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Malate Dehydrogenase from the Thermophilic Bacterium *Vulcanithermus medioatlanticus*

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Abstract—Thermostable dimeric malate dehydrogenase (MDH) was isolated from the microorganism of hydrothermal vents *Vulcanithermus medioatlanticus*. The enzyme was electrophoretically homogeneous and possessed the specific activity of 6.9 U/mg. The large molecular weight of the subunits (55 kD) is likely to provide the rigidity of the enzyme structure (the activation energy of the enzymatic reaction is 32.6 kJ/mol). The thermophilic MDH differs little from the mesophilic enzyme in terms of kinetic and regulatory characteristics.

Key words: malate dehydrogenase, purification, *Vulcanithermus medioatlanticus*, oligomeric structure, catalytic properties, thermostability

The thermophilic facultative chemolitho-heterotrophic bacterium *Vulcanithermus medioatlanticus* isolated from deep sea hydrothermal vents is capable of functioning at extremely high temperatures (up to 80°C). The adaptation of the bacterium to high temperatures is provided by different biochemical mechanisms, one of which is the synthesis of thermoenzymes, enzymes exhibiting high thermostability and thermophilicity (the optimal activity is observed at temperatures exceeded 60°C) [1-3].

According to current data, the thermostability of a protein is due not to the presence of some special amino acid residues or posttranslational modifications [4-6] but to a number of structural factors that are favorable for the formation of the electrostatic, hydrophobic, and other interactions. It was shown that the thermoenzymes cloned and expressed in mesophilic bacteria retain their unusual thermophilic properties. Consequently, they are genetically determined [1]. Thermostability and thermophilicity are mainly determined by the equilibrium between the rigidity and flexibility, as well as by the particular features of the spatial structure of the protein molecule [4, 7].

Structural properties of the important enzyme malate dehydrogenase (MDH, EC 1.1.1.37) of the thermophilic bacteria are described in a number of works. MDH isolated from mesophilic, moderately thermophilic, and extremely thermophilic bacteria is a dimeric or tetrameric protein composed of subunits of 32-36 kD [8, 9]. One of the factors of adaptation to high temperatures can be a change in the quaternary structure of MDH. For example, in the case of the enzyme from the thermophilic green sulfur bacterium *Chloroflexus aurantiacus*, only tetrameric form of the enzyme exhibits activity at high temperatures. A decrease in the temperature of cultivation results in the decomposition of the tetrameric MDH into the inactive dimers and trimers. The thermostability of the dimeric and tetrameric MDH from thermophilic and mesophilic bacteria correlates with the temperature of growth [10].

Previously, our investigations [11, 12] and the works of other authors [8] demonstrated that the rearrangement of the oligomeric structure of MDH from *Beggiatoa leptomitiformis* and *Bacillus* sp. resulted in important changes in metabolism providing the adaptation to the environmental conditions. The goal of the present work was investigation of structure-functional characteristics of MDH of the thermophilic bacterium *V. medioatlanticus*.

MATERIALS AND METHODS

In the present work, we used a pure culture of the thermophilic microorganism *V. medioatlanticus* (strain DSM 14978^T) obtained from the Laboratory of the Microbial Community (Institute of Microbiology of the Russian Academy of Sciences). The culture was cultivated in medium containing 20.0 g NaCl, 0.33 g NH_4Cl, 0.33 g KCl, 0.33 g $MgCl_2 \cdot 6H_2O$, 0.33 g $CaCl_2 \cdot 2H_2O$,

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0.33 g KH_2PO_4, 2.0 g acetate, 3.0 g $NaNO_3$, and 0.1 g of yeast extract in 1 liter of distilled water, the pH of the medium being 6.5 [13]. After sterilization under 1 atm, the medium was supplemented with 10% $NaHCO_3$ (0.125 g/liter). Directly before inoculation, solutions of vitamins and microelements (1 ml/liter) were added into the medium. The cells were centrifuged at 8000*g* for 20 min and washed with 0.05 M Tris-HCl, pH 7.0.

The amount of the enzyme consuming (for the reverse reaction) or producing (for the direct reaction) 1 μmol NADH in 1 min at 60°C was taken as the unit of the activity of MDH.

