Malate dehydrogenases in phototrophic purple bacteria

Thermal stability, amino acid composition and immunological properties*

Mahmoud A. TAYEH† and Michael T. MADIGAN‡

Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, U.S.A.

Purified malate dehydrogenases from four species of non-sulphur purple phototrophic bacteria were examined for their heat-stability, amino acid composition and antigenic relationships. Malate dehydrogenase from *Rhodospirillum rubrum*, *Rhodobacter capsulatus* and *Rhodomicrobium vannielii* (which are all tetrameric proteins) had an unusually high glycine content, but the enzyme from *Rhodocyclus purpureus* (which is a dimer) did not. *R. rubrum* malate dehydrogenase was extremely heat-stable relative to the other enzymes, withstanding 65 °C for over 1 h with no loss of activity. By contrast, malate dehydrogenase from *R. vannielii* lost activity above 35 °C, and that from *R. capsulatus* above 40 °C. Amino acid compositional relatedness and immunological studies indicated that tetrameric phototrophic-bacterial malate dehydrogenases were highly related to one another, but only distantly related to the tetrameric enzyme from *Bacillus*. This suggests that, despite differences in their thermal properties, the tetrameric malate dehydrogenases of non-sulphur purple bacteria constitute a distinct biochemical class of this catalyst.

INTRODUCTION

Malate dehydrogenase (MDH, EC 1.1.1.37) is the citric acid-cycle enzyme that catalyses the reaction

Malate dehydrogenase occurs in virtually all organisms, and surveys of the molecular properties of MDH from a variety of sources have shown that both dimeric and tetrameric forms of the enzyme exist (Murphy *et al.*, 1967a,b; Sundaram *et al.*, 1980; Smith *et al.*, 1984). MDHs from *Escherichia coli* (Murphy *et al.*, 1967a,b), *Pseudomonas* (You & Kaplan, 1975) and several other Gram-negative bacteria and from eukaryotic sources belong to the low- M_r category. Small MDHs range from 60000 to 70000 in M_r and are dimers of two identical subunits (Banaszak & Bradshaw, 1975). High- M_r MDHs include those from species of *Bacillus* (Murphy *et al.*, 1967a; Sundaram *et al.*, 1980), *Thermoactinomyces* (Smith *et al.*, 1984), and the archaeobacteria *Thermoplasma acidophilum* (Grossebuter *et al.*, 1986) and *Sulfolobus acidocaldarius* (Hartl *et al.*, 1987). Large MDHs range from 117000 to 146000 in M_r , and are tetrameric proteins consisting of four identical subunits (Banaszak & Bradshaw, 1975).

Because of the importance of citric acid-cycle reactions to the metabolism of non-sulphur purple phototrophic bacteria (Sojka, 1978), we have purified and determined the quaternary structure of MDH from selected representatives of this group (Tayeh & Madigan, 1987). The species chosen, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Rhodomicrobium vannielii* and *Rhodocyclus purpureus*, include one representative from each of the four phylogenetic groups of non-sulphur purple phototrophic bacteria defined by 16 S rRNA sequencing

(Woese *et al.*, 1984a,b). Our studies of MDH from these organisms have shown that *R. capsulatus*, *R. rubrum* and *R. vannielii* contain the tetrameric form of the enzyme, and that *R. purpureus* contains a low- M_r (dimeric) MDH (Tayeh & Madigan, 1987).

We report here on the dramatic heat-stability of MDH from one of the phototrophic species, and on the amino acid compositions of MDHs from phototrophic purple bacteria. We also present immunological evidence that supports the contention that the tetrameric MDHs of purple bacteria represent a distinct biochemical class of this catalyst.

EXPERIMENTAL

Bacterial strains, culture media and growth conditions

Rhodobacter (formerly *Rhodopseudomonas*; Imhoff *et al.*, 1984) *capsulatus* strain B100, *Rhodospirillum rubrum* strain 1.1.1, *Rhodomicrobium vannielii* strain EY33 and *Rhodocyclus purpureus* strain 6770 were obtained from our culture collection. *R. capsulatus*, *R. rubrum* and *R. vannielii* were grown in a minimal synthetic medium containing malate as sole carbon source (designated medium RCVB) as described previously (Tayeh & Madigan, 1987). *R. purpureus* was grown in medium RCVB supplemented with 20 µg of vitamin B₁₂/l and 0.01% (w/v) Difco yeast extract, and the phosphate content of the medium was doubled. Cultures were grown phototrophically (anaerobically) in completely filled screw-capped tubes (17 ml), or in bottles (1 litre), or in 20-litre glass carboys sealed under N₂/CO₂ (99:1) with rubber stoppers. *Escherichia coli* and *Bacillus megaterium* were grown aerobically in shake flasks at 37 °C in tryptic soy broth (Difco).

