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EXPERIMENTAL ARTICLES

The Role of Malate Dehydrogenase Isoforms in the Regulation of Anabolic and Catabolic Processes in the Colorless Sulfur Bacterium *Beggiatoa leptomitiformis* D-402

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Abstract—The functional role of tetrameric and dimeric isoforms of malate dehydrogenase in the carbon metabolism of the colorless sulfur bacterium *Beggiatoa leptomitiformis*, strain D-402, was studied. This strain can grow both lithotrophically and organotrophically. By use of inhibition analysis, the tetrameric isoenzyme was shown to operate in the glyoxylate cycle and the dimeric form was found to be involved in the TCA cycle. The dynamics of the dimeric isoenzyme conversion to the tetrameric isoform was found to be determined by the rate of thiosulfate oxidation. The regulation of the carbon metabolism in *Beggiatoa leptomitiformis* is supposed to be accomplished by means of structural and functional changes in the protein molecule of malate dehydrogenase.

Key words: colorless filamentous sulfur bacteria, *Beggiatoa*, chemolithoheterotrophy, mixotrophy, malate dehydrogenase, dimer, tetramer, subunits.

Representatives of the group of colorless sulfur bacteria are widespread in freshwater and marine water bodies, in areas of oceanic hydrotherms, and in anthropogenic ecosystems. These organisms are characterized by a large diversity of metabolic pathways that may vary at both the species and the strain levels [1–4]. It was previously shown that, depending on the cultivation conditions, some species can switch from organotrophic to mixotrophic or autotrophic metabolism or may oxidize sulfur compounds in reactions not linked to energy conservation. The three major factors that determine the nutrition type are the availability of an inorganic donor (sulfur compounds), the availability of oxygen, and the concentration of the organic substrate [5, 6].

In this respect, *Beggiatoa leptomitiformis* D-402 can serve as a good model object in studies of a wide range of issues connected with metabolism and pathways of its regulation.

Sulfur metabolism in strain D-402 was previously studied in detail for autotrophic, mixotrophic, and lithoheterotrophic growth. This bacterium possesses constitutive enzymes of dissimilatory sulfur metabolism and derives energy from the utilized sulfur compounds by oxidative phosphorylation in the electron transport chain (ETC). Switching from organotrophic to mixotrophic growth was found to be accompanied by substantial restructuring of the carbon metabolism [3]. The TCA cycle is known to play a major role in supplying the ETC with reducing equivalents in the form of NADH and $FADH_2$ synthesized under the influence of the corresponding dehydrogenases and in providing construction material in the form of amino acid precursors at the level of 2-oxoglutarate and oxalacetate. Studies of the enzymatic activities in the TCA cycle have shown that, in passing from heterotrophic to mixotrophic growth, the dehydrogenase activities in cells of the mixotrophic strain D-402 decrease severalfold as the ETC becomes flooded with electrons from the oxidized sulfur compounds. At the same time, the activity of the glyoxylate cycle enzymes, responsible for biosynthetic growth processes, increases by two to three times. A major role in the operation of both cycles is played by malate dehydrogenase (MDH, EC 1.1.1.37) [7].

Given that MDH is implicated in both cycles, it is important to find how the direction of their processes is controlled by this enzyme. In eukaryotic cells (animals and plants), MDH is represented by two isoenzymes—a dimer and a tetramer—which differ in their structure, functions, and localization and are separated in space. In eukaryotes, the dimeric MDH was previously shown to be located in mitochondria and involved in energy reactions of the TCA cycle, whereas the tetrameric form occurs in the cytoplasm and participates in constructive metabolic processes [7, 8]. Microorganisms do not typically have isoenzyme; in heterotrophic bac-

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teria, MDH occurs as a dimer, while a tetramer was shown in anaerobic phototrophs, where it is mostly involved in constructive metabolic reactions [9]. MDH was isolated from *Beggiatoa* cells grown under mixotrophic and organotrophic culture conditions. Using a multistep purification procedure, an enzyme preparation was obtained in the form of electrophoretically homogeneous protein [10].

