JOURNAL OF BACTERIOLOGY, Feb. 1992, p. 1307–1313
0021-9193/92/041307-07$02.00/0

Vol. 174, No. 4

Malate Dehydrogenase from *Chlorobium vibrioforme*, *Chlorobium tepidum*, and *Heliobacterium gestii*: Purification, Characterization, and Investigation of Dinucleotide Binding by Dehydrogenases by Use of Empirical Methods of Protein Sequence Analysis

COLIN CHARNOCK, UNN HILDE REFSETH, AND REIDUN SIREVÅG*

Department of Biology, Division of Molecular Cell Biology, University of Oslo, Blindern, 0316 Oslo 3, Norway

Received 18 September 1991/Accepted 3 December 1991

Malate dehydrogenase (MDH; EC 1.1.1.37) from strain NCIB 8327 of the green sulfur bacterium *Chlorobium vibrioforme* was purified to homogeneity by triazine dye affinity chromatography followed by gel filtration. Purification of MDH gave an approximately 1,000-fold increase in specific activity and recoveries of typically 15 to 20%. The criteria of purity were single bands on sodium dodecyl sulfate (SDS) and nondenaturing polyacrylamide electrophoresis (PAGE) and the detection of a single N terminus in an Edman degradation analysis. MDH activity was detected in purified preparations by activity staining of gels in the direction of malate oxidation. PAGE and gel filtration (Sephadex G-100) analyses showed the native enzyme to be a dimer composed of identical subunits both at room temperature and at 4°C. The molecular weight of the native enzyme as estimated by gel filtration was 77,000 and by gradient PAGE was 74,000. The subunit molecular weight as estimated by SDS-gradient PAGE was 37,500. N-terminal sequences of MDHs from *C. vibrioforme*, *Chlorobium tepidum*, and *Heliobacterium gestii* are presented. There are obvious key sequence similarities in MDHs from the phototrophic green bacteria. The sequences presented probably possess a stretch of amino acids involved in dinucleotide binding which is similar to that of *Chloroflexus aurantiacus* MDH and other classes of dehydrogenase enzymes but unique among MDHs.

The green phototrophic bacteria comprise two families, the *Chlorobiaceae*, or green sulfur bacteria, and the family *Chloroflexaceae*, or green gliding bacteria (29). Common to both families of green bacteria are the types of light-harvesting pigments and their structural organization into chlorosomes. Metabolically, however, the members of the two families differ significantly. Members of the family *Chlorobiaceae* as represented by the genus *Chlorobium* are obligately anaerobic and photoautotrophic, whereas members of the family *Chloroflexaceae* as represented by the genus *Chloroflexus* are facultative photoheterotrophs, capable of anaerobic photoautotrophic growth as well as aerobic heterotrophic growth in the dark. *Chlorobium vibrioforme* is a mesophilic organism which in nature is found in meromictic lakes. The natural habitat of the thermophile *Chloroflexus aurantiacus* is hot springs. A thermophilic *Chlorobium* species, *Chlorobium tepidum*, has recently been isolated from hot springs in New Zealand (33).

In spite of the great similarity with regard to pigments and fine structure, 16S rRNA molecular analyses indicate that the genera *Chlorobium* and *Chloroflexus* are phylogenetically only distantly related (8).

The heliobacteria are a group of gram-positive, green, strictly anaerobic nonsulfur bacteria, the natural habitat of which is the soil of rice fields (38). Bacteria belonging to this group have neither the internal photosynthetic membranes typical of purple bacteria nor the chlorosomes typical of green bacteria. They contain bacteriochlorophyll *g*, which is structurally related to chlorophyll *a* (7) and which presumably is associated with the cytoplasmic membrane. Data from 16S rRNA sequence analysis indicate that heliobacteria are phylogenetically related to the gram-positive eubacteria (38), and recently it has been reported that some species form endospores (21).

In order to obtain information about proteins from thermophilic sources and to do comparative and phylogenetic studies of phototrophic bacteria, we have purified and characterized malate dehydrogenase (MDH) from several bacteria. In *Chlorobium* spp., this enzyme catalyzes one of the reactions in the reductive tricarboxylic acid cycle, which is the mechanism of CO_2 fixation in this organism (6). In *Chloroflexus* spp., the reaction catalyzed by MDH is part of the tricarboxylic cycle and the glyoxylate cycle (13). High levels of MDH activity have been shown in crude extracts of *Heliobacterium gestii*, but its role in the metabolism of the cell has not yet been examined (22).

On the basis of molecular size, bacterial MDHs fall into two classes: small and large. The molecular weight (MW) of small MDHs is in the range from 60,000 to 74,000, and that of the large form is between 117,000 and 146,000. All MDHs studied so far are homogeneous in subunit composition, with a subunit MW in the range from 30,000 to 38,000 (1, 16, 17, 23, 30, 31). The small enzymes are thus dimers, and the large enzymes are thus tetramers. The large form is widespread among the purple nonsulfur bacteria, though the small form is also represented (31). The MDH of *Chloroflexus aurantiacus* is a tetramer (23).