Abbreviation used: MDH, malate dehydrogenase.

* This paper is dedicated to Professor Howard Gest for his many contributions to our understanding of citric acid-cycle function in photosynthetic bacteria.

† Present address: Forest Products Laboratory, Forest Service, USDA, Madison, WI 53705, U.S.A.

‡ To whom correspondence and reprint requests should be addressed.

Preparation of cell-free extracts, and purification and assay of MDH

Cells harvested with a Millipore High-Pressure Ultrafiltration System (Millipore Products, Bedford, MA, U.S.A.) were washed once with 50 mM-potassium phosphate (pH 7) and cell extracts prepared by disrupting thick cell suspensions with a French pressure cell (Tayeh & Madigan, 1987). MDHs from all species were purified to homogeneity as previously described (Tayeh & Madigan, 1987). Enzyme activity was assayed by measuring the initial rate of oxalacetate reduction (NADH oxidation) at 340 nm. One unit of MDH activity is defined as the amount of enzyme that oxidizes 1 μ mol of NADH/min at 25 °C. Proteins were determined by the Coomassie Blue dye-binding assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with bovine serum albumin as a standard.

Heat-stability studies

Purified MDHs were diluted in 0.1 M-potassium phosphate buffer (pH 7.5) and placed in a water bath adjusted to a temperature between 40 and 70 °C. At intervals, 100 μ l samples were removed, chilled on ice, and assayed for MDH activity at 25 °C.

Amino acid composition

Amino acid analysis of purified MDHs was carried out by the method of Dreyer & Bynum (1967), by the batch acid-hydrolysis procedure. Sample vials (each containing 33–55 μ g of protein) were dried under vacuum in a desiccator containing NaOH pellets. The pellets were then removed, and 25 ml of 6 M-HCl was added to the bottom of the desiccator. The whole container was placed in a solid- CO_2 /acetone bath for 20–30 min, evacuated and flushed with N_2 , and the samples were hydrolysed at 110 °C for 24 h. After hydrolysis, the samples were dried as before and analysed on a Beckman model 120C amino acid analyser.

Immunological techniques

Antisera against purified MDHs were prepared in female New Zealand White rabbits. Each rabbit was injected subcutaneously with 2 ml of an emulsion of 0.4–0.6 mg of purified MDH mixed with an equal volume of Freund's complete adjuvant. In subsequent injections, the enzymes were emulsified in incomplete Freund's adjuvant and injected subcutaneously at biweekly intervals. Blood withdrawn from a marginal ear vein 4 weeks after the first injection was centrifuged, and an IgG fraction was enriched by two consecutive $(\text{NH}_4)_2\text{SO}_4$ precipitations as described by Livingston (1974). The pellet from the last precipitation was suspended in 10 mM-potassium phosphate (pH 7), dialysed overnight against the same buffer, and stored in batches at –80 °C. Ouchterlony double-diffusion experiments were performed in agar plates as described by Stollar & Levine (1963) in a solution containing 1% (w/v) agarose (Sigma), 0.15 M-NaCl and 0.02% (w/v) NaN_3 in 10 mM-potassium phosphate, pH 7.5.

RESULTS

Heat-stability of MDH from phototrophic purple bacteria

In preliminary experiments to develop a purification protocol for MDHs from non-sulphur purple photo-

Fig. 1. Thermal stability of purified MDH from *Rhodospirillum rubrum*

The remaining enzyme activity was assayed after incubation for various time periods at 65 °C (○) or 70 °C (●); 100% activity represents 2300 μ mol/min per mg of protein.

trophic bacteria (Tayeh & Madigan, 1987), we observed that *Rhodospirillum rubrum* MDH was considerably heat-stable. Therefore, once in pure form, the heat-stability of MDH purified from *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodomicrobium vannielii* and *Rhodocyclus purpureus* was studied at temperatures between 40 and 70 °C. The results confirmed that MDH from *R. rubrum* was unusually heat-stable; the *R. rubrum* enzyme lost 80–90% of its original activity on heating at 70 °C for 1 h, but was completely stable to 2 h of incubation at 65 °C (Fig. 1); minimal losses were experienced after 7 h incubation at 65 °C (results not shown). MDH from the other species was not nearly as thermostable as *R. rubrum* MDH. For example, 60% of *R. capsulatus* MDH activity was lost in 20 min at 40 °C, and *R. vannielii* MDH lost 90% of its original activity after only 10 min at 40 °C; *R. purpureus* MDH was unaffected by incubation at 40 °C (Fig. 2). *R. purpureus* MDH was not as thermostable as that of *R. rubrum*, however, because *R. purpureus* MDH activity was quickly lost at a temperature of 50 °C (Fig. 3).