The activity was determined at 340 nm using a Specord UV-VIS spectrophotometer (Carl Zeiss Jena, Germany). The rate of reduction of oxaloacetate was measured in 50 mM Tris-HCl, pH 8.0, containing 1.5 mM oxaloacetate and 0.15 mM NADH. In the case of the direct reaction, the MDH activity was determined in 50 mM Tris-HCl, pH 9.0, 4 mM malate, and 1 mM NAD^+. Protein concentration was determined by the method of Lowry et al. [14].

To obtain highly purified preparations of MDH, we used a four-step purification procedure. The cells were broken by sonication on ice (2 min at 22 kHz and 500 W) using an UZDN-2T ultrasonic disintegrator (Elektron, Russia). The resulting suspension was centrifuged at 8000*g* for 10 min at 4°C, and the supernatant was passed through a G-25 Sephadex column (1.5 × 20 cm; Pharmacia, Sweden) to remove low-molecular-weight components. The volume of the applied sample did not exceed 4 ml. The proteins were eluted with 75 mM Tris-HCl, pH 8.0. Ion-exchange chromatography was performed on a DEAE-Toyopearl column (1.5 × 12 cm; Toyosoda, Japan). The proteins were eluted with 40-50 mM KCl step gradient in 50 mM Tris-HCl, pH 8.0, with a step of 2 mM. Fractions (2 ml) were collected, and the activity of the enzyme was determined. Finally, we used gel chromatography on a column (2 × 40 cm) with G-150 Sephadex (superfine; Pharmacia) equilibrated with 50 mM Tris-HCl, pH 8.0. The proteins were eluted with the same buffer. To stabilize the enzyme, 2 mM $MgCl_2$ was added into the buffers at all steps of the purification, and 1 mM EDTA on the step of extraction. All stages of the purification were performed at 0-4°C.

The molecular weight of the native enzyme was determined by gel chromatography on a G-150 Sephadex column (2 × 50 cm), and the molecular weight of the subunits was determined by SDS-electrophoresis in 12.5% polyacrylamide gel. To create the calibration curve, we used the following protein standards: bovine serum albumin, 67 kD; ovalbumin, 45 kD; cytochrome *c*, 12.5 kD.

Electrophoresis of the native MDH was performed by the method of Davis (8% polyacrylamide gel). The gels were stained for enzymatic activity using the tetrazolium method and for proteins with $AgNO_3$ [15].

RESULTS AND DISCUSSION

The results of a typical purification are presented in the table. The value of specific activity (6.9 U/mg) was significantly lower than that of MDH from other sources. The previously described bacterial enzymes from *B. leptomitiformis*, *Paracoccus denitrificans*, and *Haemophillus parasuis* exhibited specific activity of 20-24, 138, and 222 U/mg, respectively.

Electrophoresis in polyacrylamide gel of the purified enzyme with subsequent staining with $AgNO_3$ yielded a single band with $R_f = 0.68$ possessing malate dehydrogenase activity (Fig. 1).

The K_m values for oxaloacetate, NADH, malate, and NAD^+ were 48 μM, 1.4 μM, 1.7 mM, and 0.4 mM, respectively. Most meso- and thermophilic organisms exhibit higher values of K_m for MDH while using malate as the substrate, but not oxaloacetate [15-18]. It should be noted that high concentrations of oxaloacetate inhibited the enzyme with $K_i = 2.1$ mM.

Investigation of the effect of substrates of the TCA cycle on the activity of the enzyme showed that citrate inhibited the enzyme in the concentration range 0.005-0.5 mM. The character of the inhibition was competitive with respect to oxaloacetate ($K_i = 20.5$ μM). Fumarate, succinate, and isocitrate at concentrations lower than 5 mM do not affect the activity of the MDH.

