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EXPERIMENTAL ARTICLES

Regulation of Carbon Flows in the Tricarboxylic Acid Cycle–Glyoxylate Bypass System in *Rhodopseudomonas palustris* under Different Growth Conditions

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Abstract—The functional roles of the malate dehydrogenase (MDH) tetrameric and dimeric isoforms in the metabolism of the purple nonsulfur phototrophic bacterium *Rhodopseudomonas palustris*, strain f-8pt was studied with the use of specific inhibitors. It was shown that the enzyme tetrameric form allows the functioning of the glyoxylate cycle and the dimeric form provides for the operation of the tricarboxylic acid cycle.

Key words: Rhodopseudomonas, the TCA cycle, the glyoxylate cycle, malate dehydrogenase, dimer, tetramer.

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Purple bacteria are predominantly aquatic microorganisms and are widespread in fresh and marine waterbodies and in hot springs. *R. palustris* is a purple nonsulfur bacterium. It is commonly recognized that *R. palustris* is among the most metabolically versatile bacteria ever studied: it grows both in the presence of oxygen, oxidizing organic substances in the process of respiration, and in its absence, carrying out photosynthesis. The capacities of *R. palustris* for lithotrophic (with hydrogen, sulfide, and thiosulfate), organotrophic, heterotrophic, and autotrophic growth (with CO_2 assimilation via the ribulose–bisphosphate cycle) are also known [1]. Thus, the bacterium *R. palustris* f-8pt is a convenient model for studying metabolism regulation.

Earlier, it was shown that *Beggiatoa leptomitiformis* cells may contain malate dehydrogenase (MDH) isoforms that are formed from the same subunits but have either a dimeric or a tetrameric structure, are involved in differently directed metabolic flows, and differ in kinetic properties [2–5]. In anaerobic phototrophs such as *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and *Rhodomicrobium vannielii*, only the tetramer form, which allows anabolic reactions, was revealed [6]. Bacteria of the species *R. palustris* are characterized by high metabolic versatility and are capable of all types of nutrition [7]. It is known that bacteria usually do not feature isoenzymatic polymorphism, which determines multifunctionality of the malate dehydrogenase system in eukaryotic cells by means of different subcellular localization of the isoenzymes [8]. For *R. palustris*, the existence of only one MDH-encoding gene is known [9]. Along these lines, the study of the structural organization of the malate dehydrogenase enzyme system in *R. palustris* f-8pt cells grown under conditions of functioning of two different MDH-involving metabolic pathways was of interest.

MATERIALS AND METHODS

The organism and cultivation conditions. The subject of this study was the phototrophic purple nonsulfur bacterium *Rhodopseudomonas palustris* f-8pt, isolated from the Goryachii thermal brook in the Karymskii volcanic region (Kamchatka). The bacterium was grown under photo- and chemotrophic conditions, organo- and lithotrophically and hetero- and autotrophically. Pfennig medium was used for organotrophic cultivation of the bacterium [10]. For photoautotrophic growth, the medium of the same composition was used with the addition of 2 g/l of $Na_2S_2O_3 \cdot 5H_2O$. After sterilization (1 atm), $NaHCO_3$ was added to the medium (0.2 g/l for organotrophic cultivation and 1 g/l for autotrophic cultivation). Before inoculation, vitamins and trace elements were introduced into the media [10]. Cell suspensions were obtained by culture centrifugation at 8000 *g* for 15 min, washing of the cells, and resuspending in 0.05 M Tris–HCl buffer, pH 7.5.

