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Physicochemical Properties of Malate Dehydrogenase from the Bacterium *Rhodopseudomonas palustris* Strain f8pt

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Abstract—Electrophoretically homogenous isoforms of malate dehydrogenase with different quaternary structure were prepared from *Rhodopseudomonas palustris* strain f8pt cultured photolithoheterotrophically on malate and acetate. By selective inhibition of the tricarboxylic acid cycle or glyoxylate cycle, it was shown that the dimeric isoform of the enzyme is responsible for Krebs cycle functioning and the tetrameric isoform is involved in functioning of the glyoxylate cycle.

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Malate dehydrogenase (MDH, EC 1.1.1.37) is a polyfunctional enzyme responsible for both energy and constructive metabolism. We have earlier shown the role of structural–functional rearrangements in MDH in the colorless sulfur bacterium *Beggiatoa leptomitiformis* upon replacement of the organotrophic type of nutrition by the lithotrophic type [1-3]. We have shown the involvement of MDH dimer and tetramer in functioning of the tricarboxylic acid cycle (TAC) and glyoxylate cycle, respectively.

Functioning of the MDH tetramer in constructive metabolism of such phototrophic anaerobes as *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and *Rhodomicrobium vannielii* has long been known [4]. The bacterium *Rhodopseudomonas palustris* has the most variable metabolism and is capable of using various types of nutrition [5], and both the TAC and the glyoxylate cycle enzyme activities are detectable with every type of nutrition [6]. The changeover from one nutrition type to another is associated with a change in the relative roles of the TAC and glyoxylate cycle in the bacterial cells. In cultures on acetate-containing media, the glyoxylate cycle is induced in the bacterium, whereas growth on malate is associated with induction of the TAC [6, 7]. It was interesting to study the structural organization of the MDH enzymatic system of *R. palustris* strain f8pt under conditions of functioning of each metabolic pathway. As differentiated from eukaryotic cells, bacteria cannot use isoenzyme polymorphism at the cost of varied subcellular location [8]. Consequently, the involvement of MDH in different metabolic processes in bacteria can be provided by structural changes in the protein molecule [1, 3, 9]. Therefore, our work was designed to obtain homogenous preparations of MDH from *R. palustris* strain f8pt under conditions of mixotrophic growth and to study physicochemical properties of the enzyme.

MATERIALS AND METHODS

The study was performed on the purple phototrophic nonsulfur bacterium *R. palustris* strain f8pt. The bacterium was grown on Pfenning's nutrient medium [10] of the following composition (g/liter): KH_2PO_4 (0.33), $MgCl_2 \cdot 6H_2O$ (0.33), $MnCl_2 \cdot 4H_2O$ (0.33), Na_2SO_4 (0.33), KCl (0.33), $CaCl_2 \cdot 2H_2O$ (0.1), yeast extract (0.1) from Difco (USA), sodium malate (0.5) from Sigma (USA), sodium acetate (0.5) from Serva (Germany), $Na_2S_2O_3$ (2.0), distilled water, the medium pH being 7.6. After sterilization, the medium was supplemented with vitamins and microelements [11]. The cells were precipitated by centrifugation at 8000*g* for 15 min, washed in 0.05 M Tris-HCl buffer (pH 7.5), and destroyed on an ice bath with an

Abbreviations: MDH) malate dehydrogenase; TAC) tricarboxylic acid cycle; SDH) succinate dehydrogenase; BSA) bovine serum albumin.

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UZDN-2T ultrasonic disintegrator at the power of 500 W and frequency of 22 kHz for 2 min. The cellular debris was precipitated by centrifugation at 4000*g* for 5 min at 4°C.

The activity of MDH was determined with an SF-46 spectrophotometer by changes in absorption at 340 nm caused by generation or expenditure of NADH [12]. The rate of oxaloacetate reduction was determined in medium that contained 50 mM Tris-HCl buffer (pH 8.0), 1.5 mM oxaloacetate, and 0.15 mM NADH. The MDH activity in the forward reaction was determined in medium composed of 50 mM Tris-HCl buffer (pH 9.0), 4 mM malate, and 1 mM NAD^+. The MDH amount converting or producing 1 μmol NADH (in the back or forward reaction, respectively) for 1 min at 25°C was taken as the enzyme activity unit. The total amount of the protein was determined by the Lowry method [13].