Amino acid composition

Since we considered that the thermal stability of *R. rubrum* MDH may be a reflection of its amino acid composition, acid hydrolysates of homogeneous preparations of MDH from the four representative species of non-sulphur purple bacteria were prepared and the amino acid composition of each was determined (Table 1). The values reported are averages of duplicate analyses run on each sample after hydrolysis for 24 h. The number of mol of amino acid residues/mol of enzyme was calculated from average M_r values of 137000 for *R. capsulatus* and *R. rubrum* MDH, 128000 for *R. vannielii* MDH, and 57000 for *R. purpureus* MDH (Tayeh & Madigan, 1987). Half-cystine was completely absent from *R. vannielii* MDH, although detectable amounts (less than 0.3%) were found in the other three phototrophic species (Table 1). MDHs from *R. capsulatus*, *R. rubrum* and, to a lesser extent,

Fig. 2. Thermal stability of purified MDH from *Rhodobacter capsulatus* (○), *Rhodocyclus purpureus* (●) and *Rhodomicrobium vannielii* (■)

The remaining enzyme activity was assayed after incubation for various time periods at 40 °C; 100 % activity represents 1500 $\mu\text{mol/min}$ per mg of protein (*R. capsulatus*), 1700 $\mu\text{mol/min}$ per mg of protein (*R. purpureus*) or 860 $\mu\text{mol/min}$ per mg of protein (*R. vannielii*).

Fig. 3. Thermal stability of purified MDH from *Rhodocyclus purpureus*

The remaining enzyme activity was assayed after incubation for various time periods at 40 °C (○), 45 °C (●) and 50 °C (□); 100 % activity represents 1700 $\mu\text{mol/min}$ per mg of protein.

R. vannielii, had unusually high contents of glycine, nearly 20 % of the residues in the MDHs of these species (Table 1). *R. purpureus* MDH, on the other hand, had a much lower glycine content, but had a higher serine content than in MDH from the other phototrophic

species (Table 1). The acidic/basic amino acid ratio (Asp + Glu/Lys + Arg) was similar in the enzymes from all four species (1.75–2.4). Moreover, non-polar side-chain frequency, calculated as the sum of non-polar residues divided by the total number of residues (Bigelow, 1967), was nearly identical in all four phototrophic species (0.31–0.32).

Amino acid compositional relatedness of two proteins can be estimated by using the formula described by Marchalonis & Weltman (1971). A difference index, SAQ , can be calculated, which is defined as $\sum(n_{iA} - n_{iB})^2$, where n_{iA} and n_{iB} are the mole percentages of the i th type of amino acid in proteins A and B respectively (Marchalonis & Weltman, 1971). By using this formula, compositional comparisons were made of 16 amino acids (cysteine and tryptophan not included) from phototrophic purple bacterial MDHs. With this method, SAQ values < 100 indicate that the proteins being compared are compositionally related (highly related proteins showing values close to 0), whereas unrelated proteins are characterized by having SAQ values > 100.

Pair-wise comparisons of amino acids from MDHs of phototrophic purple bacteria and from other organisms are shown in Table 2. Amino acid compositional relatedness between the tetrameric MDHs of *R. capsulatus*, *R. rubrum* and *R. vannielii* revealed them to be highly related; SAQ values as low as 15 were observed (Table 2). However, compositional relatedness between these MDHs and the reported amino acid composition of MDH from *E. coli* (Murphy *et al.*, 1967b), *Bacillus subtilis* (Yoshida, 1965), *Methanospirillum hungatii* (Storer *et al.*, 1981), *Thermoplasma acidophilum* (Grossebuter *et al.*, 1986), *Saccharomyces cerevisiae* (Hägele *et al.*, 1978) and pig heart mitochondria (Noyes *et al.*, 1974) showed the tetrameric phototrophic bacterial enzymes to be highly unrelated to MDH from these sources; SAQ values were near or greater than 100 in all cases, and in many instances values greater than 200 were calculated (Table 2). SAQ values obtained in pair-wise comparisons of the dimeric MDH of *R. purpureus* and the enzyme from other phototrophic species were as follows: 124 and 158 for *R. capsulatus* and *R. rubrum* respectively, 72 for *R. vannielii*, and 41–74 for the enzyme from species other than phototrophic bacteria (Table 2). Not surprisingly, the closest relationship found for *R. purpureus* MDH was to other dimeric MDHs, in particular to the enzyme from *Methanospirillum* and *E. coli* (Table 2).