Salient structural features of dehydrogenases. Dehydrogenases possess two structural domains. The one which is closest to the N-terminal end of the polypeptide is involved in binding the coenzyme, which is the dinucleotide NAD(H) in the case of MDHs. The other domain binds the substrate proper (2, 3, 25, 34).

The dinucleotide-binding domain consists of two structur-

* Corresponding author.

ally similar halves of super secondary structure (frequently occurring combinations of secondary structure): β-strand–α-helix–β-strand–α-helix, so-called Rossmann folds (3, 25, 35). The first Rossmann fold binds the ADP moiety of the dinucleotide, which includes the adenine ring, one ribose unit, and the PP_i. The second fold binds the nicotinamide ring.

Using X-ray chrystallography, Wierenga and Hol (34) recognized a specific sequence pattern composed primarily of glycine to be involved in binding the ADP moiety. In this sequence, three glycines lie interspersed as follows: GXGXXG, where X is any residue. This motif constitutes a tight turn at the end of the first strand of a buried β-sheet (preceding the first glycine) and marks the beginning of an amphipathic α-helix which has the second glycine at its N terminus. The binding pocket constituted by the glycines allows close interaction with the ADP. The electric dipole of the amphipathic helix interacts optimally with the phosphates of the coenzyme (flavin adenine dinucleotide and NAD) in the binding process (35). The sequence of glycines appears to be highly conserved in enzymes with a requirement for NAD or flavin adenine dinucleotide (34, 36).

MDH sequences from phototrophic prokaryotes are available only for the green bacteria and include the sequences presented in this work and that of *Chloroflexus* MDH, which has been previously published (23). These MDHs have the GXGXXG motif early in their N-terminal ends. All other MDHs sequenced to date, including the complete gene-derived sequences of *Escherichia coli* (32) and *Thermus flavus* (18) lack this motif. Empirical analysis presented herein suggests a variant glycine-rich region to be the corresponding functional unit in these enzymes.

Eight other residues (or amino acid properties) have come to be recognized as conserved in positions relative to the glycine motif. These fingerprint residues have various roles in the binding of the ADP moiety and in the related structuring of the region. They may justifiably be used in locating the glycine motif along the chain (35, 36).

MATERIALS AND METHODS

Organism, growth medium, and growth conditions. *C. vibrioforme* f. sp. thiosulphatophilum NCIB 8327 (from N. Pfennig) was grown in the medium of Sirevåg and Ormerod (27), either in 62-ml screw-cap bottles (for stock cultures) or in 5- or 10-liter bottles completely filled with medium and fitted with screw caps or rubber stoppers. The incubation temperature was 30°C, and illumination was provided by two 60-W bulbs.

Preparation of cell extracts. Cells were harvested at room temperature in a Cepa continuous centrifuge and washed twice with 10 mM potassium phosphate buffer (pH 7.5). For analysis, cells were suspended in the same buffer to a concentration of 1 g (wet weight) per ml and broken by passage three times through a precooled French pressure cell (10 lb/in^2). Intact cells and cell debris were removed by centrifugation at 10,000 × *g* for 10 min at 4°C in a Sorvall RC-5B Superspeed centrifuge with an SS-34 rotor. The resulting supernatant was ultracentrifuged at 200,000 × *g* for 90 min at 4°C in a Beckman L8-55M ultracentrifuge with an SW-50.1 rotor. The pellet was discarded, and the supernatant was the cell extract.

Enzyme assays. MDH was assayed at 25°C by monitoring the rate of change of the A_{340} caused by the oxidation of NADH. The standard assay mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.2 mM oxaloacetate, 0.15 mM NADH, and enzyme in a total volume of 1 ml. All measurements were performed with a Shimadzu UV-265 recording spectrophotometer. One unit of MDH activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per min at 25°C.

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

Affinity chromatography. Cell extract was applied directly to a column of Matrix Gel Red A (binding capacity, 1 to 15 mg of protein per ml of gel; Amicon Corp., Lexington, Mass.), previously equilibrated with 10 mM sodium phosphate buffer (pH 7.5) at 4°C. The minimum volume of gel required to bind all of the applied units of MDH activity was used. This allowed the enzyme to be recovered in a small volume of eluant. The column housing the gel had dimensions of 1.6 by 20 cm. Following washing with the buffer, bound proteins were eluted with buffer containing 5 mM NAD and 10 mM malate. The fractions containing the highest activity of MDH were pooled, and sucrose was added to 20 mg/ml. Dithiothreitol was added to a final concentration of 5 mM.