Malate dehydrogenase was purified using a three-step scheme, which included fractionation with ammonium sulfate (45-80%), gel filtration through a 1.5 × 20 cm column with Sephadex G-25 (Pharmacia, Sweden), and ion-exchange chromatography on a 1.5 × 12 cm column with DEAE-Toyopearl (ToyoSoda, Japan) [14]. Elution was performed with a stepwise concentration gradient of KCl from 25 to 30 mM for MDH tetramer and from 45 to 50 mM for MDH dimer.

The quaternary structure and molecular weight of native MDH were determined by gel chromatography on a 2 × 40 cm column with Sephadex G-200 [14]. The volume of its egress (V_e) was determined. The column free volume (V_0) was determined using dextran blue (Serva). The molecular weight of the enzyme under study was determined by the formula obtained from the calibration curve:

$$\log M_r = 6.698 - 0.987(V_e/V_0).$$

Electrophoresis of native MDH was performed in 9% polyacrylamide gel using a modified Davis system [15]. Specific development was performed with tetrazolium as described in [16], and universal staining for protein was performed with $AgNO_3$ [17]. SDS-PAGE was performed in 12.5% polyacrylamide gel. Each sample contained 3-5 μg protein. For the calibration curve, standard marker proteins from Sigma were used (kD): cellulase (94.6), BSA (66.2), carbonic anhydrase (31.0), trypsin inhibitor (21.5) [18].

For inhibitory analysis we used 5 mM malonate, an inhibitor of the TAC key enzyme succinate dehydrogenase, and 3 mM itaconate, an inhibitor of the glyoxylate cycle key enzyme isocitrate lyase. These inhibitors were added into the culture medium [19].

RESULTS AND DISCUSSION

Two MDH isoforms were revealed by polyacrylamide gel electrophoresis in *R. palustris* strain f8pt under conditions of photolithoheterotrophic growth on sodium acetate and sodium malate (Fig. 1, I).

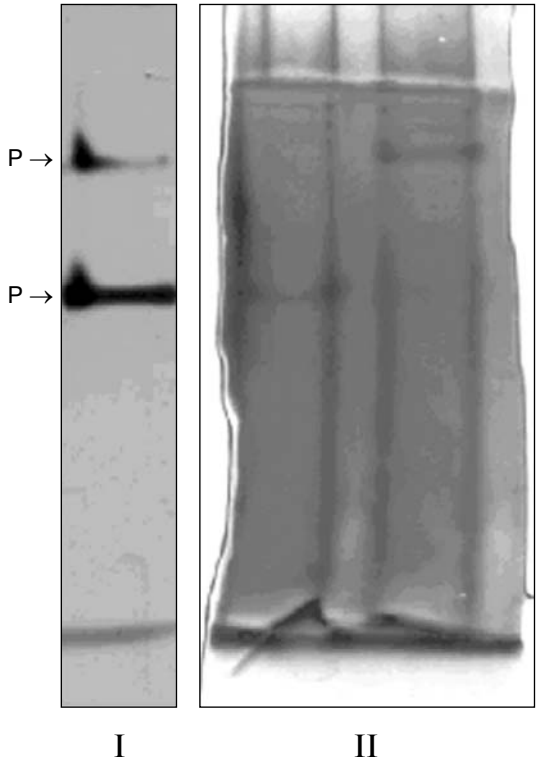

Fig. 1. Electrophoregrams of MDH purified from *R. palustris* strain f8pt cultured photolithoheterotrophically on acetate and malate. I) Specific development of MDH from homogenate of the bacteria; II) staining with $AgNO_3$ of MDH after ion-exchange chromatography on DEAE-Toyopearl (peak *1* and peak *2*). P, protein band.