Immunological relationships

Ouchterlony double-diffusion experiments were performed with crude or purified MDH preparations from phototrophic purple bacteria. Antisera prepared against MDH from *R. rubrum* formed fused precipitin lines with the homologous antigen, and formed precipitin lines with spurs with MDH from *R. capsulatus* and *R. vannielii* (Fig. 4). Cross-reactions with spur formation were also observed between anti-(*R. capsulatus* MDH) antibodies and *R. rubrum* or *R. vannielii* MDH, or between anti-(*R. vannielii* MDH) antisera and MDH from *R. rubrum* or *R. capsulatus* (results not shown). However, antisera raised to MDH from *R. capsulatus*, *R. rubrum* (Fig. 4) or *R. vannielii* did not form precipitin bands with MDH from *R. purpureus*, and also failed to react with crude cell extracts of *E. coli* or *B. subtilis* or with purified pig heart mitochondrial MDH (results not shown).

Table 1. Amino acid compositions of MDHs from non-sulphur purple bacteria

Acid hydrolysates of purified MDHs were analysed on a Beckman model 120C amino acid analyser.

Amino acid	Composition							
	(mol of residues/mol)				(mol %)			
	<i>R. capsulatus</i>	<i>R. rubrum</i>	<i>R. vannielii</i>	<i>R. purpureus</i>	<i>R. capsulatus</i>	<i>R. rubrum</i>	<i>R. vannielii</i>	<i>R. purpureus</i>
Asp	133	129	116	49	9.2	8.7	10.1	11.9
Thr	59	50	43	17	4.1	3.4	3.7	4.1
Ser	49	61	35	25	3.4	4.1	3.1	6.0
Glu	102	107	79	35	7.1	7.2	6.9	8.5
Pro	68	71	58	21	4.7	4.8	5.1	5.1
Gly	279	311	195	40	19.5	20.9	17.0	9.7
Ala	198	177	162	53	13.8	11.9	14.1	12.9
Val	110	126	90	35	7.6	8.5	7.8	8.5
Met	49	36	29	11	3.4	2.4	2.5	2.7
Ile	83	93	59	20	5.8	6.3	5.1	4.9
Leu	127	115	112	39	8.8	7.8	9.8	9.5
Tyr	19	24	27	6	1.3	1.6	2.3	1.5
Phe	53	36	30	14	3.7	2.4	2.6	3.4
His	12	15	15	5	0.8	1.0	1.3	1.2
Lys	60	94	49	20	4.2	6.3	4.3	4.8
Arg	37	40	49	22	2.6	2.7	4.3	5.3
Cys	3	4	0	2	—	—	—	—
Total residues	1441	1489	1148	414				

Table 2. Amino acid compositional relatedness of MDHs from non-sulphur purple bacteria and other species

$S\Delta Q = (n_{iA} - n_{iB})^2$, where n_{iA} and n_{iB} are the mol percentages of the i th type of amino acid in proteins A and B respectively; $S\Delta Q$ values of less than 100 indicate that the two proteins are related (Marchalonis & Weltman, 1971). Compositions of MDHs from non-phototrophic bacteria and other sources were obtained as follows: *E. coli* (Murphy *et al.*, 1967b); *B. subtilis* (Yoshida, 1965); *M. hungatii* (Storer *et al.*, 1981); *T. acidophilum* (Grossebuter *et al.*, 1986); *S. cerevisiae* (Hägele *et al.*, 1978); mitochondrial (Noyes *et al.*, 1974).

Organism	$S\Delta Q$			
	<i>R. capsulatus</i>	<i>R. rubrum</i>	<i>R. vannielii</i>	<i>R. purpureus</i>
I. Phototrophic bacteria				
<i>Rhodobacter capsulatus</i>	—	—	—	124
<i>Rhodospirillum rubrum</i>	16	—	—	158
<i>Rhodomicrobium vannielii</i>	15	36	—	72
II. Other Eubacteria				
<i>Escherichia coli</i>	130	144	97	50
<i>Bacillus subtilis</i>	175	178	136	61
III. Archaeobacteria				
<i>Methanospirillum hungatii</i>	124	133	96	41
<i>Thermoplasma acidophilum</i>	215	202	176	74
IV. Eukaryotes				
<i>Saccharomyces cerevisiae</i>	246	250	191	68
Pig heart mitochondria	179	188	140	60

DISCUSSION

Rhodospirillum rubrum MDH is unusually thermally stable. Although a mesophilic organism (Pfennig & Trüper, 1974), MDH from *R. rubrum* was unaffected by several hours of incubation at 65 °C. The only heat-stable MDHs previously described have come from

thermophilic bacteria. For example, MDHs from *Bacillus stearothermophilus* (Murphy *et al.*, 1967b) or *Thermoplasma acidophilum* (Grossebuter *et al.*, 1986) are stable at 65 °C, and lose only 50 % of initial activity after 20 min at 75 °C or after 10 min at 77 °C, respectively. The enzymes from both of these species, as for *R. rubrum* MDH, are tetramers.