Gel filtration. A column (1.4 by 50 cm) of Sephadex G-100 equilibrated with 40 mM NaCl in 20 mM phosphate buffer (pH 7.5) containing 0.02% NaN_2 was used. For determination of the MW of native MDH, the column and buffer conditions were identical to those used in the purification procedure. Purified MDH and MW standards lipoxydase (MW, 97,400), bovine serum albumin (MW, 66,000), egg albumin (MW, 45,000), lysozyme (MW, 14,000) (1 mg each) in 100 mM phosphate buffer containing 40 mM NaCl and 0.02% (wt/vol) sucrose were applied to the column. The elution volume of MDH was determined by measuring enzyme activity, and those of the protein markers were determined by monitoring the A_{280}. MDH from the gel filtration column was desalted and concentrated in 10 mM ammonium carbonate for N-terminal sequence analysis by using an ultrafiltration cartridge (Bio-Rad).

Polyacrylamide gel electrophoresis (PAGE). Gels with linear gradients of polyacrylamide (5 to 20%, wt/vol) and sucrose (0 to 16%, wt/vol) were used to determine native and subunit MDH MWs. Buffers and other conditions were as described by Laemmli (12) for homogenous gels, omitting sodium dodecyl sulfate (SDS) and heat denaturation of the samples for native gels. The position of MDH in the gel was shown either by staining the gel with Coomassie blue R-250 (5) or by incubating the gel for 5 h in an activity-staining solution composed of 20 mM malate, 0.5 mM NAD, 0.03 mg of phenazine methosulfate per ml, and 0.1 mg of *p*-Nitro Blue Tetrazolium per ml in 50 mM Tris-HCl buffer (pH 7.5).

Isoelectric focusing. The isoelectric point of MDH purified from *C. vibrioforme* was determined with Phast System TM gels and isoelectric focusing calibration proteins (Pharmacia).

Electroblotting and immunochemical analyses. Antibodies to *Chlorobium* MDH and *Chloroflexus* MDH were produced in rabbits as previously described (23). These were used to detect *Chlorobium* MDH by the procedure described by Young (40).

N-terminal amino acid sequence. The first 40 positions in the N terminus of MDH from *C. vibrioforme* were determined by means of an Edman degradation analysis, employing an automatic sequence analyzer (Applied Biosystems 477 A). Partially purified MDHs from *C. tepidum* and *H. gestii* were purified and sequenced in a two-step analysis using

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

Step	Activity (U)	Protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,112	1,778	1.2	100	1
Matrix Gel Red A	1,646	1.8	906	78	755
Sephadex G-100	376	0.3	1,201	18	1,001

high-pressure liquid chromatography and a sequence analyzer.

Amino acid composition. The sample was hydrolyzed in 6 M HCl under vacuum and analyzed in an automatic amino acid analyzer (LC-5000 Biotronic). The procedure required 8 to 20 μg of the enzyme.

Nucleotide sequence accession numbers. The sequence data presented in this paper have been submitted to EMBL and have been given the following accession numbers in the SWISS-PROT protein sequence data base: *H. gestii* MDH, P80037; *C. vibrioforme* MDH, P80038; *C. tepidum* MDH, P80039; and *Chloroflexus aurantiacus* MDH, P80040.

RESULTS

Purification of MDH. MDH was obtained efficiently and in a high state of purity by affinity chromatography followed by gel filtration. Table 1 gives a quantitative overview of the purification procedure. In the gel filtration step, MDH eluted as a single peak, which upon native-PAGE analysis revealed only a single protein band. The critical and chief stage of purification was that of affinity chromatography.

Criteria of enzyme purity. MDH was adjudged to be homogeneous, since it migrated as a single band on SDS-PAGE (Fig. 1) and on nondenaturing PAGE. Activity staining of nondenaturing gels yielded one active band for MDH, the location of which coincided with the Coomassie blue-stained band for the protein (results not shown). A further criterion of purity was the detection of a single N-terminal end in the Edman degradation analysis of the purified protein.

The position of *Chlorobium* MDH blotted onto nitrocellulose was revealed by antibodies raised both to itself and to *Chloroflexus* MDH (results not shown).

The pI of *Chlorobium* MDH as determined by isoelectric focusing was 5.95.

MW. To determine the MW of MDH from *C. vibrioforme*, two methods were used: gel filtration through a calibrated Sephadex G-100 column and gradient PAGE. There was good agreement between these methods: the data indicate that the native enzyme has an MW between 74,000 (gradient PAGE) and 77,000 (gel filtration) and a subunit MW of 37,500.

Quaternary structure. The results from native and SDS-PAGE suggest that MDH from *C. vibrioforme*, *C. tepidum*, and *H. gestii* is a dimer of identical subunits. This result is in keeping with the observation of two size classes, small (homogenous dimers) and large (homogenous tetramers), for bacterial MDHs (30).

N-terminal amino acid sequence and composition. Figure 2 shows the sequences of residues in the N terminus of MDH from *C. vibrioforme* (40 positions), *C. tepidum* (33 positions), and *H. gestii* (29 positions). Those from the two *Chlorobium* species show almost complete homology. Table 2 shows the amino acid composition of *C. vibrioforme* MDH.

