mechanisms. Previously, we have reported (8) that extracts of photoautotrophically grown *C. aurantiacus* contained levels of activity similar to those reported here.

A strong correlation between the optimum temperature for growth and MDH activity was observed. However, this is not always the case for thermophilic organisms. The activities of different enzymes isolated from a thermophilic organism might differ significantly from each other with regard to optimum temperature, which may not necessarily parallel the optimum temperature for growth. Thus, the activities of the enzymes of the glyoxylate cycle in *C. aurantiacus*, isocitrate lyase and malate synthase, have an optimum temperature of 42°C (13).

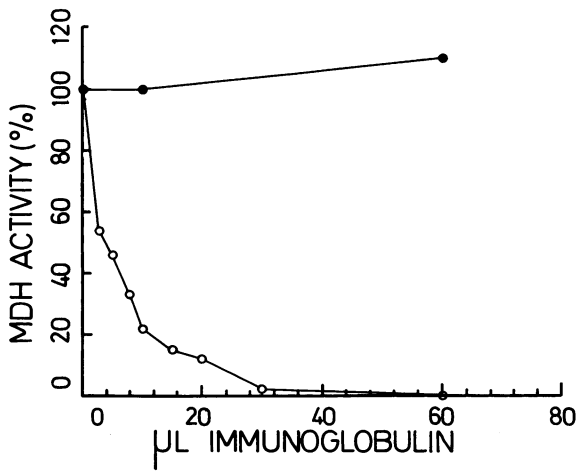

FIG. 6. Immunotitration of purified MDH from *C. aurantiacus* with immunoglobulin at 30°C. Symbols: ○, immunoglobulin from an immunized rabbit; ●, immunoglobulin from a preimmunized rabbit.

MDH was purified from *C. aurantiacus* by a two-step procedure by affinity chromatography and gel filtration. For the affinity column to be effective, it was important that binding and elution of the enzyme took place at temperatures of 50 to 55°C, which are similar to the optimum temperature both for the growth of the organism and for the activity of the enzyme (Fig. 3).

It was helpful for the purification procedure that LDH, which is eluted together with MDH from the affinity column, was retained by a freshly prepared Sepharose CL-6B column. Because the column must be fresh, it is suggested that a limited number of sites on the column material are being saturated by LDH.

MDH has been purified from several bacterial sources, as well as from plants and animals (3, 24). In all cases it has been reported that the enzyme consists of identical subunits, each of which has an MW of approximately 35,000. One exception is the MDH from *Rhodocyclus purpureus*, which has substantially smaller subunits (24). Based on the size of the native enzymes, MDH can be divided into two groups; those existing as dimers with MWs in the range of 60,000 to 65,000 and those existing as tetramers with reported MWs in the range of 117,000 to 146,000. The quaternary structure of the proteins does not seem to be correlated with their thermostability, since both dimeric and tetrameric forms are found in thermophilic bacteria (22). Even in related bacteria, both groups of MDH are represented, as was recently reported for the purple nonsulfur bacteria (24).

In order to determine the quaternary structure of MDH from *C. aurantiacus*, we measured the MW of the oligomer form by several methods. The highest value suggesting a tetrameric structure was obtained with the cross-linked enzyme at 55°C, while lower values were obtained by sedimentation equilibrium centrifugation and gel filtration of the native enzyme. The reason for the present results from sedimentation equilibrium centrifugation and gel filtration might be the lower temperature at which the measurements were made. According to this and our other findings, it

appears that temperature plays a role both in conformational changes in the protein and in the aggregation of the subunits.

With the enzyme cross-linked at 55°C, only one band with an MW corresponding to 145,000 was observed. On the other hand, cross-linking at 22°C gave rise to two additional bands, probably reflecting the fact that the enzyme exists in several different aggregation states at this temperature. Based on these findings, we conclude that in its active form at 55°C, the MDH from *C. aurantiacus* forms a tetramer. As the temperature gets lower, inactive dimeric and trimeric aggregation states of the enzyme probably increase relative to the active tetrameric state, which explains why the organism does not grow well at temperatures below 40°C.

No immunochemical homology was found between the MDH from *C. aurantiacus* and MDHs from other bacteria in immunodiffusion experiments. However, when the more sensitive ELISA and immunotitration experiments were performed, both the dimeric *E. coli* MDH and the tetrameric *B. subtilis* MDH showed cross-reactivity. Furthermore, MDH activity in extracts of the green sulfur bacterium *Chlorobium limicola* forma sp. *thiosulfatophilum* was completely inhibited by antiserum against *Chloroflexus* MDH. In immunotitration experiments the reaction between native enzymes and antiserum is being tested, and therefore, the strong inhibition observed for all the enzymes tested shows that homology exists on their surfaces.

