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EXPERIMENTAL ARTICLES

A Comparative Study of the Isocitrate Dehydrogenases of *Chlorobium limicola* forma *thiosulfatophilum* and *Rhodopseudomonas palustris*

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Abstract—The carboxylation of 2-oxoglutarate in the reductive tricarboxylic acid cycle in the obligate photolithotroph *Chlorobium limicola* forma *thiosulfatophilum* and the oxidation of isocitrate in the tricarboxylic acid cycle in the photoheterotroph *Rhodopseudomonas palustris* are catalyzed by isocitrate dehydrogenases. A comparative study of these enzymes isolated from the two bacteria showed that they virtually do not differ in enzymatic and kinetic properties.

Key words: *Chlorobium limicola*, *Rhodopseudomonas palustris*, tricarboxylic acid cycle, isocitrate dehydrogenase, isocitrate, 2-oxoglutarate.

Isocitrate dehydrogenase is involved in two enzymatic pathways: the tricarboxylic acid (TCA) cycle of heterotrophs, which oxidizes organic substrates, and the reductive TCA cycle of autotrophs, which is responsible for the assimilation of CO_2 in the green sulfur bacterium *Chlorobium limicola* [1, 2], the sulfate reducer *Desulfobacter hydrogenophilus* [3], and the hydrogen-oxidizing bacterium *Hydrogenobacter thermophilus* [4].

In *Chl. limicola*, isocitrate dehydrogenase catalyzes the reductive carboxylation of 2-oxoglutarate. In the purple nonsulfur bacterium *Rhodopseudomonas palustris*, isocitrate dehydrogenase serves another function—it catalyzes the oxidative decarboxylation of isocitrate.

The aim of the present work was to comparatively study the isocitrate dehydrogenases of these two bacteria.

MATERIALS AND METHODS

The purple nonsulfur bacterium *Rhodopseudomonas palustris* strain 285 and the green sulfur bacterium *Chlorobium limicola* forma *thiosulfatophilum* strain L were obtained from the collection of microorganisms at the Department of Microbiology, Faculty of Biology, Moscow State University. *Rps. palustris* was grown photoheterotrophically in Ormerod medium (pH 7.0) [5] with 0.3% acetate as the carbon source. *Chl. limicola* was grown in Larsen medium [6] with 0.3% $NaHCO_3$, 0.1% Na_2S, and, in some experiments, 0.1% acetate (pH 7.0). The bacteria were cultivated anaerobically at 28–30°C under illumination with an intensity of 1000 lx.

Isocitrate dehydrogenases were isolated from 10–20 g of wet cells. All the isolation steps were carried out at 4°C. Cells were separated from the medium by centrifugation at 18000 *g* for 20 min, washed with 50 mM K-phosphate buffer (pH 7.0), resuspended in the same buffer with 5 mM dithiothreitol and 10 μg/ml DNase to a cell density corresponding to 10–15 mg protein/ml, and disrupted by ultrasonic treatment at 22 kHz for 3 min. Unbroken cells and cell debris were removed by centrifugation at 35000 *g* for 60 min at 4°C. The supernatant was cleared by centrifugation at 160000 *g* at 4°C for 90 min and subjected to fractionation with ammonium sulfate. Proteins precipitated between 30 and 60% ammonium sulfate saturation were fractionated further using a (2 × 45 cm) column with Sephadex G-25 and purified by ion-exchange chromatography on a (1.5 × 14 cm) column with DEAE-32 cellulose. The column was washed with 20 mM K-phosphate buffer (pH 6.5) containing (mM) KCl, 100; dithiothreitol, 5; $MgCl_2$, 5; and $MnCl_2$, 0.5. Proteins were eluted with a linear gradient of KCl (0.1–0.4 M) in the same buffer. Active fractions eluted between 0.16 and 0.20 M KCl were pooled, desalted on a column with Sephadex G-10, and used for analysis. Purified isocitrate dehydrogenases stored in 50% glycerol at –18°C retained activity for at least 1.5–2 months.