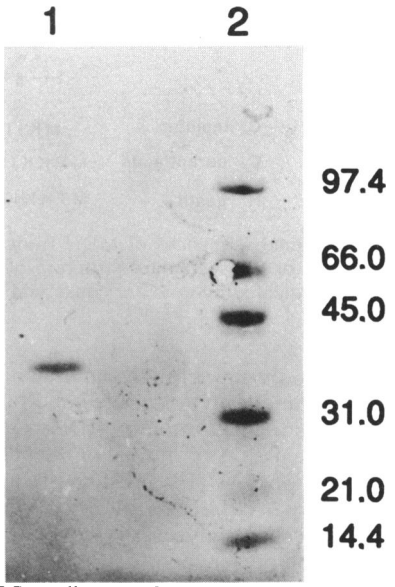


FIG. 1. SDS-gradient PAGE of purified MDH from *C. vibrioforme*. Lane 1, purified MDH; lane 2, MW standards (indicated in thousands).

The relative quantities of proline and tryptophan were not determined. The analysis failed to detect arginine, indicating either a concentration below 0.4 nM (threshold of detection) or an analytical failure. It should be noted, however, that the partial amino acid sequence has an arginine at position 18.

Catalytic properties. The specific activity of MDH in cell extracts of *C. vibrioforme* was between 1.0 and 1.5 U/mg of protein at 25°C. For the purified enzyme the value obtained was 1,200 U/mg at 25°C. This is similar to MDH activities reported for other mesophiles, including phototrophs and chemotrophs (28, 31, 39).

The K_m value for oxaloacetate reduction, as determined by double reciprocal plots, was 5.9×10^{-5} M. This value is similar to that for MDHs from both eubacterial and eukaryotic sources. The temperature and pH optima of pure preparations of MDH were 39°C and 7.4 to 7.6, respectively. For MDH in cell extracts, the corresponding values were 36°C and 7.3. So far, all MDHs examined for pH dependency show a preference for a slightly alkaline environment (10, 19, 20, 23, 41).

DISCUSSION

MDH from *C. vibrioforme* was purified to homogeneity by a two-stage procedure: triazine dye affinity chromatography followed by a Sephadex G-100 gel filtration. Affinity chromatography was the critical stage in terms of the recovery of units of activity. Similar purification procedures have been used for the isolation of MDH from a number of other bacterial sources (31). One disadvantage of the method, however, is the relatively high concentration of NAD required to elute MDH from the ligand.

Chlorobium MDH belongs to the small class of this enzyme. Dimeric and tetrameric forms are represented in both mesophilic and thermophilic bacteria (20, 23, 28, 31, 39) and within the same genus (30). The significance, if any, of the two putative size classes is still not understood. It has been suggested that large MDHs may be better suited than small ones to the physiological and biochemical demands of

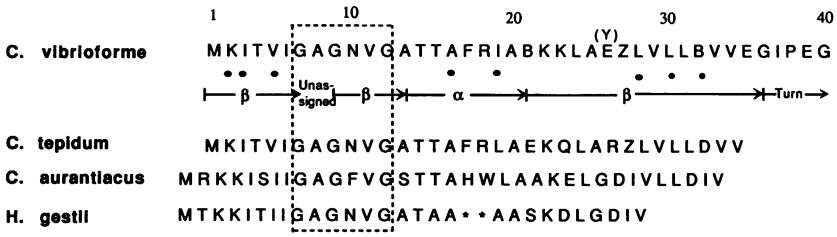


FIG. 2. The N-terminal sequences of MDH from *C. vibrioforme*, *C. tepidum*, *Chloroflexus aurantiacus*, and *H. gestii*. The Chou and Fasman prediction (4) of the secondary structure of *C. vibrioforme* MDH is presented alongside the amino acid sequence. Amino acids occupying positions indicated by dots are conserved relative to the glycine motif (35, 36).

the purple bacteria during growth on malate and other organic compounds under phototrophic conditions (31). It is possible that the dimeric *Chlorobium* MDH is an adaptation to the negative internal redox potential of the illuminated cell in which the supply of NADH is presumably large.

Binding of NAD by MDHs. The following sections of this work involve empirical analyses of the MDH sequences presented above. The presence in their N-terminal ends of a binding region for the ADP moiety of NAD is argued for. Furthermore, we propose that MDHs from the phototrophic bacteria possess a glycine motif alternative to that found in all other MDHs sequenced to date. This motif is in fact the classical pattern for dehydrogenases (34). MDH sequences from two other photosynthetic organisms (eukaryotes) are known to the authors, namely those of the marine diatom *Nitschia alba* (41) and watermelon (9). These enzymes possess the glycine motif characteristic of the majority of sequenced MDHs and not that of bacterial phototrophs. A single MDH glycine motif is thus not ascribable to all phototrophs. However, MDH sequences from purple bacteria and from cyanobacteria would be helpful in ascertaining whether a quasi-phylogeny exists for MDH from prokaryote phototrophs.