Purification of MDH from *V. medioatlanticus*

Step of purification	Total volume, ml	Protein, mg/ml	Specific activity, U/mg	Yield, %
Homogenate	3.8	207	0.063	100
Supernatant	3.3	118	0.099	89.3
Gel filtration	3.9	90	0.111	76.7
Ion-exchange chromatography	2	0.50	5.04	19.2
Gel chromatography	1.4	0.068	6.94	3.6

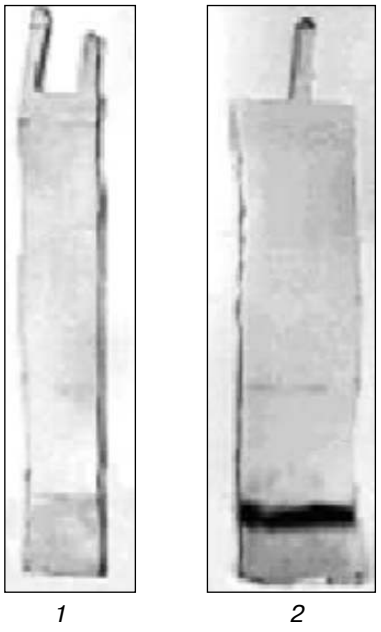

Fig. 1. Electrophoregrams of purified MDH from *V. medioatlanticus*: *1*) staining for activity; *2*) staining with $AgNO_3$.

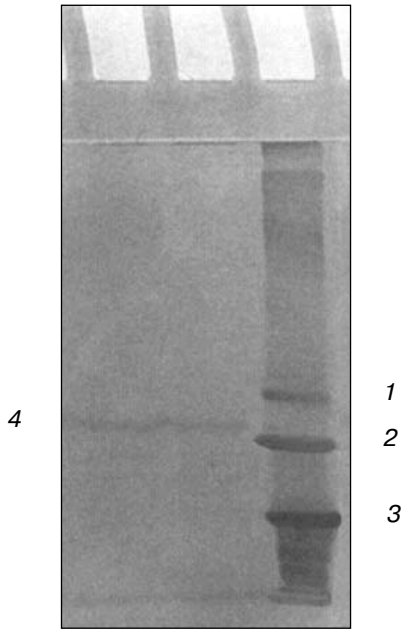

Fig. 2. SDS-PAGE of MDH from *V. medioatlanticus*: *1*) bovine serum albumin; *2*) ovalbumin; *3*) cytochrome *c*; *4*) MDH.

Ions of Mg^{2+}, Ca^{2+}, and Ba^{2+} at concentrations of 4-40 mM activate the enzyme. The Dixon plot demonstrated non-competitive character of the activation with respect to oxaloacetate (concentrations of oxaloacetate, 0.05 and 0.1 mM). Under the same conditions, Mn^{2+} (2-40 mM) and Zn^{2+} (0.05-0.5 mM) inhibited MDH from *V. medioatlanticus* competitively, the K_i value being 2.5 mM (Mn^{2+}) and 0.37 mM (Zn^{2+}). In terms of the regulatory parameters, the investigated enzyme is similar to MDH from the mesophilic bacteria *B. leptomitiformis* and *B. alba* [11]. At the same time, it should be noted that the thermophilic MDH is inhibited by ions of alkaline-earth metals non-competitively, while MDH from *Beggiatoa* is inhibited competitively.

Optimal pH values are 6.7 for oxaloacetate reduction and 8.5 for malate oxidation. According to published data, the optimal pH value for the enzyme from mesophilic organisms is higher [19].

The molecular weight of the native MDH determined by gel chromatography on Sephadex G-150 constituted 128 kD. Electrophoresis demonstrated that the MDH is composed of identical subunits of 55 kD (Fig. 2). It is interesting to note that the molecular weight of the subunits of MDH from *V. medioatlanticus* is higher than that of MDH from other sources [16, 20]. Most thermophilic bacteria have dimeric or tetrameric MDH with subunits of 32-36 kD. The exception is MDH from *Mycobacterium phlei*, being the tetramer composed of 21-kD subunits [21].