Native vertical gel electrophoresis was carried out in 7.5% PAAG slabs in a Tris–glycine system (160 V, 45 mA, 4–6 h) [7]. The isoelectric points of isocitrate dehydrogenases were determined by their isoelectric focusing in ampholytes of pH 3.5–10.0. Isocitrate dehydrogenases in gels were detected by the reduction of nitro blue tetrazolium dye in the presence of NADH or NADPH and phenazine methosulfate [8]. The

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Table 1. Purification of isocitrate dehydrogenase from *Chl. limicola* cells

Step	Protein, mg	Activity, nmol/(min ml)		Activity, nmol/(min mg protein)		Purification factor	
		ICDH	2-OGC	ICDH	2-OGC	ICDH	2-OGC
Crude extract	9420	63585	6972	6.75	0.74	1.00	1.00
Supernatant (160000 g)	4900	33550	3953	6.85	0.80	1.02	1.09
30% $(NH_4)_2SO_4$	2475	20050	1277	8.10	0.52	1.20	0.70
60% $(NH_4)_2SO_4$	1170	15046	2271	12.90	1.94	1.90	2.62
Sephadex G-25	602	12488	2349	20.74	3.90	3.10	5.27
DEAE-32 cellulose	92.5	19783	3342	278.07	36.13	41.12	49.82

Note: ICDH and 2-OGC stand for isocitrate dehydrogenase and 2-oxoglutarate carboxylase, respectively.

molecular masses of isocitrate dehydrogenases were determined by gel-filtration chromatography on a (1.5 × 150 cm) column with Sephadex G-150.

The thermostability of the enzymes was determined by their heating at different temperatures for 5 min, rapid cooling to 4°C, and assaying of residual enzymatic activities.

The enzymes were assayed either by the reduction of $NAD(P)^+$ or the oxidation of NAD(P)H, with the results expressed in nmol/(min mg protein). The absorption of reduced pyridine nucleotides was measured at 340 nm using an Ultrospec II spectrophotometer (LKB, Sweden). The activity of isocitrate dehydrogenase in the forward direction (the oxidation of isocitrate) was measured in the reaction mixture containing (mM) Tris–HCl (pH 8.0), 100; isocitrate, 0.1; $NAD(P)^+$, 3; and $MgCl_2$, 10. The activity of isocitrate dehydrogenase in the backward direction (the reductive carboxylation of 2-oxoglutarate) was measured in the reaction mixture containing (mM) potassium phosphate buffer (pH 6.5), 100; 2-oxoglutarate, 0.6; NAD(P)H, 0.5; $MnCl_2$, 1; and $NaHCO_3$, 20.

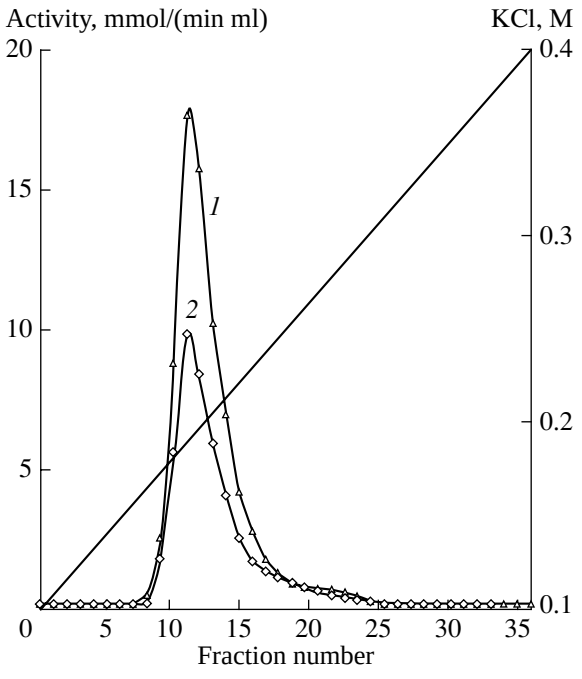

Fig. 1. Ion-exchange chromatography on DEAE-32 cellulose of the partially purified isocitrate dehydrogenase of *Chl. limicola*: *1*, isocitrate dehydrogenase activity; *2*, 2-oxoglutarate carboxylase activity.