Primary protein sequences as an indicator of phylogeny. The N-terminal sequence of MDHs may be useful in phylogenetic analysis, in that this region is highly conserved for the purpose of binding NAD. Variation in this region could therefore be significant. To test this idea, the sequences of MDHs from various eubacterial sources and those of the archaebacterium *Sulpholobus* sp. and mouse were aligned to achieve maximum homology between the first 20 residues in their N-terminal ends (Table 3). The partial amino acid sequences for actinomycetal MDHs are homologous, in accordance with results from 16S rRNA studies, which indicate that the gram-positive eubacteria form a relatively coherent phylogenetic cluster (37). It is thus somewhat surprising that *H. gestii* MDH shows no greater alignment with actinomycetal MDHs than it does with that of the *Sulpholobus* sp. or indeed *E. coli* or *T. flavus*. Comparisons of this nature are, however, by no means conclusive, and 20 amino acids are only a small part of a subunit in excess of 30 kDa. MDH from the photosynthetic bacteria shows little resemblance to MDHs from the other organisms, regardless of their group. In contrast, there is a relatively large homology between MDHs from the photosynthetic prokaryotes, including *H. gestii*. A factor other than pure phylogeny might explain these tendencies, since there is no reason to expect such an accord on the basis of 16S rRNA studies of these organisms.

TABLE 2. Amino acid composition of purified MDH from *C. vibrioforme*

Amino acid	mol%
Ala	9.8
Val	8.9
Leu	8.6
Ile	6.0
Met	4.5
Phe	2.8
Gly	0.9
Ser	7.2
Thr	11.3
Cys	1.0
Asp	5.8
Glu	12.2
Lys	6.3
Arg	0?
His	3.3

If the N-terminal regions of the different MDH sequences are examined in detail, evidence for a unique grouping of MDHs from photosynthetic prokaryotes based on NAD-binding is strengthened. Figure 2 shows that the N-terminal sequences of MDHs from the *Chlorobium* species and *H. gestii* fit exactly the predictions for an ADP-binding region in terms of a glycine motif and fingerprint residues. The reader is also referred to the MDH sequence of *Chloroflexus aurantiacus* (23), which similarly obeys the predictions (Fig. 2). Compelling empirical evidence is thus given for the presence of the ADP-binding region early in the N terminus of these four enzymes. Figure 2 also shows the results for MDH from both *Chlorobium* species when the method of Chou and Fasman (4) is applied to predict regions of secondary structure. These predictions can be compared with the well-characterized secondary structure of an ADP-binding region (1, 25, 36). There is good accord in that a β-strand has been predicted to precede the first G in the glycine motif. Similarly, the unassigned residues (8 to 10) correspond to the expected bridgehead between a β-strand and α-helix, emanating from the second G of the motif. However, the prediction of a β-strand arrangement for residues 11 to 15 is at odds with the expected pattern: the mode of ADP binding requires a run-through of α-helix incorporating about 14 residues. This suggests that the interspersing stretch of β-strand as predicted by the method of Chou and Fasman (4) is a rogue statistic. This is returned to below.

The alternating β-α-β pattern corresponding to a Rossmann fold is identified in the prediction. The method of Schiffer and Edmunson (26) for the prediction of amphipathic α-helices involves the plotting of candidate residues on a "helical wheel." Hydrophobic and hydrophilic residues along an α-helix will cluster on opposite sides of the wheel (amphipathically). If we plot the sequence of *C. vibrioforme*

TABLE 3. Number of nonalignments when the first 20 amino acid residues in the N terminus of MDH from various sources are juxtaposed to achieve maximum homology

MDH source (reference)	No. of nonalignments with MDH from:										
	C. vibrioforme	*C. tepidum*	*H. gestii*[a]	*Streptomyces* sp.	*Planomospora* sp.	*Actinoplanes* sp.	*E. coli*	*T. flavus*	*Phenylobacterium* sp.	*Sulphococcus* sp.	Mouse (24)
Chloroflexus aurantiacus (23)	9	9	5	17	16	17	15	17	16	15	16
C. vibrioforme		1	5	17	15	16	15	15	16	15	16
C. tepidum			5	17	15	16	14	15	17	15	16
H. gestii[a]				15	14	14	13	15	15	14	13
Streptomyces sp. (24)					0	4	10	6	7	16	8
Planomonospora sp. (24)						5	9	6	7	15	8
Actinoplanes sp. (24)							10	7	8	17	7
E. coli (32)								10	10	12	12
T. flavus (18)									5	15	5
Phenylobacterium sp. (24)										6	7
Sulpholobus sp. (24)											18

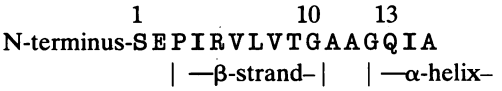
[a] Comparison over only 18 residues, since residues 19 and 20 were not determined.

