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# Purification and Physicochemical Properties of Malate Dehydrogenase from Bacteria of the Genus *Beggiatoa*

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**Abstract**—Homogeneous malate dehydrogenase (MDH) with a specific activity of 20-24 units per mg protein was purified from the sulfur bacterium *Beggiatoa leptomitiformis* strain D-402 grown organotrophically and lithotrophically and from the organotrophic bacterium *Beggiatoa alba*. MDHs from the *B. leptomitiformis* strain D-402 grown under organotrophic conditions and from *B. alba* are homodimers with the subunit molecular weight of 40 kD. Tetrameric MDH is formed in *B. leptomitiformis* strain D-402 grown under lithotrophic conditions. The dimeric and tetrameric forms of MDH from *B. leptomitiformis* D-402 display some differences in kinetic properties.

*Key words*: malate dehydrogenase, *Beggiatoa leptomitiformis* strain D-402, lithotrophy, purification, subunit structure, catalytic properties

Malate dehydrogenase (EC 1.1.1.37) is involved in catabolic and anabolic processes in plants and animals. The polyfunctional nature of an enzyme is commonly associated with isoforms having different subcellular localization [1, 2]. However, isozyme polymorphism is not characteristic of bacteria, and the involvement of MDH in different metabolic processes may be the result of structural changes in the enzyme molecule [3].

It has been shown that tetrameric MDH is involved in anabolic processes in phototrophic anaerobes [4], whereas organotrophic aerobic microorganisms contain dimeric MDH that is involved in catabolic processes [5].

Study of the structure and regulatory properties of MDH from colorless sulfur bacteria of the genus *Beggiatoa* is of certain interest. These microorganisms occupy special ecological niches characterized by simultaneous presence of two components—hydrogen sulfide and oxygen—i.e., they grow under nonequilibrium environmental conditions, which implies rapid metabolic rearrangements in them [6]. For example, depending on the environmental conditions, the sulfur bacterium *Beggiatoa leptomitiformis* strain D-402 is able to switch from one nutrition type to the other, using either organic electron donors (organotrophic growth) or reduced sulfur compounds (lithotrophic growth) as energy sources [7]. *Beggiatoa alba*, which inhabits upper biotope layers with a decreased concentration of hydrogen sulfide, can utilize only organic compounds (organotrophic growth).

In view of this, the purpose of this work was to obtain homogeneous MDH preparations from *B. leptomitiformis* strain D-402 (grown organotrophically and lithotrophically) and organotroph *Beggiatoa alba* and to study the physicochemical properties and regulatory characteristics of this enzyme.

## MATERIALS AND METHODS

The study was performed using colorless filamentous sulfur bacteria *Beggiatoa leptomitiformis* strain D-402 and *B. alba* strain DSM 1416. The medium used for organotrophic culture (pH 7.6) contained $NaNO_3$ (0.620 g/liter), $NaH_2PO_4$ (0.125 g/liter), $CaCl_2 \cdot 2H_2O$ (0.030 g/liter), $Na_2SO_4$ (0.500 g/liter), KCl (0.125 g/liter), $MgCl_2 \cdot 6H_2O$ (0.050 g/liter), peptone (0.200 g/liter), and lactate (0.200 g/liter). The medium was prepared in distilled water. After sterilization at 1 atm, the medium was supplemented with 10% $NaHCO_3$ solution (0.125 g/liter). Before inoculation, a mixture of microelements and vitamins (0.001 g/liter) was added to the solution [8]. When *B. leptomitiformis* strain D-402 was grown lithotrophically, the medium was supplemented with $Na_2S_2O_3$ (2.0 g/liter). To obtain cell suspensions, the cell cultures were centrifuged at 8000*g* for 20 min. The cells were then washed with 0.05 M Tris-HCl (pH 8.0).

*Abbreviations*: MDH) malate dehydrogenase.

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Cell extracts (homogenates) were obtained by sonication of the cells using an UZDN-2T ultrasound disintegrator (power 500 W, frequency 22 KHz) for 2 min in an ice bath. The extracts were centrifuged at 3000*g* for 5 min at 4°C, and the supernatant was used for analysis.

