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EXPERIMENTAL ARTICLES

Dependence of Malate Dehydrogenase Structure on the Type of Metabolism in Freshwater Filamentous Colorless Sulfur Bacteria of the Genus *Beggiatoa*

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Abstract—Major pathways of carbon metabolism were studied in strains D-402 and D-405 of freshwater colorless sulfur bacteria of the genus *Beggiatoa* grown organotrophically and mixotrophically. The bacteria were found to possess all the enzymes of the tricarboxylic acid (TCA) and glyoxylate cycles. When organotrophic growth changed to mixotrophic growth, the activity of the TCA cycle enzymes decreased 2- to 3-fold, but the activity of enzymes of the glyoxylate cycle increased threefold. It follows that, in the oxidation of thiosulfate, organic compounds no longer play the leading part in the energy metabolism, and most of electrons that enter the electron transport chain (ETC) derive from inorganic sulfur compounds. A connection was established between the structure and kinetic characteristics of malate dehydrogenase—an enzyme of the TCA and glyoxylate cycles—and the type of carbon metabolism in the strains studied. Malate dehydrogenase in organotrophically grown cells of strains D-402 and D-405 is dimeric, whereas in strain D-402 grown mixotrophically it is tetrameric.

Key words: malate dehydrogenase, subunit structure, tricarboxylic acid cycle, glyoxylate cycle, organotrophy, lithotrophy, *Beggiatoa.*

Freshwater filamentous sulfur bacteria of the genus *Beggiatoa* were earlier shown to possess effective mechanisms of metabolism regulation. The factors that control switching from one type of nutrition to another are the concentration of oxygen [1], the nature of the carbon source [2], and the presence in the medium of reduced sulfur compounds [1, 3]. Prokaryotes are known to have widely diverse mechanisms of metabolism regulation. There is considerable evidence suggesting that metabolism is regulated at the level of individual enzymes. One such enzyme is malate dehydrogenase (MDH, EC 1.1.1.37), which furnishes reducing equivalents for the TCA cycle and makes possible both energy and constructive metabolisms [4]. In plants and animals, MDH is represented by isoenzymes with different localization and functions. For bacteria, however, isoenzymic polymorphism is atypical, and MDHs participating in different metabolic processes may well differ in molecular structure. In many papers, malate dehydrogenase was characterized as dimer [5] or tetramer [6]. In phototrophic anaerobes, MDH is implicated predominantly in constructive metabolism and is known to have a tetrameric structure [7]. By contrast, in aerobic microorganisms characterized by organotrophic nutrition MDH participates in both constructive and energy metabolism and is dimeric [8]. Therefore, it was of great interest to examine the subunit structure and catalytic properties of MDH in colorless sulfur bacteria of the genus *Beggiatoa*, which, depending on medium conditions, switch between nutrition types and can utilize both organic electron donors and reduced sulfur compounds as the source of energy.

The goal of this work was to study and compare the structure and properties of MDH in the freshwater *Beggiatoa* strains D-402 and D-405 differing in the type of carbon metabolism. In this connection, the activity of enzymes of the tricarboxylic acid (TCA) and glyoxylate cycles was studied in strain D-402 cells grown organotrophically and mixotrophically. We also studied and compared physicochemical properties of homogeneous MDH preparations from cells both strains cultured under organotrophic and lithoheterotrophic conditions.

MATERIALS AND METHODS

Two strains of freshwater filamentous colorless sulfur bacteria of the genus *Beggiatoa* were studied. Strain D-402 is capable of organoheterotrophic, lithoheterotrophic, mixotrophic, and lithoautotrophic growth [1, 3], while strain D-405 can grow only organotrophically [9]. The bacteria are maintained in the collection

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of microorganisms of the Institute of Microbiology, Russian Academy of Sciences.

Composition of nutrient media. Microorganisms were cultured on nutrient medium composed of the following (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. Upon medium sterilization at 121°C for 15 min, 10% sterile solutions of $NaHCO_3$ (0.125 g/l) and $Na_2S_2O_3$ (2.0 g/l) were added to the medium. Before inoculation, a solution of trace elements and vitamins (1.0×10^{-3} g/l) was added [9].

Obtaining cell suspensions and enzyme extracts. Suspensions of bacterial cells were obtained by centrifugation of cultures for 20 min at 8000 *g*. The cells were then washed in 0.05 M Tris–HCl buffer (pH 8.0). Cell extracts (homogenates) were obtained by disrupting cells for 2 min in an ice bath with an UZDN-2T ultrasonic disintegrator operated at 22 kHz (500 W). Supernatant was obtained by centrifugation of cell extracts for 5 min at 9000 *g* at 4°C.