The amino acid composition of the MDH from *C. aurantiacus* (Table 2) does not explain its thermostability. Half-cystine residues were detected only in trace amounts, but this is common to several MDHs (3). Comparison of the amino acid composition of *Chloroflexus* MDH with those of the mesophilic *E. coli* MDH (3, 25) and the extremely thermophilic *T. flavus* MDH (18) revealed only small differences. This is in agreement with the idea that thermostability in proteins is due to subtle changes in the molecules, such as the exchange of one amino acid for another, or to stabilization by other molecules in the cell (1). Compared with the overall amino acid composition, however, the N-terminal end of *Chloroflexus* MDH is enriched with hydrophobic amino acids (Fig. 5). This is in accord with the results obtained by Schär and Zuber (19), who found an increase in the number of intrinsic hydrophobic domains in the proteins as the thermostability increased in an analysis of different thermophilic and mesophilic LDHs.

The complete nucleotide sequence of the MDH gene from the extremely thermophilic bacterium *T. flavus* was recently determined, and the amino acid sequence thus deducted was compared with the N-terminal amino acid sequence of *E. coli* MDH (16). The latter was determined by amino acid and nucleotide sequence analyses (23, 25). Homology between the enzymes of these two species is distinctly lower than that between *T. flavus* MDH and porcine cytoplasmic MDH.

To compare the amino acid sequence of the N-terminal end of *Chloroflexus* MDH with that of other known MDHs with regard to functionally similar amino acids, we have used the program SIMPLIFY (4). In this program all the amino acids are placed in one of six categories according to the following properties: A, neutral, weakly hydrophobic; D, hydrophilic, acid amine; H, hydrophilic, basic; I, hydrophobic; F, hydrophobic, aromatic; and C, cross-link-forming. The results of the comparison of *Chloroflexus* MDH with *E. coli* and *T. flavus* MDHs, after simplification, are given in Fig. 7. In the first case (Fig. 7a) more than 63% of the amino acids showed similarity, and in the second case (Fig. 7b) approximately 50% did. The similarity between the N-terminal end of the MDH from *E. coli* and the MDH from *T. flavus* was 67% (Fig. 7c). Thus, the N-terminal ends of the three different MDHs have a high degree of similarity with regard to functionally similar amino acids.

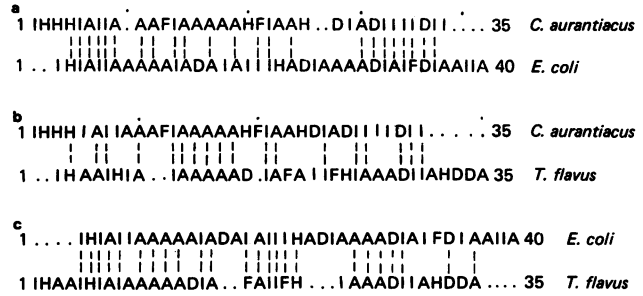

FIG. 7. Comparison of the amino acid sequences from the N-terminal end of MDHs from *C. aurantiacus*, *E. coli*, and *T. flavus* by using simplifications based on amino acids with similar properties (3). The following categories of amino acids were used: A, neutral, weakly hydrophobic; D, hydrophilic, acid amine; H, hydrophilic, basic; I, hydrophobic; F, hydrophobic, aromatic; C, cross-link-forming.

ACKNOWLEDGMENTS

We thank K. Sletten for determining the amino acid composition and the N-terminal amino acid sequence. We also thank T. B. Christensen for carrying out the analytical ultracentrifugation.

This study was supported by grant D.70.49.098 from the Norwegian Research Council for Science and the Humanities (NAVF).