The enzyme activity was determined spectrophotometrically at 340 nm [9]. The rate of oxaloacetate enzymatic reduction was measured in medium containing 50 mM Tris-HCl (pH 8.0), 1.5 mM oxaloacetate, and 0.15 mM NADH. The MDH activity in the direct reaction was determined in medium containing 50 mM Tris-HCl (pH 9.0), 4 mM malate, and 1 mM $NAD^+$. The enzyme amount that catalyzed conversion of 1 μmole of substrate in 1 min at 25°C was taken as an activity unit (U). The protein content was determined by the method of Lowry et al. [10].

The enzyme was purified according to a four-stage scheme (cell extraction, protein fractionating with ammonium sulfate (45-80%), gel-filtration on a column with Sephadex G-25, and ion-exchange chromatography on a column with DEAE cellulose). MDH from *B. leptomitiformis* strain D-402 and *B. alba* were eluted from DEAE cellulose with step-wise (40-50 mM) and continuous KCl gradients, respectively.

The molecular weight of native protein was determined by gel chromatography [11]. The enzyme solution was passed through columns (2 × 45 cm) filled with Sephadex G-200 (superfine), and its final volume ($V_e$) was measured. The void volume of the column ($V_o$) was determined using Dextran blue. The molecular weight ($M_r$) of the enzyme was determined by the equation: $\log M_r = 6.698 - 0.987(V_e/V_o)$.

The molecular weight of native MDH from *B. leptomitiformis* strain D-402 cultured in the presence of sodium thiosulfate was also determined by gel chromatography on Toyopearl HW-65 using marker proteins catalase (248 kD), peroxidase (44 kD), egg albumin (45 kD), and cytochrome *c* (12.3 kD).

The proteins were separated by SDS-PAGE (12.5% polyacrylamide). Each specimen contained 3 to 5 μg protein. The calibration curve was plotted using standard marker proteins—cellulase (94.6 kD), BSA (66.2 kD), egg albumin (45 kD), carbonic anhydrase (31 kD), and lysozyme (14.4 kD) [12]. Native MDH was subjected to electrophoresis by the method of Davis with some modifications [13]. MDH was specifically detected with Tetrazolium as described in [14]. Protein was detected on electrophoregrams using silver staining [15].

## RESULTS AND DISCUSSION

The results of a typical purification of MDH from *B. leptomitiformis* strain D-402 are shown in Table 1. We obtained MDH preparations from *B. leptomitiformis* strain D-402 cultured both organotrophically (specific activity 24.5 U/mg) and lithotrophically (specific activity 20.4 U/mg) and from *B. alba* (specific activity 23.8 U/mg). The key purification stage yielding homogeneous MDH preparations is ion-exchange chromatography on DEAE cellulose. The electrophoregrams of the purified preparations contained a single protein band with malate dehydrogenase activity.

The specific activities obtained were much lower than those characteristic of plant and animal MDHs. Interestingly, the enzyme from chloroplasts had the closest value of specific activity [16]. MDHs from *Paracoccus denitrificans* and *Haemophilus parasuis* with specific activities 138 and 222 U/mg, respectively, are described in the literature [5, 17]. The MDH specific activity apparently varies significantly depending on the enzyme source and the isolation conditions.

Preparation of highly purified MDH preparations from *B. leptomitiformis* strain D-402 and *B. alba* allowed us to study the physicochemical properties of this enzyme (Table 2). The molecular weight of native MDH from *B. leptomitiformis* strain D-402 determined by gel chromatography on Sephadex G-200 was 84 ± 2 and 165 ± 3 kD for the bacteria cultured organotrophically and lithotrophically, respectively. In the latter case, the molecular weight of the enzyme was also determined by gel chromatography on Toyopearl HW-65 (172 ± 4 kD)

**Table 1.** Purification of malate dehydrogenase from *B. leptomitiformis* strain D-402 cultured in the presence of sodium thiosulfate

| Purification stage | Total volume, ml | Protein amount, mg | Specific activity, U/mg | Yield, % |
|---|---|---|---|---|
| Homogenate | 9.5 | 246.10 | 0.166 | 100 |
| Supernatant | 8.5 | 56.60 | 0.542 | 75 |
| Fractionating with $(NH_4)_2SO_4$ (45-80%) | 1.5 | 6.21 | 2.636 | 40 |
| Gel filtration through Sephadex G-25 | 4 | 5.06 | 2.975 | 37 |
| Ion-exchange chromatography on DEAE cellulose | 1.5 | 0.20 | 20.43 | 10 |