Elsewhere, we showed that *Beggiatoa* cells contained two structural isomers of MDH—a dimer and a tetramer—which was not previously known in prokaryotic organisms [11]. The dimer was found under organotrophic growth conditions and the tetramer, under mixotrophic growth. Their considerable distinction was established by the analysis of kinetic properties and inhibition assays. However, the roles that the MDH dimer and tetramer play in metabolism of *Beggiatoa leptomitiformis* D-402 were not fully understood.

The goal of this work was to study the roles of different isoforms of MDH in the regulation of metabolic processes in *Beggiatoa leptomitiformis* D-402.

MATERIALS AND METHODS

The organism and culture conditions. The freshwater filamentous colorless sulfur bacterium *Beggiatoa leptomitiformis*, strain D-402, grown organotrophically and mixotrophically was studied.

The nutrient medium for organotrophic growth was composed of (g/l) $NaNO_3$, 0.620; NaH_2PO_4, 0.125; $CaCl_2 \cdot 2H_2O$, 0.030; Na_2SO_4, 0.500; KCl, 0.125; $MgCl_2 \cdot 6H_2O$, 0.050; peptone, 0.200; lactate, 0.200; and distilled water; the medium pH was 7.6. For lithotrophic growth of *Beggiatoa leptomitiformis*, the same medium was used supplemented with $Na_2S_2O_3$, 2.0 g/l. Upon sterilization (1 atm), a 10% solution of $NaHCO_3$, 0.125 g/l, was added to the medium. Prior to inoculation, the media were supplemented with a solution of trace elements and vitamins [12].

The cell suspension was obtained by culture centrifugation at 8000 *g* for 20 min. The cells were washed in 0.05 M Tris–HCl buffer (pH 8.0) and then resuspended in the same buffer.

Enzyme activity assays. The activities of MDH, isocitrate lyase (IL), and isocitrate dehydrogenase (IDH) were determined as described elsewhere [13].

The total protein was determined by the method of Lowry *et al.* [14]. Thiosulfate was determined by the method of iodometric titration.

Purification and studies of MDH properties. To obtain high-purity MDH preparations, a multistep purification procedure was developed, consisting of enzyme extract isolation, gel filtration in a column with Sephadex G-25 (Pharmacia, Sweden) to remove low-molecular-weight admixtures, and ion-exchange chromatography on a column with DEAE-cellulose with elution by a stepwise KCl gradient (40–50 mM). MDH gel chromatography was performed on a column with Sephadex G-200 (Pharmacia, Sweden).

Determining the molecular mass of the native enzyme. The molecular mass of the native malate dehydrogenase was determined by the method of gel filtration through Sephadex G-200 [15]. The enzyme was passed through a column (2 × 45 cm) filled with Sephadex G-200 (ultrafine) and its volume V_e at the column outlet was measured. The free column volume V_o was determined by using blue dextran. The molecular mass of the enzyme under study was estimated from the relationship

$$\log Mr = 6.698 - 0.987(V_e/V_o).$$

Inhibition assays. In inhibition experiments, rotenone (2 mmol/l) and itaconate (2.5 and 5 mmol/l) were added to the culture medium.

RESULTS

Using multistep purification, MDH enzyme preparations from the sulfur bacterium *Beggiatoa leptomiti-*

Table 1. Physicochemical and kinetic characteristics of MDH isoenzymes from *B. leptomitiformis*

Isoenzymes	Specific activity, μmol/(min mg protein)	Purity index	Molecular mass, kDa	Molecular mass of subunits, kDa	K_m, μM				pH optimum	
					oxalacetate	NADH	malate	NAD^+	oxalacetate	malate
Dimer	24.49 ± 0.73	131	84 ± 1.5	40 ± 2	56 ± 0.15	48 ± 0.22	320 ± 12.23	120 ± 5.90	7.4 ± 0.1	9.7 ± 0.1
Tetramer	20.43 ± 0.61	123	165 ± 3	40 ± 2	20 ± 0.05	17 ± 0.10	670 ± 31.14	530 ± 20.12	8.8 ± 0.2	10.2 ± 0.2

Table 2. Dynamics of transition the tetrameric MDH isoenzyme to the dimeric form upon mixotrophic growth

Enzyme	24 h		48 h		72 h		120 h	
	E	%	E	%	E	%	E	%
Tetramer	2.555	100	1.088	63	0.090	13	0.035	12.7
Dimer	–	–	0.652	37	0.597	87	0.244	87.3