The bacterium *R. palustris* is characterized by a mobile type of metabolism depending on the culture conditions, and this allowed us to reveal two isoforms of the enzyme under conditions of photolithoheterotrophic growth. For many purple nonsulfur bacteria (*R. capsulatus*, *R. rubrum*, *R. vannielii*), only the tetrameric form of the enzyme is specific [4].

By three-step purification, preparations of two MDH isoforms were obtained with the specific activities of 1.61 and 1.55 U/mg protein from photolithoheterotrophic cultures of *R. palustris* on sodium acetate and sodium malate, respectively (Table 1). Ion-exchange chromatography on DEAE-Toyopearl was a crucial step in the purification, which allowed us to separate the MDH isoforms. Electrophoresis of the purified preparations on polyacrylamide gel and subsequent universal staining of proteins and specific development revealed single protein bands with R_f values of 0.11 and 0.34 (Fig. 1). The specific activities of the resulting homogenous MDH isoforms from *R. palustris* were lower than those of similar enzymes from *Beggiatoa leptomitiformis*. Thus, the

Table 1. Purification of MDH from *R. palustris* strain f8pt grown photolithoheterotrophically on sodium acetate and sodium malate

Step of purification	Total volume, ml	Protein, mg/ml	Specific activity, U/mg	Yield, %
Homogenate	12.5	604	0.035	100
Supernatant	8.8	277	0.038	48.5
Fractionation with $(NH_4)_2SO_4$	4	92	0.065	28.0
Gel filtration	3	38	0.103	18.1
Ion-exchange chromatography:				
peak 1	2	0.50	1.61	3.8
peak 2	2	0.49	1.55	3.5

specific activities of the dimeric and tetrameric isoenzymes from *B. leptomitiformis* were 24.5 and 20.4 U/mg protein, respectively. The specific activity of MDH isolated from the organotrophically growing bacterium *Leucothrix mucor* was 1.2 U/mg protein, whereas under the lithotrophic growth it was only 0.56 U/mg protein. Hence, the specific activity of MDH strongly varies depending on the source of the enzyme isolation [2].

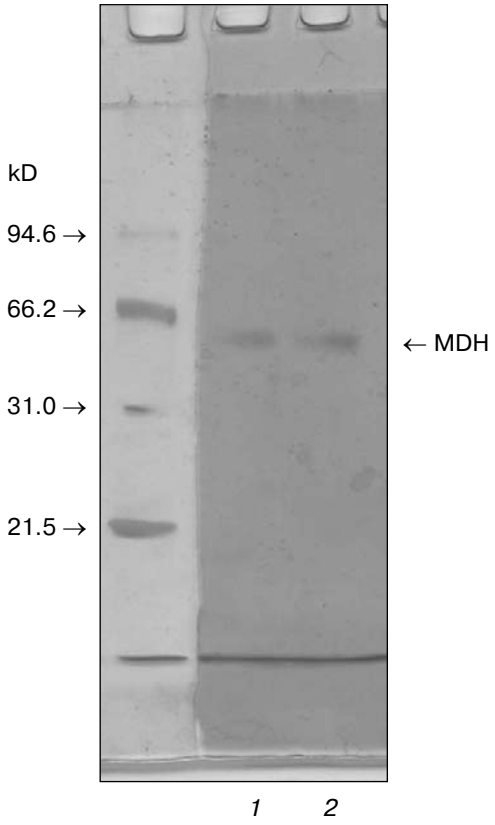

Fig. 2. Determination of molecular weight of the MDH subunit by SDS-PAGE: *1*) subunit of MDH dimer; *2*) subunit of MDH tetramer.