**Table 2.** Physicochemical and catalytic properties of MDH

| Characteristic | *B. leptomitiformis* strain D-402 (lithotroph) | *B. leptomitiformis* strain D-402 (organotroph) | *B. alba* (organotroph) |
|---|---|---|---|
| Molecular weight of native enzyme, kD | 165 ± 3 | 84 ± 2 | 70 ± 1 |
| Subunit weight, kD | 40 ± 1 | 40 ± 1 | 39 ± 2 |
| Number of subunits | 4 | 2 | 2 |
| pH optimum of activity (oxaloacetate) | 8.8 ± 0.2 | 8.0 ± 0.2 | 9.2 ± 0.2 |
| pH optimum of activity (malate) | 10.2 ± 0.2 | 10.3 ± 0.2 | 10.6 ± 0.2 |
| $K_m$ for oxaloacetate, μM | 20 ± 1 | 56 ± 3 | 30 ± 2 |
| $K_m$ for NADH, μM | 17 ± 1 | 48 ± 3 | 22 ± 1 |
| $K_m$ for malate, μM | 670 ± 21 | 320 ± 19 | 230 ± 16 |
| $K_m$ for $NAD^+$, μM | 530 ± 18 | 120 ± 6 | 190 ± 9 |
| $K_i$ for citrate, μM | 0.0 | 140 ± 9 | 85 ± 6 |

(Fig. 1). The molecular weight of MDH from the organotroph *B. alba* was 70 ± 1 kD.

The molecular weight of MDH subunits was determined by SDS-PAGE (Fig. 2). Comparison of the weight of subunits and native enzyme showed that MDH from *B. leptomitiformis* strain D-402 grown lithotrophically is a tetramer comprised of identical subunits. However, MDH from *B. leptomitiformis* strain D-402, which were grown organotrophically, and MDH from *B. alba* are comprised of two identical subunits. According to the published data, MDH is a dimer [18, 19] or a tetramer [20, 21]. Some researchers have reported the existence *in vivo* of MDH oligomers comprised of six and even eight subunits [22, 23].

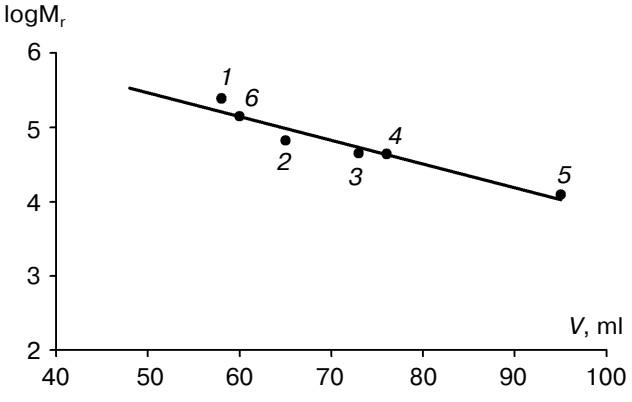


**Fig. 1.** Determination of molecular weight of native MDH from *B. leptomitiformis* strain D-402 cultured lithotrophically by gel chromatography: *1*) catalase; *2*) BSA; *3*) egg albumin; *4*) peroxidase; *5*) cytochrome *c*; *6*) MDH.

It was shown earlier that, during adaptation to hydrogen sulfide-containing biotopes, *B. leptomitiformis* strain D-402 preferentially accepts the electrons from hydrogen sulfide rather than from organic matter. In this case, oxidation is performed along a shortened electron-transport chain, thus excluding the production of hydrogen peroxide (which destroys microorganisms lacking catalase) [24]. For this reason, the lithotrophic nutrition type dominates in *B. leptomitiformis* strain D-402 grown under the conditions of a hydrogen sulfide-containing biotope. The tetrameric MDH form in these bacteria is apparently involved in anabolic processes, providing the bacterium (via the Krebs cycle) with organic acids, such as malate, as a carbon source rather than as an energy source. Apparently, the tetrameric MDH form is better adapted for these physiological conditions than the dimeric form. Conversely, MDH from *B. leptomitiformis* strain D-402 and *B. alba* that utilize only organic electron donors as an energy source (i.e., use the Krebs cycle as an oxidative pathway) are comprised of two subunits.