Methods for determining enzyme activity. The activity of TCA and glyoxylate cycle enzymes was

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Table 1. Specific activity (nmol/(min/mg protein)) of the TCA and glyoxylate cycle enzymes in *R. palustris* f-8pt grown under different conditions

Enzymes	Cultivation conditions					
	Phototrophic				Chemotrophic	
	Acetate	Succinate	Acetate, succinate	Thiosulfate, bicarbonate	Succinate	Acetate, succinate
Malate dehydrogenase	46.3 ± 1.4	71.1 ± 2.1	83.6 ± 2.5	55.10 ± 1.65	29.8 ± 0.9	28.00 ± 0.84
Isocitrate dehydrogenase	5.40 ± 0.16	21.60 ± 0.65	32.9 ± 1.0	4.20 ± 0.13	37.5 ± 1.1	26.00 ± 0.78
Succinate dehydrogenase	1.10 ± 0.03	10.20 ± 0.31	8.90 ± 0.27	2.20 ± 0.07	2.10 ± 0.06	1.70 ± 0.05
Fumarate hydratase	17.1 ± 0.5	42.60 ± 1.28	37.4 ± 1.1	16.2 ± 0.5	28.0 ± 0.8	45.9 ± 1.4
Citrate synthase	13.8 ± 0.4	15.4 ± 0.5	16.30 ± 0.49	7.80 ± 0.23	8.30 ± 0.25	5.7 ± 0.2
Malate synthase	14.80 ± 0.45	0.80 ± 0.02	9.8 ± 0.3	30.0 ± 0.9	0.50 ± 0.01	8.80 ± 0.26
Isocitrate lyase	10.60 ± 0.31	0.500 ± 0.015	9.10 ± 0.25	15.8 ± 0.5	0.0	4.30 ± 0.13

determined by the methods described in [11–15]. The total amount of protein was determined by the method of Lowry et al. [16].

MDH purification. In order to obtain highly purified MDH preparations, a purification scheme was developed that included obtaining of enzyme extract, gel filtration on a Sephadex G-25 column (Pharmacia, Sweden), fractionation with ammonium sulfate (45–80%), and ion-exchange chromatography on a DEAE-Toyopearl column.

Determination of the molecular mass of native enzyme. In order to determine the molecular mass of native MDH, gel chromatography through Sephadex G-200 (Pharmacia, Sweden) was used [17].

Electrophoresis. Electrophoresis of native MDH was carried out in polyacrylamide gel (9%) according to a modified Davis method. The tetrazolium method was used for specific development [18]. Electrophoresis in the presence of sodium dodecyl sulfate was used for determining the molecular mass of the MDH subunit [19].

Inhibitory analysis. In inhibition experiments, the specific inhibitors itaconate (isocitrate lyase inhibitor) and malonate (succinate dehydrogenase inhibitor) were added to the cultivation medium at a concentration of 5 mmol/l.

RESULTS

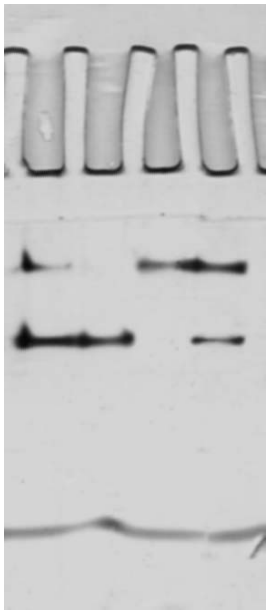
The activity of the TCA and glyoxylate cycle enzymes was found in the cells of *R. palustris* f-8pt; the dependences of the enzyme activities on growth conditions, including the growth substrate used, were revealed (Table 1). On the whole, the highest specific activity of the TCA cycle enzymes was observed during photoorganotrophic growth on succinate and acetate, whereas cultivation on acetate only decreased the activity of the TCA cycle enzymes and increased the activity of isocitrate lyase and malate synthase. The activity of the key enzymes of the glyoxylate cycle was highest during photoautotrophic growth and lowest during photo- and chemoorganotrophic growth on succinate.