MDH, assuming an α-helix to originate in the second G of the motif (residue 9), and continue through the short β-strand predicted by the Chou and Fasman method, a helical wheel is obtained with only a single misplaced residue (data not shown). This suggests that the short interspersing β-strand of the Chou and Fasman prediction (4) is a statistical artifact.

Figure 3 shows the hydrophobicity profile of *C. vibrioforme* MDH, according to the method of Miller et al. (15). The method of Chou and Fasman (4) predicts residues 37 to 40 to constitute a turn in the sequence, and these residues occupy a region of hydrophilicity (negative hydrophobicity) in the profile. Turns are almost always on the surface of a protein. The buried β-strand preceding the first G of the glycine motif, which is a characteristic of an ADP-binding region, is represented by a segment of hydrophobicity. The trace describes a region either on the surface or shallowly buried corresponding in position to the functional glycines. This topology would be necessary for access of NAD to the binding site.

A composite of the results of empirical analyses of the *C. vibrioforme* MDH sequence provides very strong argument for the involvement of the N terminus of the enzyme in the binding of NAD.

A recent analysis of cytoplasmic pig heart MDH by X-ray crystallography provides support for the ideas put forward in the present work (3). This analysis showed a short β-strand beginning just beyond the N terminus (third residue) of this enzyme, followed by a short break in secondary structure (residues 11 to 12). An α-helix originates from the glycine residue in position 13, and the residues in positions 10 (glycine) and 12 (alanine) are implicated in the direct binding of the ADP moiety. The situation can be summarized thus:



The early location in the N terminus and secondary structural features of the ADP-binding region of the pig heart MDH are as for the MDH sequences in the present work. However, neither in this segment of pig heart MDH nor elsewhere in the enzyme sequence is there a glycine motif GXGXXG. As was noted above, it is also missing in the complete nucleotide sequences of *E. coli* MDH (32) and *T. flavus* MDH (18). Instead, in common with every MDH sequence known to date (with the exception of those from prokaryotic phototrophs), the pig heart MDH sequence has a glycine motif early in the N terminus (residues 10 to 16), which can be represented as GAXGXXG/A. We propose in addition that this is also the glycine motif of yeast alcohol dehydrogenase (36).

If a different glycine motif is the functional unit in the majority of characterized MDHs, the fingerprint residues should also be conserved in positions relative to it. Table 4 shows MDH sequences which have been scored for glycine motif and fingerprint residues according to the method of Wierenga et al. (35, 36). Four points are given for the possession of glycine residues arranged in the ADP-binding motif proposed above. A further eight points are obtained if a residue with the required amino acid property is located in each of the fingerprint positions. The analysis gives either perfect scores or a single mismatch. Attempts to score with any other G-rich region, such as AXGXXG as was suggested for yeast alcohol dehydrogenase (36), resulted in lower tallies in each instance.

It is beyond the scope of this work to conjecture on the evolutionary origin of the two putative ADP-binding motifs presented. At present, few K_m (NAD) values are available for MDHs, and although this enzyme has been purified from several purple bacteria (31) and a *Chloroflexus* sp. (23), the K_m (NAD) for MDH is known for only one prokaryotic phototroph, the cyanobacterium *Coccochloris penicystis*

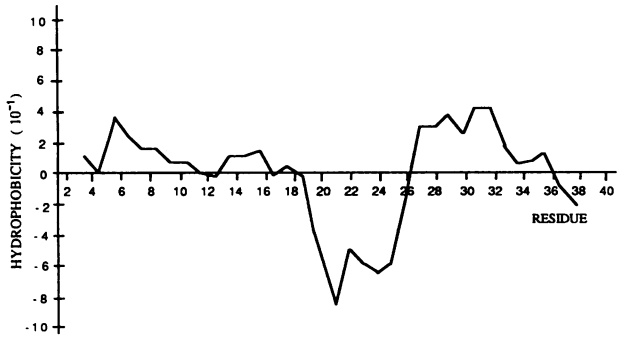


FIG. 3. Hydrophobicity plot of the N-terminal end (33 amino acids) of *C. vibrioforme*, according to Miller et al. (15).

TABLE 4. Appraisal of a variant glycine motif for the binding of the ADP portion of NAD

Glycine motif	Source of MDH (reference)[a]	Score[b]
GASGGIG	m pig (2)	11
GAAGGIG	m yeast (11)	12
GAAGGIG	m melon (9)	9/9[c]
GAAGQIA	c pig (24)	9/9
GAGGGIG	c yeast (11)	9/9
GAAGGIG	c melon (9)	9/9
GAAGQIA	c mouse (24)	9/9
GAAGGIG	c diatom (41)	9/9
GAAGGIG	*E. coli* (32)	12
GAAGQIG	*T. flavus* (18)	11
GAAGNIG	*Phenylobacterium* sp. (24)	8/8
GAAGQIG	*Streptomyces* sp. (24)	9/9
GAAGQIG	*Acintomycetes* sp. (24)	8/8
GAAGLGG	ADH c yeast (36)	11

[a] c, cytoplasmic; m, mitochondrial.