Enzyme activity assays. The activities of enzymes of the TCA and glyoxylate cycles were determined using an SF-26 spectrophotometer by conventional methods described elsewhere [10]. The activity of MDH in the direct reaction was determined using a reaction medium composed of 50 mM Tris–HCl buffer, pH 9.0; 4 mM malate; and 1 mM NAD^+. The amount of enzyme catalyzing the conversion of 1 μmol of the substrate in 1 min at 25°C was taken as the activity unit.

The rate of organic acid decarboxylation was determined as described elsewhere [9]. The total protein was determined by the Lowry method.

MDH purification and studies of its properties. MDH was purified in five steps: obtaining of an enzyme extract; precipitation of protein with ammonium sulfate (45–80%); gel filtration on a Sephadex G-25 column; ion-exchange chromatography on a column with DEAE-cellulose with the elution by stepped KCl gradient (40–50 mM); and chromatography on a Sephadex G-200 column.

The molecular mass of native protein was measured by gel chromatography [11]. The enzyme was passed through a 2×45 cm column filled with Sephadex G-200 (fine grade), and its effluent volume V_e was measured. The free volume V_o of the column was determined using blue dextran. The molecular mass of the enzyme under study was determined by the relationship $\lg M_r = 6.698 - 0.987(V_e/V_o)$.

The SDS–PAAG (sodium dodecyl sulfate–polyacrylamide gel) electrophoresis was performed using a concentration of polyacrylamide gel of 12.5%. Each sample contained 3–5 μg protein. Silver nitrate was used for staining [12]. The calibration curve was obtained using standard marker proteins (kDa): cellulase, 94.6; BSA, 66.2; ovalbumin, 45; carbon anhydrase, 31; and lysozyme, 14.4 [13].

Electrophoresis of native MDH followed the modified Davis method [14]. The tetrazolium method was used for specific MDH detection [15]. Protein was detected by staining with silver nitrate [12].

RESULTS

Carbon metabolism in *Beggiatoa* strain D-402 cells grown organotrophically and mixotrophically. The activities of enzymes of the TCA and glyoxylate cycles determined in strain D-402 cells grown on a medium containing organic compounds in the presence and absence of thiosulfate are given in Table 1. As shown elsewhere, this strain can grow mixotrophically in the presence of thiosulfate [2]. In organotrophically grown cells of strain D-402, most of the enzymes of the TCA cycle showed high activity, comparable to those recorded in strain D-405, which grows only organotrophically [9], and in typical heterotrophic bacteria. The only TCA cycle enzyme that showed low activity was aconitate hydratase. The activity of fumarate hydratase in homogenate of organotrophically grown cells was twofold lower than in homogenate of lithotrophically grown cells.

The activity of dehydrogenases of the TCA cycle during mixotrophic growth was only one half to one-fifth of that recorded under organoheterotrophic conditions. This reduction in activity is apparently associated with changes taking place in the TCA cycle function: its role in the energy metabolism declines, while its contribution to the constructive metabolism increases.

The activities of isocitrate lyase and malate synthase—the key enzymes of the glyoxylate cycle—were also observed to vary markedly with cultivation conditions: they were two to three times higher during mixotrophic growth than during organotrophic growth. The obtained results indicate that the carbon metabolism in cells is altered notably in the presence of reduced carbon compounds: the TCA cycle now operates primarily as an anabolic pathway and the dehydrogenase site is shunted by the glyoxylate cycle, which also provides intermediates for biosynthetic reactions.

To substantiate our hypothesis regarding the operation of the complete TCA cycle and glyoxylate cycle in the bacteria studied, rates of organic acids decarboxylation were determined by the radioisotopic method combined with the inhibitory analysis. The results obtained with 1,4-[^{14}C]-succinate employed as a substrate are presented in Table 2. The rate of decarboxylation dropped down by 78–85% when thenoyltrifluoroacetone, which inhibits succinate dehydrogenase, was added at a concentration of 2 mM and by 85–89% when arsenite (2 mM), inhibiting the activity of 2-oxoglutarate dehydrogenase, was used. These findings indicate that the TCA cycle is closed and active. The use of itaconate (5 mM), which inhibits isocitrate lyase, decreased the rate of decarboxylation by 36–56%. The

Table 1. Activities of enzymes of the TCA and glyoxylate cycles in cells of *Beggiatoa* strain D-402 grown mixotrophically and organotrophically