Homogeneous MDH preparations were obtained from *R. palustris* f-8pt cells using multistage purification procedure. It was revealed by means of ion-exchange chromatography through DEAE-Toyopearl and gel chromatography through Sephadex G-200 that MDH was represented by two isoforms with molecular mass (Mr) values of 90 and 180 kDa. The Mr of the single subunit, determined by electrophoresis in the presence of sodium dodecyl sulfate, constituted 47 kDa. Hence, the enzyme dimeric and tetrameric isoforms functioned in the cells; however, the presence of one or another isoform depended on the composition of the cultivation medium. The presence of the two forms of the enzyme was confirmed by an electrophoretic study that included specific MDH development (figure).

Experiments on the influence of inhibitors on the ration of MDH forms under different cultivation conditions showed that, when the bacteria grew in the presence of itaconate (the inhibitor of isocitrate lyase, one of the key enzymes of the glyoxylate cycle), only the dimeric MDH isoform could be revealed in the cells, while cultivation of the bacteria in the presence of malonate (the inhibitor of succinate dehydrogenase, one of the key enzymes of the TCA cycle) resulted in the presence of only the tetrameric form of the enzyme. The degree of inhibition of the TCA and glyoxylate cycles was judged from the change in the activity of certain enzymes in cells grown in the presence of the inhibitors, as compared to normal cultivation conditions (Table 2).

DISCUSSION

Analysis of the changes in the specific activity of the TCA and glyoxylate cycle enzymes upon cultivation of phototrophic purple nonsulfur bacteria under different conditions showed that the TCA cycle enzymes were



Electrophoresis of the MDHs isolated from *R. palustris* f-8pt cells grown under different conditions: (*1*) photoorganotrophically on acetate and succinate; (*2*) chemoorganotrophically on succinate; (*3*) photoorganotrophically on acetate; and (*4*) chemoorganotrophically on acetate and succinate.

constitutive, whereas the key enzymes of the glyoxylate cycle proved to be inducible (Table 1). Apparently, the TCA cycle, being the most important catabolic pathway, is also necessary for the formation of precursors for amino acid biosynthesis. Therefore, the TCA cycle enzymes are synthesized virtually irrespectively of growth conditions. The glyoxylate bypass, as an anaplerotic reaction sequence, is obligately necessary during growth on acetate for replenishing the TCA cycle intermediates expended for biosynthetic purposes [20].

We paid special attention to malate dehydrogenase, since this enzyme allows the proceeding of both catabolism and anabolism. Earlier, we revealed functioning of MDH isoforms differing in molecular mass in *B. leptomitiformis* cells grown under different conditions [4].

The methods of gel chromatography and denaturing electrophoresis showed that, in *R. palustris* cells, dimeric and tetrameric forms of the enzyme function; the presence of one or the other isoform is determined by the type of nutrition and the nature of the growth substrate, and, in turn, determines the predominance of the TCA cycle or the glyoxylate cycle (Table 3). The presence of two enzyme isoforms was revealed when the bacteria grew on medium containing acetate + succinate, i.e., under conditions requiring the functioning of both cycles, as well as during chemotrophic growth on acetate, when the TCA cycle is necessary for providing the cells with energy and the glyoxylate cycle compensates for the loss of TCA cycle intermediates expended on biosynthetic needs. Only the dimeric form was revealed when the bacteria were cultivated in the presence of succinate in the dark or in the light. The presence of the malate dehydrogenase dimer as the only isoform correlated with the absence of the glyoxylate bypass. In the case of growth on succinate in the dark, succinate utilization is linked to the reactions of the TCA cycle, whose functioning allows the bacterium both to oxidize organic compounds and to form from them precursors for further biosynthetic processes [20]. When growth occurs on succinate photosynthetically, the TCA cycle only works to meet the biosynthetic needs of the cell, because the bacteria get the energy by means of photosynthetic light transformation. Tetrameric isoform functioning was revealed during phototrophic growth on medium with acetate, when constructive metabolism occurs at the expense of the operation of the glyoxylate cycle, as well as under photoautotrophic conditions, when the Calvin cycle intermediates are the sources of the TCA cycle substrates [1, 7].