Protein concentration was determined by the method of Bradford *et al.* [9], with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Purification and molecular properties of isocitrate dehydrogenase of *Chl. limicola*. As can be seen from Table 1 and Fig. 1, isocitrate dehydrogenase and 2-oxoglutarate carboxylase activities are not separated upon the purification of enzyme from *Chl. limicola* cells, indicating that both activities are due to the same protein. This inference is confirmed by native PAAG electrophoresis (data not shown). The electrophoretic mobility of proteins with the NADPH-dependent isocitrate dehydrogenase activity depended neither on the growth conditions (either autotrophic or heterotrophic) of the cells from which they were isolated nor on the purification step. Isoenzymes differing in electrophoretic mobility or capable of using NADH as the electron donor were not detected. Therefore, *Chl. limicola* cells have one isocitrate dehydrogenase, which catalyzes both dehydrogenase and carboxylase reactions. Analogous results were obtained upon the purification of isocitrate dehydrogenase from *Rps. palustris* cells (data not shown).

According to the data of gel filtration on Sephadex G-150, the molecular masses of isocitrate dehydrogenases from *Chl. limicola* and *Rps. palustris* cells are 64.4 ± 0.8 and 69.1 ± 0.8 kDa, respectively (Fig. 2). The isoelectric points of these enzymes are 5.8 ± 0.2 and 5.2 ± 0.2, respectively. After heating at 50°C for 5 min, both enzymes lost all activity (Fig. 3). The optimal pH values of the *Chl. limicola* isocitrate dehydrogenase in the forward and backward directions are 8.1 and 6.7, respectively. For the isocitrate dehydrogenase from

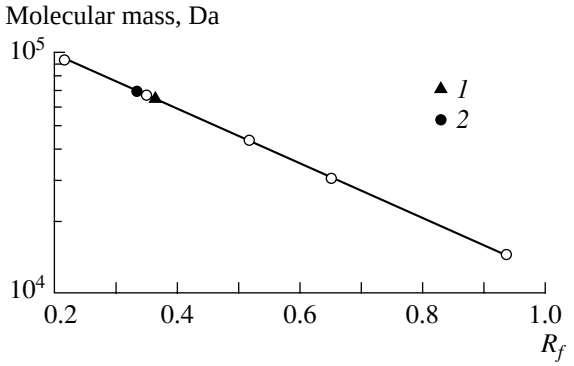

Fig. 2. The determination of the molecular mass of isocitrate dehydrogenases from (*1*) *Chl. limicola* and (*2*) *Rps. palustris* by gel filtration on a column with Sephadex G-150, using α-albumin (14400 kDa), carboanhydrase (30000 kDa), ovalbumin (43000 kDa), bovine serum albumin (67000 kDa), and phosphorylase B (94000 kDa) as the molecular markers.

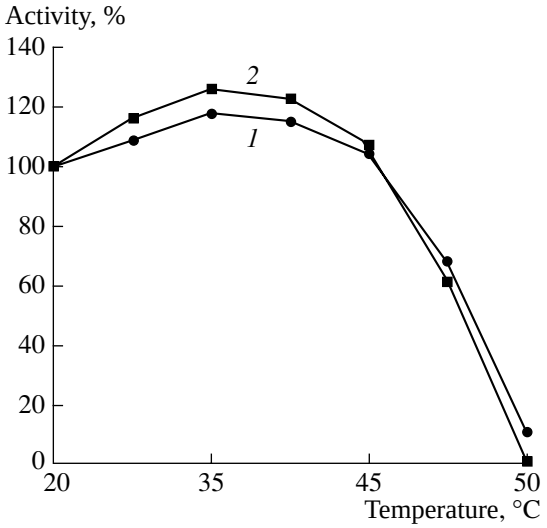

Fig. 3. The thermal inactivation of isocitrate dehydrogenases from (*1*) *Chl. limicola* and (*2*) *Rps. palustris.*

Rps. palustris, these values are 8.3 and 6.1, respectively (Fig. 4).