Note: E denotes μmol NADH/(min mg protein).

formis D-402 grown mixotrophically and organotrophically were obtained. Their respective specific activities were 20.4 ± 4.1 U/mg protein (purity, 123) and 24.4 ± 4.8 U/mg protein (purity, 131). The MDH from organotrophically grown cells (24 h) was shown to be dimeric and that from mixotrophically cultured organisms, tetrameric. The physicochemical and kinetic parameters of the obtained MDH isoforms were found to differ significantly (Table 1). The time variation of the relative activities of the two MDH isoenzymes in mixotrophically grown *B. leptomitiformis* D-402 is given in Table 2 (for 24, 48, 72, and 120 h).

One can see that the tetrameric isoenzyme alone was active during the first 24 h of *B. leptomitiformis* D-402 cultivation, accounting for 100% of the MDH activity. After 48 h, both the tetrameric and dimeric MDH were present in cells, as demonstrated by the results of gel chromatography through Sephadex G-200, the enzyme elution from which showed two peaks. The contents of these isoenzymes at this point in time were 63 and 37%, respectively. After that, the share of the tetrameric enzyme continued to decline and that of the dimeric isoform to increase. After 5 days of growth, the tetrameric isoform constituted only 12.7%, whereas the content of the dimeric MDH was as high as 87.3% (Table 2; Fig. 1).

Special experiments were carried out to study the effect of adding extra thiosulfate to the nutrient medium after 48 and 72 h of culture growth. This was found to result in elimination of the dimeric isoenzyme but preservation of the activity in bacteria of the tetrameric form (Figs. 2 and 3).

The freshwater strain *B. leptomitiformis* D-402 is capable of mixotrophic growth in the presence of reduced sulfur compounds and organic compounds [5]. We found that the rate of thiosulfate oxidation by *B. leptomitiformis* D-402 was not constant over 120 h of culturing. The oxidation rate was highest during the first 24 h (30 mg/l $S/S_2O_3^{2-}$) and declined afterwards (20–0 mg/l $S/S_2O_3^{2-}$).

In separate experiments, the influence of rotenone, inhibiting the first ETC complex, and itaconate, inhibiting isocitrate lyase in the glyoxylate cycle, on the occurrence of MDH isoforms was studied. Only the tetrameric enzyme was detected in cells grown in the presence of rotenone and only the dimeric enzyme in cells cultured in the presence of itaconate (Fig. 4).

The results obtained are in agreement with findings on thiosulfate oxidation by bacteria in the presence of itaconate or rotenone. No thiosulfate oxidation was observed in the presence of itaconate, and, in the presence of rotenone, the oxidation rate of $S/S_2O_3^{2-}$ was 25–35 mg/l. The activities of the enzymes IDH and IL in the presence of itaconate were, respectively, 0.27 and 0.002 μmol/(min mg protein) and in the presence of rotenone, 0.09 and 0.035 μmol/(min mg protein).

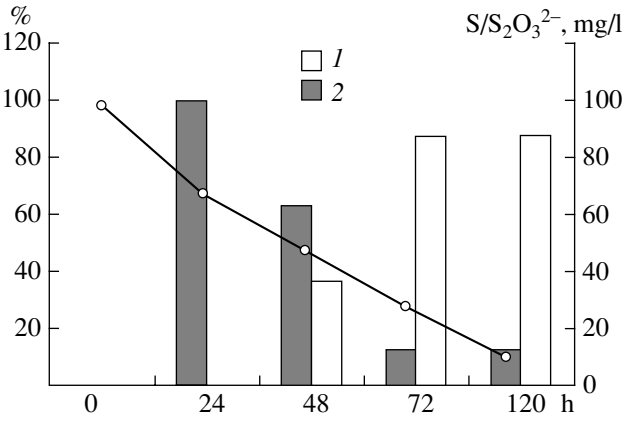

Fig. 1. Dynamics of the activities of the (*1*) dimeric and (*2*) tetrameric MDH isoenzymes in mixotrophically cultured *B. leptomitiformis*. The ordinate is the activity of each of the isoenzymes as a percent of the total MDH activity. The curve shows the thiosulfate oxidation rate as a function of time.