Table 2. Specific activity (nmol/(min mg protein)) of the TCA and glyoxylate cycle enzymes in *R. palustris* f-8pt grown in the presence of inhibitors

Enzymes	Cultivation conditions					
	Light, anaerobically (acetate + succinate)			Darkness, aerobically (acetate + succinate)		
	Without inhibitors	Itaconate	Malonate	Without inhibitors	Itaconate	Malonate
Malate dehydrogenase	83.6 ± 2.5	60.3 ± 1.8	48.90 ± 1.47	28.00 ± 0.84	19.8 ± 0.6	9.6 ± 0.3
Isocitrate dehydrogenase	32.9 ± 1.0	28.8 ± 0.9	12.00 ± 0.36	26.00 ± 0.78	11.4 ± 0.34	0.4 ± 0.01
Succinate dehydrogenase	8.90 ± 0.27	4.30 ± 0.13	0.40 ± 0.01	1.70 ± 0.05	1.0 ± 0.04	0.7 ± 0.02
Fumarate hydratase	37.4 ± 1.1	47.8 ± 1.4	0.0	45.9 ± 1.4	71.3 ± 2.2	8.5 ± 0.2
Citrate synthase	16.30 ± 0.49	13.9 ± 0.4	21.70 ± 0.65	5.7 ± 0.2	7.6 ± 0.23	1.3 ± 0.04
Malate synthase	9.8 ± 0.3	0.0	5.40 ± 0.16	8.80 ± 0.26	0.0	4.1 ± 0.12
Isocitrate lyase	9.10 ± 0.25	1.10 ± 0.03	9.60 ± 0.30	4.30 ± 0.13	0.8 ± 0.02	2.5 ± 0.07

Table 3. Regulation of the carbohydrate metabolism of *R. palustris* f-8pt under different growth conditions

Cultivation conditions		Activity of the glyoxylate bypass enzymes, nmol/(min mg protein)		MDH quaternary structure	Functioning metabolic pathways
		Isocitrate lyase	Malate synthase		
Light	Acetate	10.6	14.8	Tetramer	Glyoxylate bypass
	Succinate	0.5	0.8	Dimer	TCA cycle
	Acetate, succinate	9.1	9.8	Tetramer and dimer	TCA cycle and glyoxylate bypass
	Acetate, succinate, itaconate	1.1	0.0	Dimer	TCA cycle
	Acetate, succinate, malonate	9.6	5.4	Tetramer	Glyoxylate bypass
	Photoautotrophic	15.8	30.0	Tetramer	Glyoxylate bypass and Calvin cycle
Darkness	Acetate	4.7	8.5	Tetramer and dimer	TCA cycle and glyoxylate bypass
	Succinate	0.0	0.5	Dimer	TCA cycle
	Acetate, succinate	4.3	8.8	Tetramer and dimer	TCA cycle and glyoxylate bypass
	Acetate, succinate, itaconate	0.8	0.0	Dimer	TCA cycle
	Acetate, succinate, malonate	2.5	4.1	Tetramer	Glyoxylate bypass

The use of inhibitory analysis, which gives the opportunity to selectively switch off individual metabolic pathways, allowed the functional role of the MDH dimeric and tetrameric isoforms to be determined. When the bacterium was grown on the acetate and succinate in the presence of malonate (TCA cycle inhibitor), only the tetrameric form of the enzyme was revealed in the cells, while in the presence of itaconate (glyoxylate cycle inhibitor), only the MDH homodimer was revealed.

Thus, structural and functional changes in the malate dehydrogenase system were revealed in *R. palustris* cells, which are able to switch over their metabolic flows during growth under different conditions. It was shown that, in this bacterium, the dimeric form of the enzyme ensures the proceeding of the TCA cycle reactions, and the MDH tetrameric form provides for functioning of anabolic pathways. Pronounced correlation was revealed between the induction of the glyoxylate cycle and the formation of the MDH tetrameric isoform.

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