The kinetic properties of isocitrate dehydrogenases. The activities of isocitrate dehydrogenases in the crude and purified preparations from *Chl. limicola* and *Rps. palustris* cells measured in the forward and backward directions completely depended on the presence of $NADP^+$ and NADPH, respectively, in the reaction mixtures. In purified preparations, isocitrate dehydrogenase activity was proportional to the protein concentration up to a value of 50 μg protein/ml (*Rps. palustris*) or 15 μg protein/ml (*Chl. limicola*). For maximum activity, the reaction of the carboxylation of 2-oxoglutarate required high concentrations of Mg^{2+} or Mn^{2+} (Table 2). In this case, the enzymes from the two bacteria differed insignificantly in the requirements for these ions. The oxidation of isocitrate also required either Mg^{2+} or Mn^{2+} ions, the requirements being considerably lower (by about an order of magnitude) than in the case of the carboxylation reaction (Table 2). Furthermore, the enzyme from *Chl. limicola* required concentrations of Mg^{2+} and Mn^{2+} ions an order of magnitude higher than did the enzyme from *Rps. palustris.*

The kinetic parameters (K_m for 2-oxoglutarate, NADPH, and HCO_3^-) of isocitrate dehydrogenases from *Chl. limicola* and *Rps. palustris* measured in the backward reaction (the carboxylation of 2-oxoglutarate) were close (Table 3). In contrast, the kinetic parameters measured in the forward reaction (the oxidation of isocitrate) were 3 to 4 times lower for the enzyme from *Chl. limicola* than for the enzyme from *Rps. palustris.* It should be noted that both isocitrate dehydrogenases exhibited a very low affinity for HCO_3^- ($K_m \approx 10$–15 mM).

The influence of various effectors on isocitrate dehydrogenases. The energy-rich compounds ATP and ADP, taken at a concentration of 2.5 mM, slightly inhibited (by 25–30%) the activities of the isocitrate dehydrogenases in the forward and backward reactions. This inhibiting effect, however, can be explained by the ability of ATP and ADP to bind to the necessary ions Mg^{2+} and Mn^{2+}, which are present in the reaction mixtures at concentrations comparable with those of the adenine nucleotides. As for AMP, it did not exert any effect on the activity of isocitrate dehydrogenases as measured in both reactions.

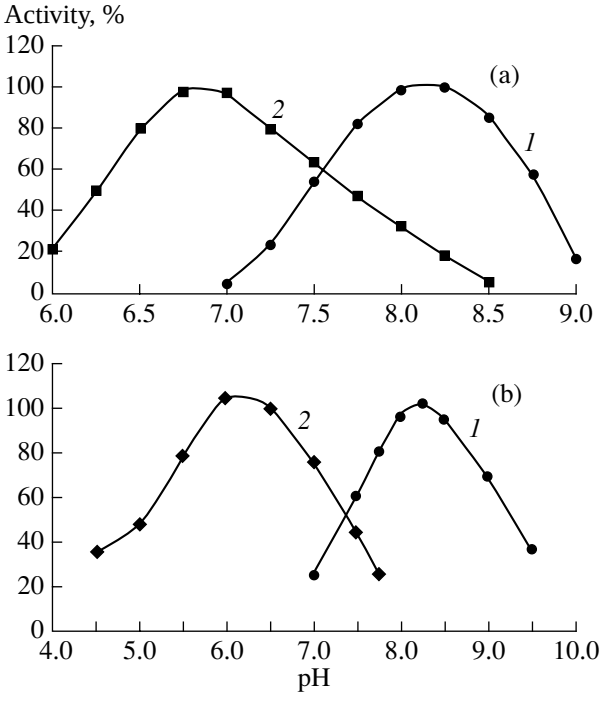

Fig. 4. The effect of pH on the (*1*) isocitrate dehydrogenase and (*2*) 2-oxoglutarate carboxylase reactions catalyzed by the isocitrate dehydrogenases isolated from (a) *Chl. limicola* and (b) *Rps. palustris* cells.

