Isolation and Partial Characterization of the Membrane-Bound NADH Dehydrogenase from the Phototrophic Bacterium *Rhodopseudomonas capsulata*

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NADH Dehydrogenase, Purification, Localization, Inhibitors, *Rhodopseudomonas capsulata*

Chemotrophically grown cells of *Rhodopseudomonas capsulata* contain at least three different pyridine nucleotide dehydrogenases,

i) a soluble, found in the supernatant (144000 × *g*) of cell free extracts, NADH-dependent,
ii) a membrane-bound, NADH-dependent, and
iii) a soluble, found in the supernatant NADPH dependent.

The membrane-bound NADH dehydrogenase (E.C. 1.6.99.3) has been solubilized by sodium deoxycholate treatment of membranes and purified 75 fold by column chromatography on Sephadex G-150 and DEAE cellulose in the presence of sodium cholate. The native enzyme has an apparent molecular mass (M_r) of 97000, containing polypeptides of M_r of about 15000. The pH optimum was at 7.5. The enzyme was specific for NADH. The Michaelis constant for NADH and DCIP were 4.0 and 63 μM, respectively.

The enzyme was inactivated by FMN, riboflavin and NADH. In contrast, the soluble NADH-dehydrogenase (i) was activated by FMN.

Introduction

Some species of the Rhodospirillaceae are facultative phototrophic bacteria, *i.e.* they produce ATP either by cyclic photophosphorylation under anaerobic conditions in the light (phototrophic) or by oxidative phosphorylation under aerobic conditions in the dark (chemotrophic). The organisms therefore have both photosynthetic and respiratory membrane bound electron transport systems [1]. In our laboratory membrane differentiation has been studied in cells of *Rhodopseudomonas capsulata* and other Rhodospirillaceae in order to understand regulation of membrane biosynthesis and differentiation on the molecular basis [2]. Light intensity and oxygen partial pressure are the major external factors which influence the level of respiratory and photosynthetic electron transport chain components in the membrane system [2].

In this article NADH dehydrogenases of *Rps. capsulata* are partially characterized. NADH dehydrogenase (1.6.99.3) is known as an iron-sulfur flavoprotein in the respiratory electron transport system. Horio *et al.* purified two NADH dehydrogenases from anaerobically light-grown cells of *Rhodospirillum rubrum* that react with different electron acceptors and are activated by FMN [3]. They suggested two electron pathways involving NADH in the light-grown cells [4]. Boll also showed to different NADH dehydrogenases in light-grown cells, one the soluble enzyme, is activated by FMN [5, 6] and the other, the membrane-bound one is not activated by FMN [7].

From the NADH dehydrogenases of *Rhodopseudomonas palustris* and *Rps. capsulata* only the effect of growth conditions on the enzyme activity in membrane fractions is known [8, 9]. In this paper we describe solubilization, isolation and some properties of the membrane-bound NADH dehydrogenase from chemotrophically grown cells of *Rps. capsulata.*

Materials and Methods

Culture conditions

Rhodopseudomonas capsulata, strain 37 b_4, was cultivated aerobically in the dark in a 12 l Microferm

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Abbreviations: DCIP, 2,6-dichlorophenol indophenol; DOC, deoxycholate; DTE, Dithioerythritol; SDS, sodium dodecyl sulfate; MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; *Rps., Rhodopseudomonas; R., Rhodospirillum*

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New Brunswick fermentor under stirring (500 rpm) and gassing with air. The Medium RÄH [10] with 0.25% (w/v) sodium malate as carbon source and 0.05% yeast extract was used. The cells were harvested and washed twice with 50 mM Tris-HCl buffer (pH 7.5) and stored at −80 °C.