Table 2. Physicochemical characteristics of MDH isoforms from *R. palustris* strain f8pt

Parameter	Value	
	for dimer	for tetramer
Molecular weight, kD	90 ± 4	180 ± 7
K_m (oxaloacetate), μM	30 ± 1	22 ± 1
K_m (NADH), μM	23.0 ± 0.5	17.0 ± 0.5
K_m (malate), μM	530 ± 20	555 ± 15
K_m (NAD), μM	85 ± 3	270 ± 8
pH optimum of activity:		
oxaloacetate	8.9	9.0
malate	10.2	9.7

During gel chromatography on Sephadex G-200, MDH was eluted as two peaks corresponding to molecular weights of 90 and 180 kD. SDS-PAGE of the protein revealed molecular weight of the subunit to be 47 kD in both cases (Fig. 2). Note that the subunit size considerably varies and is 39 kD in *L. mucor* and 58 kD in *Vulcanithermus medioatlanticus* [20, 21]. Data of gel chromatography and SDS-PAGE have shown that MDH in *R. palustris* is present in two isoforms, dimer and tetramer, as is also specific for other bacteria capable of mixotrophic growth [1-3].

Homogenous preparations of the enzyme have different catalytic characteristics of the MDH dimer and tetramer (Table 2). The K_m value for oxaloacetate of the dimer was 30 ± 1 μM and of the tetramer 22 ± 1 μM. This difference can be physiologically important. The K_m values for malate did not differ within the limits of determination error.

Results of experiments with modified nutrient media and specific inhibitors have shown different functional roles of dimeric and tetrameric isoforms of MDH. In the bacteria grown in the presence of itaconate (an inhibitor

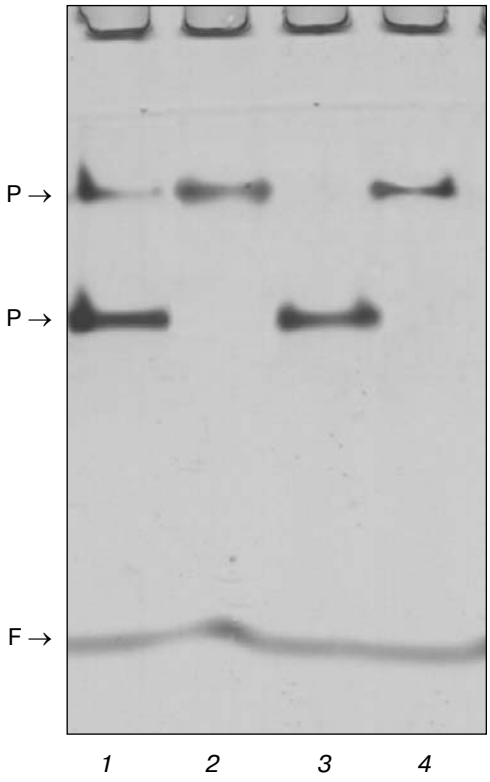

Fig. 3. Electrophoregrams of MDH purified from the bacterium cultured photolithoheterotrophically on acetate and malate: *1*) without inhibitors; *2*) in the presence of malonate; *3*) in the presence of itaconate; on acetate: *4*) without inhibitors. P, protein band; F, front of bromophenol blue dye.

of the glyoxylate shunt), only MDH homodimer has been found in the cells. The shutdown of the glyoxylate cycle by itaconate was indicated by absence or a sharply decreased activity of the cycle key enzyme isocitrate lyase. In nutrient media providing for the "shutdown" of the TAC or a sharp decrease in its functions (during the growth of the bacteria on acetate as the only source of organic substance or in the presence of the SDH inhibitor malonate) only MDH tetramer was detected in *R. palustris* strain f8pt (Fig. 3). It was earlier shown that the nutrition type induced in colorless sulfur bacteria synthesis of either dimer or tetramer of the enzyme under study. Thus, the functional role has been confirmed for the MDH dimer and tetramer, similarly to earlier shown for *B. leptomitiformis* and *B. alba* [1].

Thus, isolation of two homogenous MDH isoforms from the bacterium *R. palustris* strain f8pt cultured under conditions of mixotrophic growth revealed significant features of the enzyme structure. MDH seems to be a mixture of dimer and tetramer responsible for different functions. The dimer of MDH is involved in TAC functioning, whereas the MDH tetramer provides for the functioning of the glyoxylate cycle.

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