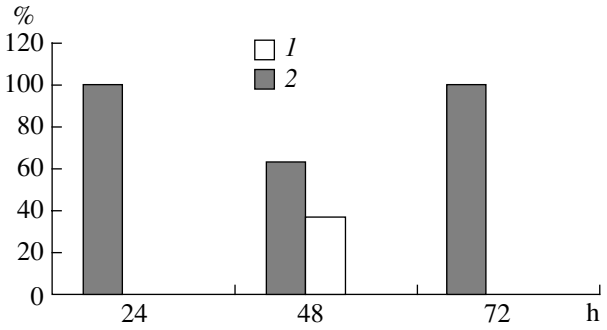

Fig. 2. Dynamics of the activities of the MDH isoenzymes of *B. leptomitiformis* with thiosulfate added to the medium 48 h after the beginning of growth. For notation, see Fig. 1.

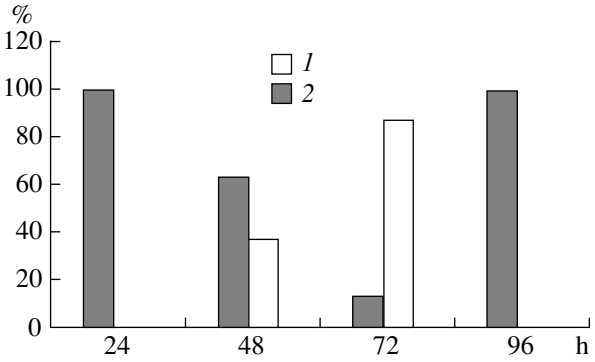

Fig. 3. Dynamics of the activities of the MDH isoenzymes of *B. leptomitiformis* with thiosulfate added to the medium 72 h after the beginning of growth. For notation, see Fig. 1.

DISCUSSION

The occurrence in *B. leptomitiformis* of the two malate dehydrogenase isoenzymes was previously shown to be determined by the type of nutrition of this organism

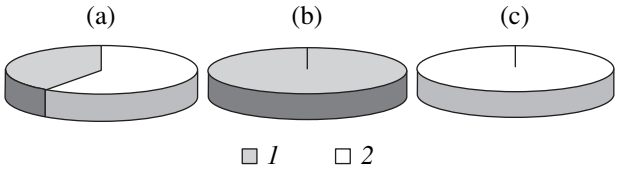

Fig. 4. Relationship between the (*1*) dimeric and (*2*) tetrameric MDH isoenzymes in *B. leptomitiformis* observed after 48 h of mixotrophic growth with thiosulfate (a) in the absence of inhibitors, (b) in the presence of itaconate, and (c) in the presence of rotenone.

[11, 13]. Organotrophic nutrition causes it to have the dimeric isoenzyme, whereas, under lithotrophic growth, the tetrameric MDH becomes functional in these bacteria.

Based on the results obtained, the functional roles of the tetrameric and dimeric malate dehydrogenase isoenzymes can be inferred. The observed dynamics of the dimer-to-tetramer transition in the course of culture growth suggests that the two isoenzymes are involved in different biochemical processes.

The relative content of the dimeric isoenzyme increased as the concentration of thiosulfate in the medium decreased because of its utilization, and this indicates that the dimeric MDH isoenzyme participates in the TCA cycle. Having depleted the inorganic source of electrons, the bacteria switch to heterotrophic growth, and the relative share of the TCA cycle in the energy metabolism of mixotrophs increases. When new portions of thiosulfate are added to the medium, a very fast change in the MDH structure takes place, with the tetrameric isoenzyme activity reaching 100% within only 24 h; this did not depend on the growth phase in which thiosulfate was added.

It follows that thiosulfate acts as a factor regulating the relative abundances of the MDH dimeric and tetrameric isoenzymes. The physicochemical and kinetic characteristics of these isoenzymes also suggest their participation in different processes.