Enzyme assay

Assays for NADH, NADPH and succinate dehydrogenases were usually performed at 25 °C by following the decrease of absorbance at 578 nm in the Eppendorf photometer using DCIP as an electron acceptor. The reaction mixture for NADH or NADPH dehydrogenase contained 100 mM potassium phosphate buffer (pH 7.5), 0.6 mM N-ethylmaleimide, 2 mM KCN, 70 μM DCIP, enzyme and 0.15 mM NADH or NADPH in a total volume of 1.0 ml. For soluble NADH dehydrogenase assay 10 μM of FMN was added to the reaction mixture. Succinate dehydrogenase activity was measured in a system containing 90 mM potassium phosphate buffer (pH 7.5), 0.6 mM N-ethylmaleimide, 2 mM KCN, 10 mM sodium succinate, enzyme, 70 μM DCIP and 0.4 mM PMS in a total volume of 1.0 ml. The reagents were added in these order. One unit of enzyme activities was defined as the amount of enzyme catalyzing the reduction of 2 μmol of DCIP (20.6 mM^{-1} cm^{-1} at 578 nm) [5] per min at 25 °C. N-ethylmaleimide was added to the reaction mixture to remove excess DTE in the enzyme solution, which reduce DCIP nonenzymatically. The other nonenzymatic reduction of DCIP was determined and substracted. NADH dehydrogenase activities with menadione, potassium ferricyanide and cytochrome *c* as electron acceptors were monitored at 366 nm (3,3 mM^{-1} cm^{-1}), 405 nm (1.0 mM^{-1} cm^{-1}) and 555 nm (21 mM^{-1} cm^{-1}), respectively [5].

Analytical methods

Protein was determined using the method of Lowry *et al.* [11] with bovine serum albumin as a standard or the spectrophotometric method of Kalb and Bernlohr [12].

Slab gel electrophoresis in 7.5% polyacrylamide gel containing 0.1% Triton X-405 and 15% polyacrylamide gel containing 0.1% SDS was performed by the modified method of Laemmli [13]. Protein bands were stained with 0.25% Coomassie blue R-250 and NADH dehydrogenase-activity staining was carried out according to the DCIP-MTT linked method of Kaplan and Beutler [14].

Enzyme solubilization

The membrane and soluble fractions were prepared as follows. The frozen cells were thawed and resuspended with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM DTE and then were disrupted by two passages through the French pressure cell at 70000 kPa. Whole cells and debris were removed by centrifugation at 25500 × *g* for 20 min. The resulting supernatant, crude extract, was further centrifuged at 144000 × *g* for 90 min. The clear supernatant was used as the soluble fraction. The sediment was washed once with the same buffer as described above and used as the membrane fraction.

The membrane fraction was suspended in 50 mM Tris-HCl buffer (pH 7.5) supplemented with 10 μM DCIP, 0.1 mM sodium ascorbate and 0.2 mM DTE, and then the concentrated solution of detergent or chaotropic agent was added dropwise to the suspension under stirring. After incubation for 40 min at room temperature, the solution was centrifuged at 144000 × *g* for 90 min and the supernatant was collected as the solubilized fraction.

Purification

All operations were carried out at 0−6 °C. Unless otherwise stated, 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM DTE, 0.1 M NaCl and 0.5% sodium cholate was basically used. The following is a typical purification procedure of the enzyme.

Sephadex G-150 column chromatography

The solubilized enzyme solution (8 ml) was applied on a column (2.2 × 91 cm), preequilibrated with the basic buffer system. Main active fraction were collected and immediately concentrated to a volume of less than 10 ml in an Amicon ultrafiltration cell with a UM 10 Diaflo membrane.

DEAE-cellulose column chromatography

The concentrated enzyme was further applied to a DEAE-cellulose column (Whatman DE 52, 2 × 10 cm), previously equilibrated with the basic buffer system.

Results and Discussion

Distribution of NADH, NADPH and succinate dehydrogenases

The crude extract of the aerobic-dark grown cells of *Rps. capsulata* contained both NADH and NADPH dehydrogenase activity. After centrifugation of the crude extract of 144000 × *g* for 90 min, NADH dehydrogenase occured in both the soluble and membrane fractions, but succinate dehydrogenase was found mainly in the membrane fraction and NADPH dehydrogenase in the soluble one, respectively (Table I). NADH dehydrogenase activity in the membrane fraction and NADPH dehydrogenase activity in the soluble fraction were not affected by the addition of FMN (10 μM) to the reaction mixture, but the activity of NADH dehydrogenase in the soluble fraction decreased to about 40% if the reaction mixture was depleted of FMN. These data suggest the presence of at least three different pyridine nucleotide dehydrogenases in the chemotrophically grown cells of *Rps. capsulata, i.e.* membrane-bound and soluble NADH dehydrogenases and soluble NADPH dehydrogenase. Phototrophically grown cells of *R. rubrum* contain similar membrane-bound and soluble NADH dehydrogenases [5, 6]. The soluble NADPH dehydrogenase seems to be similar to $NADP^+$ reductases from several photosynthetic bacteria such as *Thiocapsa roseopersicina* [12], *Rps. palustris* [16], *Rps. sphaeroides* [17] and *R. rubrum* [18, 19].