Enzymes	Specific activity, nmol/(min mg protein)	
	$-S_2O_3^{2-}$	$+S_2O_3^{2-}$
Citrate synthase (EC 4.1.3.7)	ND	8.5
Aconitate hydratase (EC 4.2.1.3)	15.0	15.0
Isocitrate dehydrogenase (EC 1.1.1.42)	**90.2**	**32.8**
2-Oxoglutarate dehydrogenase (EC 1.2.4.2)	ND	12.0
Succinate dehydrogenase (EC 1.3.99.1)	**97.1**	**36.0**
Fumarate hydratase (EC 4.2.1.2)	42.3	97.0
Malate dehydrogenase (EC 1.1.1.37)	**264.0**	**113.0**
Isocitrate lyase (EC 4.1.3.1)	**2.1**	**6.8–7.5**
Malate synthase (EC 4.1.3.2)	**6.5**	**10.4**

Table 2. Decarboxylation of organic acids of the TCA cycle in the suspension of strain D-402 cells grown under different nutrition conditions (experiments with 1,4-[^{14}C]-succinate)

Growth conditions	Specific activity, nmol/(min mg protein)						
	No inhibitor	+TTFA*	Inhibitor, %	+Arsenite	Inhibitor, %	+Itaconate	Inhibitor, %
Mixotrophic	2.4×10^4	5.2×10^3	78	3.9×10^3	85	1.8×10^4	37
	3.5×10^4	6.5×10^3	85	3.7×10^3	89	1.5×10^4	49
Organotrophic	1.7×10^4	5.6×10^3	68	6.2×10^3	63	1.8×10^4	0
	1.7×10^4	3.3×10^3	76	–	–	1.4×10^4	0

* TTFA stands for thenoyltrifluoroacetone.

Table 3. Purification of malate dehydrogenase from cells of strain D-402 grown on medium with thiosulfate

Purification stage	Net volume, ml	Net activity, EU	Amount of protein, mg	Specific activity, EU/mg	Purification degree	Yield, %
Homogenate	9.5	40.9	246.1	0.16	1	100
Supernatant	8.5	30.7	56.6	0.5	3.2	75
Fractionation with 45–80% $(NH_4)_2SO_4$	1.5	16.4	6.2	2.6	15.8	40
Gel filtration on Sephadex G-25	4	15.1	5.1	2.9	17.9	37
Ion-exchange chromatography on DEAE-cellulose	1.5	4.1	0.2	20.43	123	10

Note: The specific MDH activity, the degree of purification, and the enzyme yield for organotrophically grown strains D-402 and D-405 were, respectively, 24.5, 131, 20.9 and 14.6, 94, 14.

latter effect was observed only with mixotrophically grown bacteria.

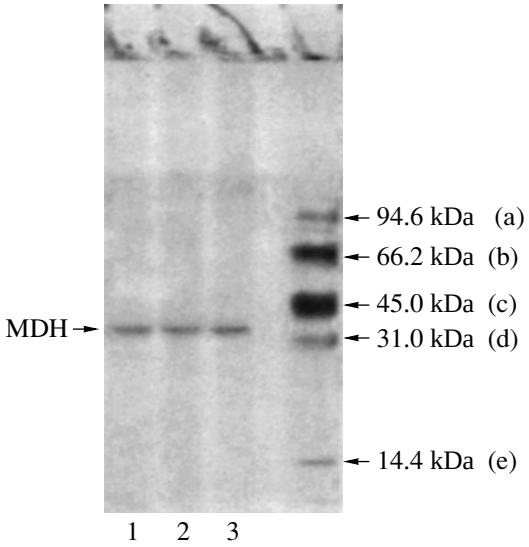
As shown elsewhere [9], the activity of aconitate hydratase in strain D-402 and in strain D-405 is low because this enzyme requires the presence of Fe(II) ions for its operation. Its activity increased by an order of magnitude and even more if the homogenate was preincubated with Fe(II) at a concentration of 10 mg/l. The increased activity of fumarate hydratase during mixotrophic growth as compared to organotrophic growth is likely to be caused by the accumulation in the bacterial cells and growth medium of hydrogen peroxide, which, as we showed for strain D-405 [9, 16], inhibits fumarate hydratase.