Fig. 4. Immunodiffusion analyses of phototrophic bacterial MDHs

The centre well of the agar plate contained antiserum (140 μ g) to *Rhodospirillum rubrum* MDH. The outer wells contained the following: A, 45 μ g of purified *R. capsulatus* MDH; B, 54 μ g of purified *R. purpureus* MDH; D, 66 μ g of purified *R. vannielii* MDH; C, E, F, 60 μ g of purified *R. rubrum* MDH.

Since analyses of the tetrameric MDHs of phototrophic purple bacteria showed their amino acid compositions to be quite similar, it is unlikely that the heat-stability of *R. rubrum* MDH can be explained by any unusual amino acid composition. And, although the replacement of non-essential cysteine residues by different amino acids in the MDHs of certain thermophilic bacteria has been postulated to prevent thermal inactivation (Saiki *et al.*, 1978), this also cannot account for the thermal properties of *R. rubrum* MDH; MDH from this organism was found to have the highest cysteine content of all four phototrophic bacteria tested (*R. vannielii* MDH totally lacked cysteine, and was the most thermally labile of the enzymes tested). The thermal stability of *R. rubrum* MDH relative to the enzyme from other phototrophic species is probably due to subtle sequence differences in the *R. rubrum* MDH molecule. For example, Yutani *et al.* (1977) have explained the increase in thermostability of tryptophan synthase from certain mutant strains of *E. coli* as the result of a single amino acid replacement in the α -subunit. Similarly, Perutz & Raidt (1975) have compared the amino acid sequence of ferredoxins from two clostridia, *Clostridium thermosaccharolyticum* (which makes a very heat-stable ferredoxin) with that of *Clostridium tartarivorum* (which makes a very heat-labile ferredoxin), and found that the protein from the two species differed by only two amino acid substitutions. The substitutions, of course, were critical ones, as they allowed the formation of additional salt bridges, which stabilized the ferredoxin from the thermophilic species

(Perutz & Raidt, 1975). In the present case, because antibodies to MDH from other phototrophic purple bacteria still recognize *R. rubrum* MDH, it is likely that considerable amino acid sequence homology between tetrameric MDHs from phototrophic bacteria exists, even if the *R. rubrum* sequence varies in a few locations to account for its thermal-stability properties.

A striking feature of tetrameric MDHs from phototrophic bacteria is their unusually high glycine content, which is at least twice that reported from compositional analyses of MDHs from other sources, including other tetrameric forms of the enzyme (Murphy *et al.*, 1967b; Yoshida, 1965). The functional significance of the high glycine contents of tetrameric phototrophic bacterial MDHs is at present unclear. It obviously does not confer thermal stability on the proteins, because of the wide range of thermal stabilities observed. However, the high glycine contents of the tetrameric MDHs may dictate the unique immunological properties of these molecules. All phototrophic bacterial tetrameric MDHs were found to cross-react, but no cross-reaction was observed between the tetrameric enzymes of phototrophic bacteria and that of *Bacillus*; MDH from species of the latter organism has less than half the glycine content of phototrophic bacterial MDHs (Murphy *et al.*, 1967b; Yoshida, 1965). The amino acid compositional studies and the immunological results thus suggest that the tetrameric MDHs of phototrophic purple bacteria represent a unique biochemical class of this catalyst.

From studies of proteins with known amino acid sequence (and thus composition), Marchalonis & Weltman (1971) and Cornish-Bowden (1979, 1981, 1983) have shown that sequence homology between proteins can be predicted from knowledge of their amino acid compositions. Because compositional and immunological analyses of tetrameric MDHs from phototrophic purple bacteria show them to be significantly related, it is likely that these proteins will show significant amino acid sequence homology, dictated by the fact that nearly one in every five residues is glycine. If significant sequence homology exists, perhaps it is because tetrameric MDHs of phototrophic purple bacteria have evolved to accommodate the diverse metabolic properties of these organisms (Madigan & Gest, 1979).

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