Solubilization of membrane-bound NADH dehydrogenase

Deoxycholate (DOC), sodium cholate, Triton X-100 and 405, Briji 35 and 58, $NaClO_4$, NaCl, NaI and lauryl dimethylamine oxide were tested for their ability to solubilize the membrane bound NADH dehydrogenase under preservation of enzyme activity. The enzyme was effectively solubilized by Triton X-100 (1–2 mg protein), sodium cholate (5–10 mg/mg protein) and DOC (2–3 mg/mg protein). The activity was maintained in the 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM DTE supplemented with 0.5% sodium cholate, 0.1% DOC or Triton X-405. A maximum of solubilization and a recovery of about 140% of the original membrane-bound enzyme activity was obtained with a DOC concentration of 2 mg/mg protein (Fig. 1). Routinously the membranes were treated with DOC (2 mg/mg protein) for 40 min at room temperature and 0.5% sodium cholate was added to the buffer system as the best stabilizing detergent for the enzyme.

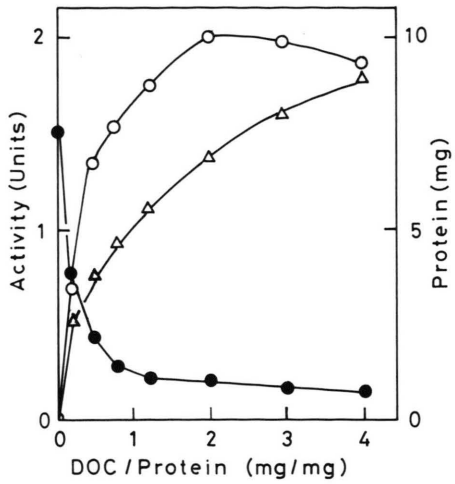

Fig. 1. Deoxycholate-solubilization of membrane-bound NADH dehydrogenase as a function of DOC/membrane protein ratio. Membrane fraction (10 mg, 1.5 units) was incubated with various concentration of DOC at room temperature for 40 min, and then centrifuged at 144000 × *g* for 90 min at 4 °C. Enzyme activity and protein were determined in the supernatant fluid as the solubilized fraction and in the sediment. (○–○), NADH dehydrogenase activity in the supernatant fluid; (●–●), the activity in the sediment; and (△–△), protein in the supernatant fluid.

Table I. Distribution of NADH dehydrogenase. Each fractions were separated as described in Materials and Methods. Wet weight of cells used was about 22 g.

Fractions	Volume	Total protein	Succinate dehydrogenase		NADH dehydrogenase		NADPH dehydrogenase	
			Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
	[ml]	[mg]	[units]	[units/mg]	[units]	[units/mg]	[units]	[units/mg]
Crude extract	46	554	39.6	0.071	58.6	0.106	72.6	0.131
Membrane fraction	8.9	157	25.9	0.165	23.6	0.150	0.027	0.002
Soluble fraction	68	375	4.95	0.013	31.0	0.083	79.3	0.211