Table 4. Molecular mass and subunit structure of MDH in different microorganisms

Microorganism	Molecular mass, kDa	Number of subunits	Subunit molecular mass, kDa
B. alba (organotrophic growth) [17]	70	2	39
Beggiatoa, strain D-405 (organotrophic growth)	95	2	39
Beggiatoa, strain D-402 (organotrophic growth)	84	2	40
Beggiatoa, strain D-402 (mixotrophic growth)	165	4	40
Rhodocyclus purpureus [7]	57	2	28
Paracoccus denitrificans [8]	80	2	40
Pseudomonas stutzeri [5]	66.5	2	34
Chloroflexus aurantiacus [6]	120	4	30
Rhodospirillum rubrum [7]	140	4	37
Rhodobacter capsulatus [7]	130	4	35

Purification of MDH and study of its physicochemical properties. MDH is known to be the most important regulatory enzyme of the TCA cycle. By employing multistage purification, homogeneous MDH preparations were obtained from *Beggiatoa* strains D-402 and D-405 and their physicochemical and regulatory properties were studied (Table 3).

The molecular mass (determined by gel chromatography on Sephadex G-200) of native enzymes isolated from organotrophically grown strains D-405 and D-402 was, respectively, 95 ± 1.9 and 84 ± 1.5 kDa, and that of the enzyme from lithotrophically grown strain D-402 was 165 ± 3 kDa (Table 4). Molecular masses of enzyme subunits were determined by the method of SDS–PAAG electrophoresis. MDH was found to be composed of several isologous subunits with a molecular mass of 39–40 kDa (figure).



SDS–PAAG electrophoresis of purified MDH from *Beggiatoa* representatives: (1) strain D-405 grown organotrophically and strain D-402 grown (2) organotrophically and (3) mixotrophically. The marker proteins are (a) cellulase; (b) BSA, (c) ovalbumin; (d) carbonic anhydrase; and (e) lysozyme.

By studying the effect of the concentration of hydrogen ions on the MDH activity in different *Beggiatoa* strains, the rates of oxalacetate reduction and malate oxidation were shown to peak at alkaline pH values.

The values of K_m with respect to oxalacetate (Table 5), determined by the Lineweaver–Burk method, were 30 ± 1.9 μM for MDH from strain D-405, 56 ± 3.3 μM for MDH from cells of strain D-402 (organotrophic growth), and 20 ± 1.4 μM for MDH from the same strain grown lithoheterotrophically; thus, the enzyme had a high affinity to oxalacetate. The obtained values of K_m with respect to malate showed lower MDH affinity to this substrate. These values were 384 ± 19.3 μM for MDH from strain D-405, 320 ± 18.9 μM for MDH from strain D-402 cells grown organotrophically, and 670 ± 21.2 μM for MDH from strain D-402 cells grown with thiosulfate. High concentrations of oxalacetate were found to inhibit the activity of MDH. The substrate inhibition constant K_{si} (for oxalacetate) for MDH from strains D-405 and D-402 grown organotrophically equaled 3.1 ± 0.1 and 2.95 ± 0.09 mM, respectively, and, for strain D-402 grown with thiosulfate, it was 3.65 mM.

The influence of several substrates of the TCA cycle on the MDH activity was studied. Citrate was shown to competitively inhibit the activity of MDH from organotrophically grown cells of strains D-405 and D-402. The inhibition constants K_i are given in Table 5. However, at the concentrations studied (0.005–0.5 mM), citrate failed to affect the activity of MDH from cells of strain D-402 grown mixotrophically. Fumarate, succinate, and isocitrate, used at concentrations 0.005–5 mM, did not influence the activity of MDH.

Table 5. Kinetic parameters and physicochemical properties of MDH in representatives of the genus *Beggiatoa* grown under different cultivation conditions

Properties	Organotrophic growth			Mixotrophic growth
	B. alba, strain DSM 1416	Strain D-405	Strain D-402	Strain D-402
Optimal pH (oxalacetate)	9.2	8.5	8.0	8.8
Optimal pH (malate)	10.6	10.5	10.3	10.2
K_m, μM (oxalacetate)	30 ± 1.9	47 ± 2.1	56 ± 3.3	20 ± 1.4
K_m, μM (NADH)	22 ± 1.3	40 ± 2.0	48 ± 2.5	17 ± 1.1
K_m, μM (malate)	230 ± 14.7	384 ± 19.3	320 ± 18.9	670 ± 21.2
K_m, μM (NAD^+)	190 ± 9.3	101 ± 5.1	120 ± 6.4	530 ± 18.5
K_i, μM (citrate)	85 ± 5.9	73 ± 4.8	140 ± 9.1	0.0

DISCUSSION

The data on the rates of organic acid decarboxylation and on the effect of inhibitors show that, in strain D-402 grown organotrophically, the TCA cycle is implicated both in anabolic and catabolic processes, whereas, during mixotrophic growth, the TCA cycle most likely operates as an anabolic pathway. It appears that the flow of electrons deriving from oxidized organic and sulfur compounds and getting into the electron transport chain reduces the flux of electrons from NADH, which, in turn, shifts the organic substrate utilization balance towards biosynthetic processes. During mixotrophic growth, the glyoxylate cycle is effective, contributing to constructive metabolism and acting as a shunt for the dehydrogenase site of the TCA cycle.