Table 2. The concentrations of Mg^{2+} and Mn^{2+} ions (in mM) necessary for the maximum activities of the isocitrate dehydrogenase and 2-oxoglutarate carboxylase reactions catalyzed by the isocitrate dehydrogenases isolated from *Chl. limicola* and *Rps. palustris* cells

	Chl. limicola	*Rps. palustris*
Isocitrate dehydrogenase		
Mg^{2+}	40	2
Mn^{2+}	10	1
2-Oxoglutarate carboxylase		
Mg^{2+}	250	200
Mn^{2+}	50	20

Table 3. The Michaelis constants for substrates of the isocitrate dehydrogenase and 2-oxoglutarate carboxylase reactions catalyzed by the isocitrate dehydrogenases isolated from *Chl. limicola* and *Rps. palustris* cells (μM)

Substrate	*Chl. limicola*	*Rps. palustris*
Isocitrate dehydrogenase		
Isocitrate	1.4	9.0
$NADP^+$	6.5	16.5
2-Oxoglutarate carboxylase		
2-Oxoglutarate	304.0	397.7
NADPH	10.3	33.8
$NaHCO_3$	11680.0	13820.0

Table 4. The effect of reaction products (expressed as a percent of the control without inhibitor) on the isocitrate dehydrogenase and 2-oxoglutarate carboxylase reactions catalyzed by the isocitrate dehydrogenases isolated from *Chl. limicola* and *Rps. palustris* cells

Substrate, mM	*Chl. limicola*	*Rps. palustris*
Isocitrate dehydrogenase		
$NaHCO_3$		
10.0	72	91
2-Oxoglutarate		
1.5	50	50
15.0	1	5
NADPH		
0.5	58	34
2-Oxoglutarate carboxylase		
Isocitrate		
0.1	50	20
1.2	3	1
$NADP^+$		
4.0	63	65

Table 5. The effect of glyoxylate and oxaloacetate (expressed as a percent of the control) on the activities of isocitrate dehydrogenases isolated from *Chl. limicola* and *Rps. palustris*

Substrate (2.5 mM)	*Chl. limicola*	*Rps. palustris*
Glyoxylate	99.3	97.3
Oxaloacetate	81.4	84.5

In contrast, the reaction products (2-oxoglutarate for the forward reaction and isocitrate for the backward reaction) were found to be potent inhibitors of the isocitrate dehydrogenase and 2-oxoglutarate carboxylase reactions, respectively (Table 4). The carboxylation reactions of the isocitrate dehydrogenases from *Chl. limicola* and *Rps. palustris* were inhibited by 50% at isocitrate concentrations of 0.1 and 0.02 mM, respectively, and by 100% at an isocitrate concentration of 1.2 mM. At the same time, the isocitrate dehydrogenases from *Chl. limicola* and *Rps. palustris* did not differ in sensitivity to 2-oxoglutarate: both enzymes were inhibited by 50 and 100% at 2-oxoglutarate concentrations equal to 1.5 and 15 mM, respectively. According to data available in the literature [10], the inhibitory action of 2-oxoglutarate and isocitrate on the activity of isocitrate dehydrogenase is due to kinetic peculiarities in the formation and breakdown of the enzyme–substrate complexes during the forward and backward reactions catalyzed by this enzyme. The effect of bicarbonate ion, $NADP^+$, and NADPH on the forward and backward reactions of isocitrate dehydrogenase is less pronounced.