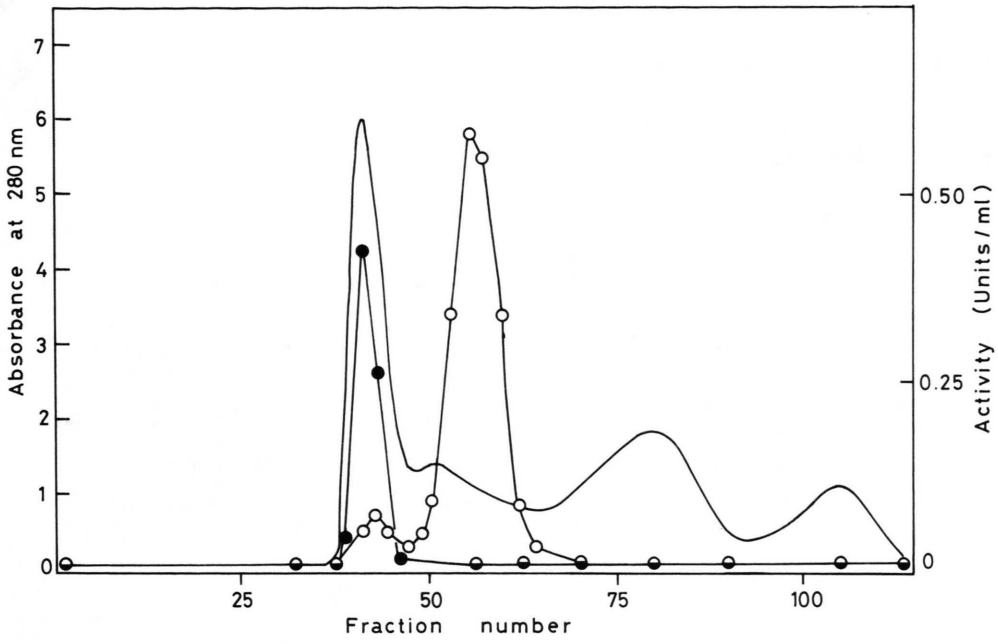

Fig. 2. Sephadex G-150 chromatography of NADH dehydrogenase. The solubilized enzyme solution was applied to a Sephadex G-150 column (2.2 × 91 cm) preequilibrated with the basic buffer system. Fractions of 3.3 ml were collected and the flow rate was about 12 ml/h. (○–○), NADH dehydrogenase activity; (●–●), succinate dehydrogenase activity; and (–), absorbance at 280 nm which was measured by use of a LKB UVICORD II.

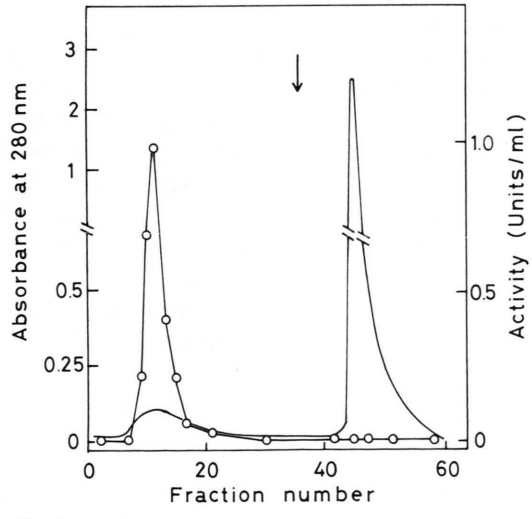

Fig. 3. DEAE-cellulose chromatography of NADH dehydrogenase. The enzyme solution obtained from Sephadex G-150 chromatography was concentrated, and then applied to a DEAE-cellulose column (2 × 10 cm) preequilibrated with the basic buffer system. Fractions of 4.0 ml were collected and the flow rate was about 30 ml/h. The arrow points where the concentration of NaCl in the elution buffer system was changed from 0.1 to 0.5 M. (○–○), NADH dehydrogenase activity and (——), absorbance at 280 nm.

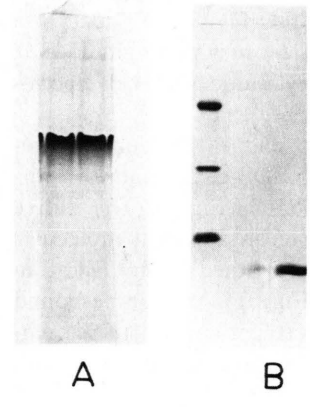

Fig. 4. Polyacrylamide gel electrophoresis of purified NADH dehydrogenase. (A) Electrophoresis was carried out at 4 °C. Acrylamide concentration: 7.5% for separating and 3% for stacking gels, using 0.1% Triton X-405 in the gel and the buffer systems. Gel is shown after staining with Coomassie blue. The staining for NADH dehydrogenase activity is at the same position. (B), 15% separating and 3% stacking polyacrylamide gel electrophoresis in the presence of 0.1% SDS. The enzyme preparation was previously treated with 1% SDS and 1% 2-mercaptoethanol at 65 °C for 45 min and then submitted to SDS polyacrylamide electrophoresis; first lane marker proteins, from top to bottom: ovalbumin, heart lactate dehydrogenase, β-lactoglobulin.