A number of works showed no direct dependence between the thermal stability and the oligomeric composition of the MDH thermoenzyme. For example, both the dimeric MDH from the thermophilic archaea *Methanothermus fervidus* and *Archaeoglobus fulgidus* [16, 22] and the tetrameric MDH from *Ch. aurantiacus* [23] exhibit high thermostability. Malate dehydrogenase from *V. medioatlanticus* is stable within a wide range of temperature (Fig. 3). During the time of the experiment, at 25-60°C MDH retains its activity virtually completely. Incubation of the enzyme at 70°C for 1 min results in its partial inactivation. At 85°C, the enzyme completely loses activity during 1 min.

A high thermostability of MDH is characteristic for many thermophilic microorganisms. Malate dehydrogenase from *Bacillus* sp. completely retains its activity after 10 min of incubation at temperatures up to 55°C [24]. Malate dehydrogenase from *Bacillus candolyticus* [25] lost 50% of its activity after 1 min of incubation at 59°C.

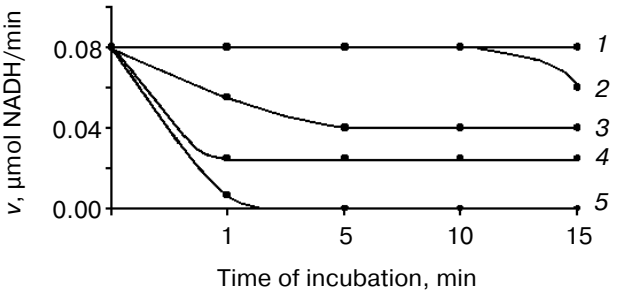

Fig. 3. Thermostability of the preparation of MDH from *V. medioatlanticus*: *1*) at 25 and 50°C; *2*) at 60°C; *3*) at 70°C; *4*) at 80°C; *5*) at 85°C.

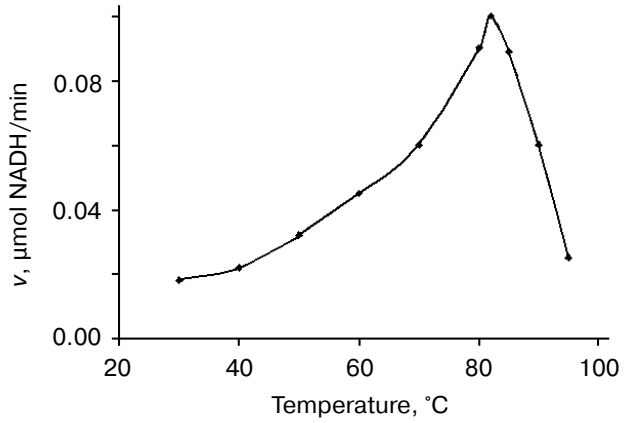

Fig. 4. Dependence of the rate of the reaction catalyzed by MDH from *V. medioatlanticus* on temperature.

Similar data on thermostability were obtained for MDH from *Rhodospirillum rubrum* [20] and *Ch. aurantiacus* [23]. Malate dehydrogenase from mesophilic organisms exhibits less stability [15].

The optimal temperature for the activity of MDH from *V. medioatlanticus* is 82°C (Fig. 4). The value is lower for MDH from mesophilic bacteria [6]. The Arrhenius plot for the reduction of oxaloacetate by MDH from *V. medioatlanticus* was linear (not shown). The calculated value of the activation energy is 32.6 kJ/mol. This value is higher than that for MDH from mesophilic organisms (24.7 kJ/mol) [26]. In the opinion of Hochachka and Somero [6], this suggests "slacking" of the enzyme–substrate complex with increase in temperature. Consequently, for an enzyme functioning at relatively high temperatures more energy is required to stabilize the enzyme–substrate complex than at low temperatures.

Thus, the isolation of electrophoretically homogeneous MDH from *V. medioatlanticus* allowed demonstration of its resistance to irreversible inactivation at high temperatures and thermophilicity. An important feature of the enzyme is its heavy subunits forming the dimeric molecule of MDH. The ability of the thermoenzyme to function at high temperatures is likely due to its structural stability, which suggests restrictions in its flexibility. The increased rigidity of the molecule that is expressed in a high value of the activation energy (compared to mesophilic bacteria) is usually achieved by the increased compactness and formation of additional electrostatic and hydrophobic bonds (the necessary condition for the functioning of MDH at extremely high temperatures).

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