Interestingly, a similar dependence of the MDH subunit structure on the nutrition type was also discovered in phototrophic organisms belonging to the same systematic group [25]. Hunter and Hellman [26] also detected dimeric and tetrameric MDH forms in *Trypanosoma cruzi* and showed that the dimer is a mitochondrial isozyme, whereas the tetramer is a glyoxysomal enzyme. It was also shown that, in plants, the isozyme with etioplast localization is a tetramer, whereas the mitochondrial isozyme is a dimer [16].

The kinetic and regulatory characteristics of MDH to some extent confirm the possible involvement of the tetrameric and dimeric forms of the enzyme in different biochemical processes.

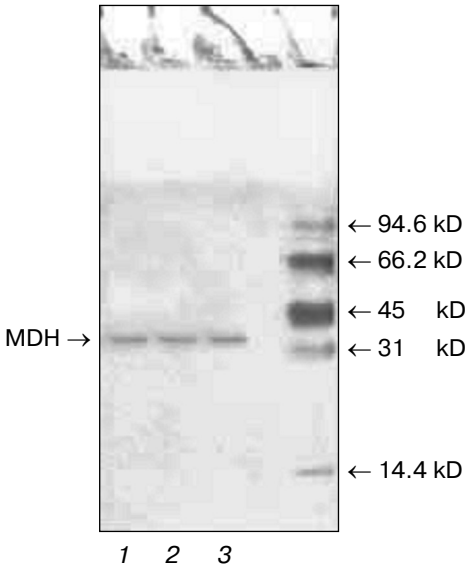


**Fig. 2.** SDS-PAGE of purified MDH from *B. alba* (*1*) and *B. leptomitiformis* strain D-402 cultured lithotrophically (*2*) and organotrophically (*3*).

The $K_m$ for oxaloacetate was 56 ± 3 and 20 ± 1 μM for MDH from *B. leptomitiformis* strain D-402 cultured in the presence and absence of sodium thiosulfate, respectively, which is indicative of a high affinity of the enzyme for oxaloacetate. The $K_m$ values for malate are indicative of a lower affinity of the enzyme for this substrate: 320 ± 5 and 670 ± 13 μM for MDH from *B. leptomitiformis* grown organotrophically and lithotrophically, respectively.

The $K_m$ values obtained in this study are consistent with the data obtained by other researchers. MDH isolated from different sources is characterized by a high affinity for oxaloacetate (e.g., $K_m$ for mitochondrial MDH from spinach leaves is 5.7 μM; from *Thermoplasma acidophilum*, 5.6 μM; from *Toxocara canis*, 53 μM) and a lower affinity for malate [27]. Apparently, the differences between the kinetic parameters of the reactions of malate oxidation and oxaloacetate reduction can be used by the cell for selective performance of one or another biochemical transformation.

We found that oxaloacetate at high concentrations inhibits the enzyme. The substrate inhibition constant ($K_{si}$) was 2.95 ± 0.1 and 3.65 ± 0.1 mM for MDH from *B. leptomitiformis* strain D-402 grown organotrophically and lithotrophically, respectively.

Comparison of the kinetic parameters of MDHs from the bacteria studied shows that $K_m$ for oxaloacetate determined for MDH from *B. leptomitiformis* strain D-402 grown organotrophically was greater than that determined for *B. leptomitiformis* strain D-402 grown lithotrophically. The opposite picture was observed in the case of malate oxidation. This fact indirectly confirms the assumption that some reactions of the reverse Krebs cycle occur in *B. leptomitiformis* strain D-402 cultured in the presence of sodium thiosulfate.

The kinetic and regulatory characteristics of MDH from *B. alba* and *B. leptomitiformis* strain D-402 cultured organotrophically are similar.

We also studied the effect of some substrates used in the Krebs cycle on the MDH activity. We found that citrate inhibited the activity of the dimeric MDH from *B. leptomitiformis* strain D-402 in a competitive manner ($K_i$ = 140 μM) but had no effect (at the concentrations used) on the tetrameric MDH from *B. leptomitiformis* strain D-402. The enzyme was insensitive to fumarate, succinate, and isocitrate at the concentrations used (0.005-5 mM).

Thus, we demonstrated that bacteria of the genus *Beggiatoa* contain dimeric and tetrameric MDH. The bacteria with organotrophic nutrition type contain dimeric MDH, which is probably involved in catabolic processes (the Krebs cycle). The tetrameric MDH functions in the bacteria cultured under lithotrophic conditions, possibly being involved in anabolic processes (supply of organic acids as a carbon source).

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