[b] The maximum score of 12 included glycine motif (4 points), basic or hydrophobic residue (1 point), small or hydrophobic residue (6 points), acidic residue (1 point). Some small leeway is tolerated for the fingerprint residues: of the eight recognized positions at which a particular amino acid property is preferred, six or more coincidences invariably denoted an ADP-binding Rossmann fold. For lowers scores the relationship rapidly broke down. The glycine motif and the acid residue are strictly conserved in all instances (35, 36). ADH, alcohol dehydrogenase.

[c] Some of the sequenced lengths are too short to include all the fingerprint residues.

(19). The MDH from *Coccochloris penicystis* has not been sequenced; however, its K_m (NAD) of 24 μM is similar to the value of 200 μM which was obtained with MDH from the thermophile *Thermoleophilum album* (20), which has the glycine motif we have proposed for the majority of MDHs. A thorough analysis of K_m (NAD) for MDHs is pertinent to see whether a correlation between catalytic efficiency and the primary structure of the ADP-binding region emerges. However, specific activities of MDH obtained from various prokaryotic sources, including phototrophs and heterotrophs, are available (23, 28, 30, 31, 39), and these do not appear to partition along lines of trophic type or genus.

For phototrophic organisms it would be useful to study fluctuations in the level of MDH in cells grown with different light intensities. This would go some way towards ascertaining a possible relationship between the expression of the MDH gene and photosynthetic activity.

ACKNOWLEDGMENTS

We thank Knut Sletten for determination of the amino acid composition and the N-terminal amino acid sequences.

This study was partially supported by grant 450.89/006 from the Norwegian Research Council for Science and Humanities (NAVF) and from The Nansen Foundation.

REFERENCES

1. **Banaszak, L. J., and R. A. Bradshaw.** 1975. Malate dehydrogenases, p. 369–396. *In* P. D. Boyer (ed.), The enzymes, vol. XI. Academic Press, Inc., New York.
2. **Birktoft, J. J., and L. J. Banaszak.** 1983. The presence of a histidine-aspartic acid pair in the active site of 2-hydroxy acid dehydrogenases. J. Biol. Chem. **258:**472–482.
3. **Birktoft, J. J., G. Rhodes, and L. J. Banaszak.** 1989. Refined crystal structure of cytoplasmic malate dehydrogenase at 2.5-Å resolution. Biochemistry **28:**6065–6081.
4. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47:**45–148.
5. **Duhamel, R. C., E. Meehan, and K. Brendel.** 1980. Metachromatic staining with Coomassie brilliant blue R 250 of the proline-rich calf thymus histone H I. Biochim. Biophys. Acta **626:**432–442.
6. **Evans, M. C. W., B. B. Buchanan, and D. I. Arnom.** 1966. A new ferredoxin dependent carbon reduction cycle in a photosynthetic bacterium. Proc. Natl. Acad. Sci. USA **55:**1420–1425.
7. **Gest, H., and J. L. Favinger.** 1983. *Heliobacterium chlorum* an anoxygenic brownish-green photosynthetic bacterium containing a new form of bacteriochlorophyll. Arch. Microbiol. **136:**11–16.
8. **Gibson, J., W. Ludwig, E. Stackebrandt, and E. R. Woese.** 1985. The phylogeny of the green photosynthetic bacteria: absence of a close relationship between *Chlorobium* and *Chloroflexus*. Syst. Appl. Microbiol. **6:**152–156.
9. **Gietl, C., F. Lottspeich, and B. Huck.** 1986. Sequence homologies between glyosomal and mitochondrial malate dehydrogenases. Planta **169:**555–558.
10. **Grim, F. C., and D. G. Doherty.** 1901. Properties of the two forms of malate dehydrogenase from beef heart. J. Biol. Chem. **236:**1980–1985.
11. **Kopetzi, E., K.-D. Entian, F. Lottspeich, and D. Mecke.** 1987. Purification procedure and N-terminal amino acid sequence of yeast malate dehydrogenase isoenzymes. Bichim. Biophys. Acta **912:**398–403.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
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. **Miller, S., J. Janin, A. M. Lesk, and C. Chothia.** 1987. Interior and surface of monomeric proteins. J. Mol. Biol. **196:**641–656.
16. **Murphey, W. H., C. Barnaby, F. J. Lin, and N. O. Kaplan.** 1967. Malate dehydrogenases. II. Purification and properties of *Bacillus subtilis*, *Bacillus stearothermophilus* and *Escherichia coli* malate dehydrogenases. J. Biol. Chem. **242:**1548–1559.
17. **Murphey, W. H., G. B. Kitto, J. Everse, and N. O. Kaplan.** 1967. Malate dehydrogenases. I. A survey of molecular size measured by gel filtration. Biochemistry **6:**603–609.
18. **Nishiyama, M., N. Matsubara, K. Yamamoto, S. Iijima, T. Uozumi, and T. Beppu.** 1986. Nucleotide sequences of the malate dehydrogenase gene of *Thermus flavus* and its mutation directing an increase in enzyme activity. J. Biol. Chem. **261:**14173–14183.
19. **Norman, E. G., and B. Colman.** 1991. Characterization of a malate dehydrogenase in the cyanobacterium *Coccochloris penicystis*. Arch. Microbiol. **156:**28–33.
20. **Novotny, J. F.** 1990. Characterization of the malate dehydrogenase from *Thermoleophilum album* NM. Arch. Microbiol. **154:**304–307.
21. **Ormerod, J. G., T. Nesbakken, and Y. Torgersen.** 1990. Phototrophic bacteria that form heat resistant endospores, p. 935–938. *In* M. Baltscheffsky (ed.), Current research in photosynthesis, vol. IV. Kluwer, Dordrecht, The Netherlands.
22. **Pickett, M. W., and D. J. Kelly.** 1991. Carbon metabolism in heliobacteria. Abstr. VII Int. Symp. Photosynthet. Prokaryotes, Amherst, Mass.
23. **Rolstad, A. K., E. Howland, and R. Sirevåg.** 1988. Malate dehydrogenase from the thermophilic green bacterium *Chloroflexus aurantiacus*: purification, molecular weight, amino acid composition, and partial amino acid sequence. J. Bacteriol. **170:**2947–2953.
24. **Rommel, T. O., H.-K. Hund, A. R. Speth, and F. Lingens.** 1989. Purification and N-terminal amino acid sequence of bacterial malate dehydrogenases from six Actinomycetales strains and from *Phenylobacterium immobile*, strain E. Biol. Chem. Hoppe-Seyler **370:**763–768.
25. **Rossman, M. G., D. Moras, and K. W. Olsen.** 1974. Chemical and biological evolution of a nucleotide-binding protein. Nature (London) **273:**443–446.