LITERATURE CITED

1. **Argos, P., M. G. Rossmann, U. M. Grau, H. Zuber, G. Frank, and J. D. Tratschin.** 1979. Thermal stability and protein structure. Biochemistry **18**:5698–5703.
2. **Avrameos, S., T. Ternynck, and J.-L. Guesdon.** 1978. Coupling of enzyme to antibodies and antigens. Scand. J. Immunol. **8**(Suppl. 7):7–23.
3. **Banaszak, L. J., and R. A. Bradshaw.** 1975. Malate dehydrogenases, p. 369–396. *In* P. D. Boyer (ed.), The enzymes, vol. XI. Oxidation-reduction, part A. Academic Press, Inc., New York.
4. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
5. **Dietz, A., and T. Lubrano.** 1967. Separation and quantitation of lactic dehydrogenase isoenzymes by disc electrophoresis. Anal. Biochem. **20**:246–257.
6. **Duhamel, R. C., E. Meezan, and K. Brendel.** 1980. Metachromatic staining with Coomassie brilliant blue R 250 of the proline-rich calf thymus histone H1. Biochim. Biophys. Acta **626**:432–442.
7. **Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blackemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrsen, K. N. Chen, and C. R. Woese.** 1980. The phylogeny of prokaryotes. Science **209**:457–463.
8. **Holo, H., and R. Sirevåg.** 1986. Autotrophic growth and CO_2 fixation of *Chloroflexus aurantiacus*. Arch. Microbiol. **145**:173–180.
9. **Hudson, L., and F. C. Hay.** 1980. Practical immunology, p. 1–3. Blackwell Scientific Publications, Ltd., Oxford.
10. **Iijima, S., T. Saiki, and T. Beppu.** 1980. Physiochemical and catalytic properties of thermostable malate dehydrogenase from an extreme thermophile, *Thermus flavus* AT 62. Biochim. Biophys. Acta **613**:1–9.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Lam, E.** 1986. Nearest-neighbor relationships of the constituent

polypetides in plastoquinol-plastocyanin oxidoreductase. Biochim. Biophys. Acta **848**:324–332.
13. **Løken, Ø., and R. Sirevåg.** 1982. Evidence for the presence of the glyoxylate cycle in *Chloroflexus*. Arch. Microbiol. **132**:276–279.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Madigan, M. T., S. R. Peterson, and T. D. Brock.** 1974. Nutritional studies on *Chloroflexus*, a filamentous, photosynthetic, gliding bacterium. Arch. Microbiol. **100**:97–103.
16. **Nishiyama, M., N. Matsubara, K. Yamamoto, S. Iijima, T. Vozumi, and T. Beppu.** 1986. Nucleotide sequence of the malate dehydrogenase gene of *Thermus flavus* and its mutation directing an increase in enzyme activity. J. Biol. Chem. **261**:14178–14183.
17. **Pierson, B., and D. Castenholz.** 1974. A phototrophic gliding filamentous bacterium of hot springs, *Chloroflexus aurantiacus* gen. and sp. nov. Arch. Microbiol. **100**:5–24.
18. **Saiki, T., S. Iijima, R. Tohda, T. Beppu, and K. Arima.** 1978. Purification and properties of malate dehydrogenase and isocitrate dehydrogenase from an extreme thermophile, *Thermus flavus* AT-62, p. 287–303. *In* S. M. Friedman (ed.), Biochemistry of thermophily. Academic Press, Inc., New York.
19. **Schär, H.-P., and H. Zuber.** 1979. Structure and function of L-lactate dehydrogenases from thermophilic and mesophilic bacteria. Hoppe-Seyler's Z. Physiol. Chem. **360**:795–807.
20. **Sirevåg, R.** 1975. Photoassimilation of acetate and metabolism of carbohydrate in *Chlorobium thiosulfatophilum*. Arch. Microbiol. **104**:105–111.
21. **Sletten, K., J. B. Natvik, G. Husby, and J. Juul.** 1981. The complete amino acid sequence of a prototype immunoglobulin-light-chain-type amyloid-fibril protein AR. Biochem. J. **195**:561–572.
22. **Sundaram, T. K., I. P. Wright, and A. E. Wilkinson.** 1980. Malate dehydrogenase from thermophilic bacteria. Molecular size, subunit structure, amino acid composition, immunochemical homology, and catalytic activity. Biochemistry **19**:2017–2022.
23. **Sutherland, P., and L. McAlister-Henn.** 1985. Isolation and expression of the *Escherichia coli* gene encoding malate dehydrogenase. J. Bacteriol. **163**:1074–1079.
24. **Tayeh, M. A., and M. T. Madigan.** 1987. Malate dehydrogenase in phototrophic purple bacteria: purification, molecular weight, and quaternary structure. J. Bacteriol. **169**:4196–4202.
25. **Vogel, R. F., K.-D. Entian, and D. Mecke.** 1987. Cloning and sequence of the mdh structural gene of *Escherichia coli* coding for malate dehydrogenase. Arch. Microbiol. **149**:36–42.