Purification

The solubilized NADH-dehydrogenase was eluted from the Sephadex column as one major peak (Fig. 2). The collected and concentrated fraction was applied to DEAE-cellulose column and eluted with the void volume (Fig. 3). No further activity was eluted with buffer containing concentrations of NaCl higher than 0.1 M. The enzyme was absorbed on the column and eluted with buffer containing 0.1 M NaCl when the equilibration buffer did not contain NaCl, but the recovery of the enzyme activity was low.

During these steps the enzyme was enriched about 75-fold with a yield of 75% (Table II). In order to obtain a good yield dilution of the enzyme has to be avoided and the enzyme purified from the membrane within two days.

Properties of the membrane-bound NADH dehydrogenase

Previous to polyacrylamide electrophoresis the detergent in the buffer system of the purified enzyme solution was exchanged from 0.5% sodium cholate to 0.1% Triton X-405 in an ultrafiltration cell. After staining with Coomassie blue one major and a very thin minor band were observed (Fig. 4 A). The major band contained NADH-DCIP-MTT reductase activity. The highly purified enzyme migrated as a single band in a polyacrylamide slab gel electrophoresis (Fig. 4 B).

The relative molecular mass of the subunit, migrating as a single band in SDS-electrophoresis, was estimated to be about 15500 (Fig. 5). The native enzyme was found to have an apparent molecular mass of 97000 ± 5000, determined by gel filtration on Sephadex G-150 (Fig. 6). Membrane-bound NADH dehydrogenase therefore appears to be composed of six subunits of the same molecular weight.

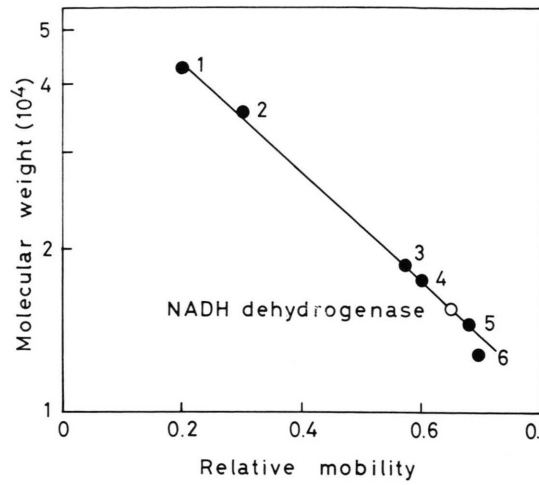

Fig. 5. Estimation of the molecular mass of subunits of NADH dehydrogenase by SDS gel electrophoresis. The enzyme and marker proteins were treated as described in the legend of Fig. 4 B and submitted to polyacrylamide electrophoresis with a 15% separating and 3% stacking slab gel (0.1 × 10 × 11.5 cm). The marker proteins used were: 1, ovalbumin; 2, heart lactate dehydrogenase; 3, β-lactoglobulin; 4, myoglobin; 5, lysozyme; and 6, cytochrome c.

The molecular mass of this enzyme (97000 ± 5000) is somewhat larger than that of the membrane-bound enzyme (75000) from *R. rubrum* [7].

The purified enzyme exhibited an optimum of activity at pH 7.5 (Fig. 7). The enzyme was specific for NADH as an electron donor, and inert for NADPH (below 0.1% of activity for NADH). In this respect the enzyme from the chemotrophically grown *Rps. capsulata* is different from that of phototrophically grown *R. rubrum*, which can utilize NADPH as an electron donor [7].

In addition to DCIP, either ferricyanide, menadione or cytochrome c can be used as an electron acceptor for oxidation of NADH.