26. **Schiffer, M., and A. B. Edmundson.** 1968. Correlation of amino acid sequence and conformation in tobacco mosaic virus. Biophys. J. **8**:29–39.
27. **Sirevåg, R., and J. G. Ormerod.** 1970. Carbon dioxide fixation in green sulfur bacteria. Biochem. J. **120**:399–408.
28. **Smith, K., T. K. Sundaram, M. Kernick, and A. E. Wilkinson.** 1982. Purification of bacterial malate dehydrogenases by selective elution from a triazinyl dye affinity column. Biochim. Biophys. Acta **708**:17–25.
29. **Stanier, R. Y., N. Pfennig, and H. G. Trüper.** 1981. Introduction to the photosynthetic prokaryotes, p. 197–220. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes, vol. 1. Springer-Verlag, New York.
30. **Sundaram, T. K., I. P. Wright, and A. E. Wilkinson.** 1980. Malate dehydrogenase from thermophilic and mesophilic bacteria. Molecular size, subunit structure, amino acid composition, immunological homology and catalytic activity. Biochemistry **19**:2017–2022.
31. **Tayeh, M. A., and M. T. Madigan.** 1987. Malate dehydrogenase in phototropic purple bacteria: purification, molecular weight, and quaternary structure. J. Bacteriol. **169**:4196–4202.
32. **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.
33. **Wahlund, T. M., C. R. Woese, R. W. Castenholz, and M. T. Madigan.** 1991. A thermophilic green sulfur bacterium from a New Zealand hot spring: *Chlorobium tepidum* sp. nov. Arch. Microbiol. **156**:81–90.
34. **Wierenga, R. K., and W. G. J. Hol.** 1983. Predicted nucleotide-binding properties of p 21 protein and its cancer-associated variant. Nature (London) **302**:842.
35. **Wierenga, R. K., M. C. H. Maeyer, and W. G. J. Hol.** 1985. Interaction of pyrophosphate moieties with alpha-helixes in dinucleotide binding proteins. Biochemistry **24**:1346–1357.
36. **Wierenga, R. K., P. Terpstra, and W. G. J. Hol.** 1986. Prediction of the occurrence of the ADP-binding fold in proteins using an amino acid sequence fingerprint. J. Mol. Biol. **187**:101–107.
37. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
38. **Woese, C. R., B. A. Debrunner-Vassbrinck, and H. Oyaizy.** 1985. Gram-positive bacteria: possible photosynthetic ancestry. Science **229**:162–165.
39. **You, K.-S., and N. O. Kaplan.** 1975. Purification and properties of malate dehydrogenase from *Pseudomonas testosteroni*. J. Bacteriol. **123**:704–716.
40. **Young, P. R.** 1989. An improved method for the detection of peroxidase-conjugated antibodies on immunoblots. J. Virol. Methods **24**:227–235.
41. **Yueh, A. Y., C.-S. Chung, and Y.-K. Lai.** 1989. Purification and molecular properties of malate dehydrogenase from the marine diatom *Nitschia alba*. Biochem. J. **258**:221–228.