The intermediates of the TCA cycle (other than isocitrate and 2-oxoglutarate), phosphoenolpyruvate, 2- and 3-phosphoglycerate, pyruvate, and the amino acids aspartate, alanine, and asparagine synthesized from the TCA cycle intermediates, as well as glycolate, acetate, and propionate, did not affect the activity of the isocitrate dehydrogenases in either direction.

Glyoxylate and oxaloacetate added individually at a concentration of 2.5 mM only slightly inhibited the isocitrate dehydrogenases of *Chl. limicola* and *Rps. palustris* (Table 5). At the same time, these compounds added together turned out to be potent inhibitors. For instance, the 50% inhibition of the isocitrate dehydrogenase reaction was observed in the presence of 0.05 μM glyoxylate and 2.5 mM oxaloacetate or 2.5 mM glyoxylate and 0.05 μM oxaloacetate (Fig. 5). The complete inhibition of this reaction was observed in the presence of 1 μM glyoxylate or oxaloacetate against the background of 2.5 mM oxaloacetate or glyoxylate. As is known from experiments with the $NADP^+$-dependent isocitrate dehydrogenase of *Escherichia coli*, the strong inhibitory effect of glyoxylate and oxaloacetate is due to the competitive inhibition of the isocitrate dehydrogenase reaction by oxalomalate, which is formed from glyoxylate and oxaloacetate through their condensation [10–12].

Thus, the carboxylation of 2-oxoglutarate in the reductive TCA cycle of *Chl. limicola* is catalyzed by an enzyme that does not considerably differ (in physicochemical, biochemical, and kinetic properties) from the isocitrate dehydrogenase of *Rps. palustris*, whose key function is the oxidative decarboxylation of isocitrate. The ability of $NADP^+$-dependent isocitrate dehydrogenase to catalyze the forward reaction in the TCA cycle of *Rps. palustris* and the backward reaction in the

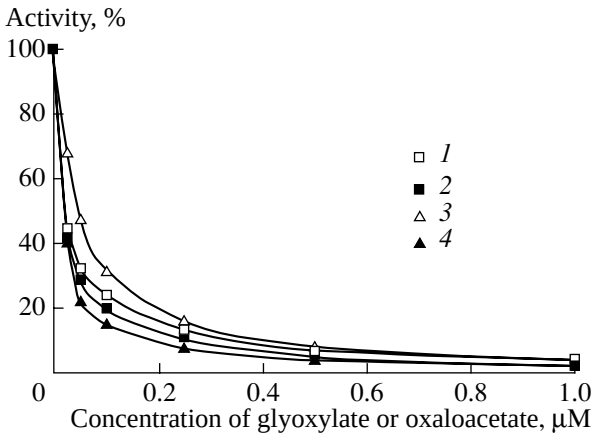

Fig. 5. The effect of glyoxylate and oxaloacetate on the activity of isocitrate dehydrogenases from (*1, 2*) *Chl. limicola* and (*3, 4*) *Rps. palustris*: (*1, 3*) 2.5 mM oxaloacetate + glyoxylate; (*2, 4*) 2.5 mM glyoxylate + oxaloacetate.

reductive TCA cycle of *Chl. limicola* autotrophically fixing CO_2 is determined by the peculiar thermodynamic characteristic of the isocitrate dehydrogenase reaction ($\Delta G'_0$ = –4.6 kJ/mol), which allows it to proceed easily in both directions [13]. The failure to reveal specific effectors of the carboxylase reaction in *Chl. limicola* in our study may indicate that the reductive carboxylation of 2-oxoglutarate in the obligate photoautotroph *Chl. limicola* is catalyzed by an $NADP^+$-dependent isocitrate dehydrogenase that does not considerably differ from the $NADP^+$-dependent isocitrate dehydrogenases of the photoheterotroph *Rps. palustris* and the heterotroph *E. coli* [14].

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