Table II. Purification scheme of membrane-bound NADH dehydrogenase from *Rps. capsulata.*

Steps[a]	Volume [ml]	Total protein [mg]	Total activity [units]	Specific activity [units/mg]	Recovery [%]	Purification fold
Membrane fraction	7.5	122	12.4	0.102	100	1
DOC-solubilized fraction	9.8	73	16.8	0.229	135	2.3
Sephadex G-150	31	6.2	10.8	1.73	87	17
DEAE-cellulose	29	1.2	9.24	7.70	75	75

[a] About 8 g of wet weight cells were used.

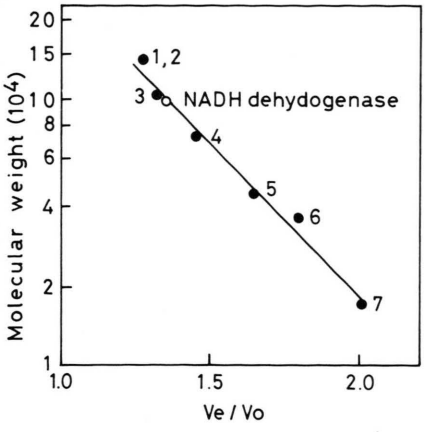

Fig. 6. Estimation of the molecular weight of NADH dehydrogenase by Sephadex G-150 chromatography. A column (1.7 × 64 cm) of Sephadex G-150 was equilibrated with the basic buffer system. The standard proteins (1–3 mg) used were: 1, heart lactate dehydrogenase; 2, yeast alcohol dehydrogenase; 3, yeast hexokinase; 4, bovine serum albumin; 5, ovalbumin; 6, β-lactoglobulin; and 7, myoglobin.

Michaelis constants for NADH and DCIP of the enzyme were estimated to be 4.0 and 63 μM, respectively, by the method of Velick and Vavra [20].

The purified soluble NADH dehydrogenase of *Rps. capsulata* (T. Ohshima and G. Drews, unpublished) and *R. rubrum* [5, 6] is known to be activated by flavin compounds, especially FMN, whereas the membrane-bound enzyme of *Rps. capsulata* was not influenced by addition of FMN to the assay mixture.

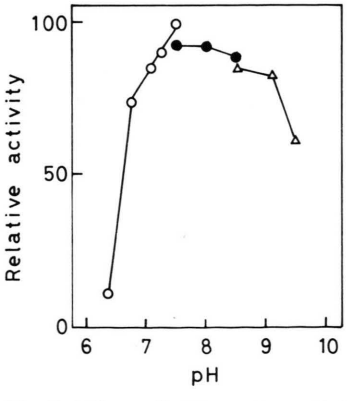

Fig. 7. Effect of pH on the activity of NADH dehydrogenase. Enzyme activity was assayed as described in Materials and Methods, and 0.1 M KH_2PO_4–K_2HPO_4 (○–○), Tris-HCl (●–●) and glycylglycine-NaOH (△–△) buffers were used.

Table III. Effect of some compounds on NADH dehydrogenase. The activity was measured as described in Materials and Methods after preincubation of the enzyme with the compounds at 25 °C for 10 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, 0.2 mM DTE and 0.5% sodium cholate. The concentrations given in the table were selected to demonstrate the effect of inhibition or non-inhibition.

Compounds	Concentration [mM]	Relative activity
None	–	100
ATP	1	96
ADP	1	103
AMP	1	106
FAD	0.025	80
FMN	0.025	20
Riboflavin	0.025	16
NADH	0.025	4.9
NADPH	0.025	92
NAD^+	0.050	87
$NADP^+$	0.050	100
EDTA	1	100
Sodium azide	2	99
Sodium arsenate	5	132
Sodium arsenate	12.5	134
2,2′-Dipyridyl	1	100
2,2′-Dipyridyl	2.5	87
O-Phenanthroline	1	72

However, the solubilized membrane-bound enzyme was strongly inactivated by FMN (incubation at 37 °C for 10 min) and riboflavin. In addition, the substrate, NADH, also inactivated the enzyme, but FAD, NAD, NADPH and $NADP^+$ did not inactivate (Table III). In contrast sodium arsenate increased the activity of the enzyme. A similar inactivation by NADH was observed with the membrane-bound enzyme from *R. rubrum* [7]. The mechanisms of such inactivation and activation is still unknown.

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