By using the multistage purification method, homogeneous preparations of MDH were obtained and physicochemical and regulatory properties of enzymes isolated from different strains of filamentous sulfur bacteria were studied.

Using gel chromatography on Sephadex G-200 and SDS–PAAG electrophoresis, the molecular masses of the native MDH and its subunits were determined. It was found that, depending on growth conditions, MDH isolated from cells of the same strain could be either tetrameric or dimeric. For example, MDH from cells of strain D-402 grown mixotrophically is an isologous tetramer. At the same time, the enzyme from the same strain D-402 and from strain D-405 cultured organotrophically is composed of two similar subunits.

The dimeric form of MDH was earlier shown in *B. alba* grown organotrophically. Its properties are similar to those of the dimeric MDH from strains D-402 and D-405 grown organotrophically (Tables 4 and 5). It is very interesting that both dimeric and tetrameric forms of MDH did occur in representatives of one genus. According to some reports, the tetrameric form of the enzyme may implicated in constructive metabolism and the dimeric may responsible for energy processes [7]. In bacteria of the genus *Beggiatoa*, the actual occurrence of either the tetrameric or dimeric form of MDH seems to be determined by the nutrition type. For eukaryotes, it was also shown that dimeric and tetrameric MDH may be involved in different metabolic processes. Hunter *et al.* [17] found dimeric and tetrameric MDH isoforms in *Trypanosoma cruzi* epimastigotes and argued that the dimer was a mitochondrial isoenzyme (MDH implicated in catabolism) and the tetramer was a glyoxysomal isoenzyme (implicated in anabolism). It was also shown that, in plants, the isoenzyme located in etioplast is a tetramer (participating mostly in biosynthetic reactions), whereas the isoenzyme contained in mitochondria is a dimer [18].

The obtained kinetic and regulatory characteristics of malate dehydrogenase suggest that the tetrameric and dimeric varieties of the enzyme could be implicated in different biochemical processes.

The kinetic constant K_m with respect to oxalacetate for MDH of strains D-405 and D-402 grown organotrophically was higher than that for MDH occurring in cells of strain D-402 grown mixotrophically. The reverse pattern was observed with malate oxidation. The MDH from the bacteria that utilized organic compounds (i.e. dimeric MDH) showed higher affinity to malate.

Investigation of the effect of certain substrates of the TCA cycle on the MDH activity in the organisms studied showed that citrate competitively inhibited the activity of MDH having the dimeric structure and isolated from both strains grown organotrophically. The observed competition mechanism may be related to the structural similarity between the substrate and the inhibitor (both having a hydroxyl group). At the same time, citrate in the concentrations tested failed to influence the activity of the four-subunit enzyme from cells of strain D-402. The enzymes were resistant to fumarate, succinate, and isocitrate over the concentration range 0.005 to 5 mM.

In this study, malate dehydrogenase was obtained in a homogeneous state from cells of *Beggiatoa* strains differing in their nutrition type. As a result, we were able to show that these enzymes differed in their subunit structure and catalytic characteristics. Organotrophic microorganisms have a dimeric MDH, which

participates in energy metabolism. A tetrameric MDH is operative in cells of strain D-402 grown mixotrophically. It catalyzes anabolic reactions; in particular, it is involved in the supply of organic acids for anabolic purposes. The data obtained in the study of physicochemical properties of MDH are in fair agreement with the change in the activities of the enzymes of the TCA and glyoxylate cycles observed when organotrophic growth changes to mixotrophic growth. The activities of dehydrogenases of the TCA cycle decreased 2- to 5-fold, and the activities of enzymes of the glyoxylate cycle increased markedly (Table 1), testifying to a serious decline in the role of the TCA cycle in the energy metabolism. The MDH transition from the dimeric form to the tetrameric one effectively curtails the flow of electrons into the electron transport chain and reduces the rate of oxidative reactions in the TCA cycle.

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