By using two specific inhibitors—itaconate (inhibiting the key enzyme in the glyoxylate cycle) and rotenone

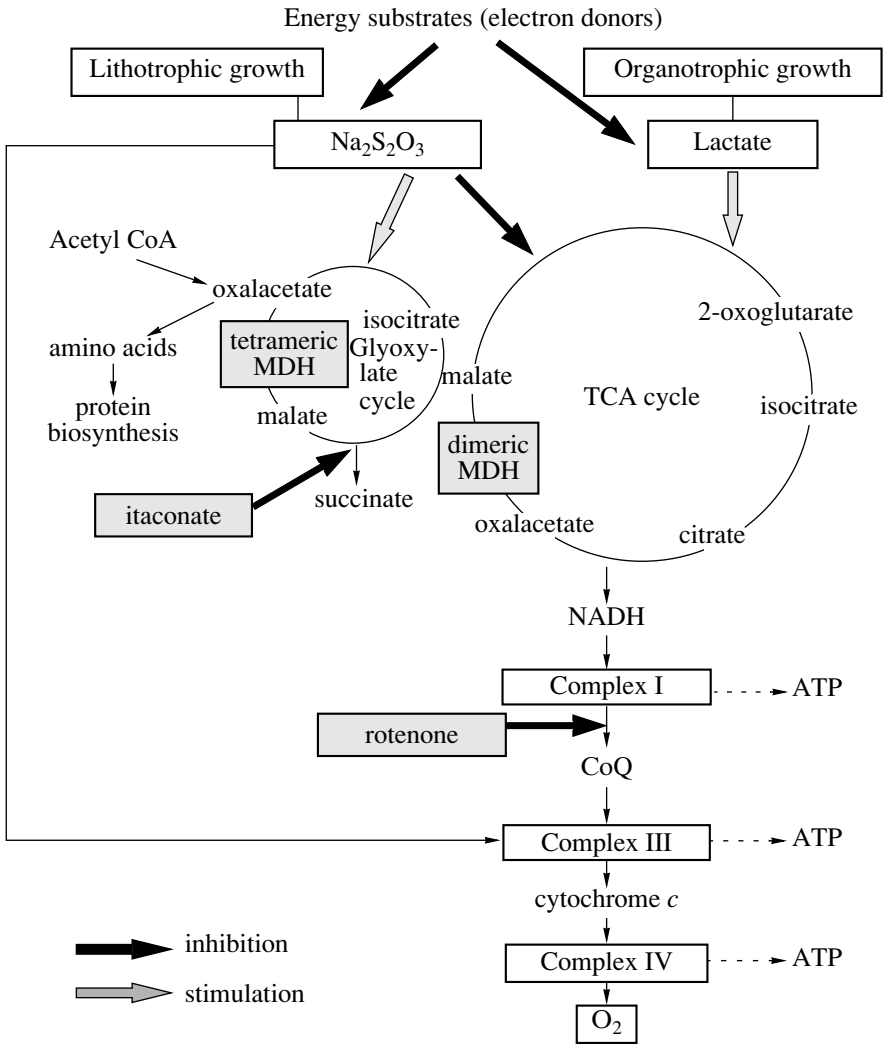

Fig. 5. Hypothetical scheme of MDH participation in switching of major metabolic pathways in sulfur bacteria of the genus *Beggiatoa* under different growth conditions.

(inhibiting NADH dehydrogenases)—interesting results were obtained that suggest different roles of the dimeric and tetrameric isoenzymes of malate dehydrogenase. When bacteria were cultured in the presence of itaconate, their glyoxylate cycle was inhibited, and so their energy metabolism was effected by means of the TCA cycle alone. Interestingly, only the dimeric form of the enzyme under study was found in *B. leptomitiformis* under such conditions, and no thiosulfate oxidation was observed.

With rotenone added to the nutrient medium containing thiosulfate, only the tetrameric isoenzyme was found in cells, and the rate of thiosulfate oxidation was fairly high (Fig. 2).

It follows that, under lithotrophic growth conditions, malate dehydrogenase is functional in the glyoxylate cycle. This conclusion is also supported by a very low activity of isocitrate lyase in the presence of itaconate and by high catalytic capacity of IDH.

An opposite activity pattern of these enzymes is observed upon addition of rotenone.

Based on the obtained evidence, a model of metabolism regulation in sulfur bacteria of the species *B. leptomitiformis* occurring at the level of the malate dehydrogenase enzyme system was developed (Fig. 5). This model specifies the mechanism responsible for regulating major metabolic flows in *B. leptomitiformis* under different growth conditions. The switching and regulation of metabolism in *B. leptomitiformis* occurs, apparently, by means of structural and functional changes in the MDH protein molecule.

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