Purification and Partial Characterization of the Soluble NADH Dehydrogenase from the Phototrophic Bacterium *Rhodospseudomonas capsulata*

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Soluble NADH dehydrogenase was purified to homogeneity from chemotrophically grown cells of *Rhodospseudomonas capsulata* by ammonium sulfate fractionation, AH-Sepharose 4B chromatography and FMN-Sepharose 6B affinity chromatography. The enzyme contains a single polypeptide chain of an apparent Mr of 37,000, suggesting that the subunit structure is different from that of the membrane-bound enzyme. The purified soluble NADH dehydrogenase requires flavin compounds, e.g., FMN, FAD and riboflavin, for activity. Addition of FMN and FAD, but not riboflavin, to the enzyme solution stabilized the enzyme. The pH optimum for activity was at 7.5. The enzyme was specific for NADH as an electron donor while NADPH was inert. Menadione, ferricyanide, cytochrome c and DCIP served as an electron acceptor. The Michaelis constants for NADH, DCIP, FMN, and cytochrome c were 45, 2.9, 7.9 and 15 |μM|, respectively. Many properties of soluble NADH dehydrogenase were substantially different from those of the membrane-bound enzyme, suggesting different functions.

Introduction

Most species of the facultative phototrophic bacteria of *Rhodospirillaceae* can produce energy either by cyclic photophosphorylation under aerobic condition (light (phototrophic)) or by oxidative phosphorylation under aerobic condition in the dark (chemotrophic). These bacteria have both photosynthetic and respiratory electron transport systems, which are influenced by light intensity and oxygen partial pressure as the major external factors [1, 2]. We have been interested in understanding the structural basis and the regulation of function of both electron transport systems. Membrane-bound NADH dehydrogenase (EC 1.6.99.3) is one major entrance of electrons and protons to the respiratory chain. In a previous paper, we showed the presence of at least three different pyridine nucleotide dehydrogenases in chemotrophically grown cells of *Rhodospseudomonas capsulata*, i.e., membrane-bound and soluble NADH dehydrogenases and soluble NADPH dehydrogenase, and described the solubilization, isolation and some properties of the membrane-bound enzyme [3].

In the present paper, isolation and partial characterization of soluble NADH dehydrogenase from chemotrophically grown cells of *Rps. capsulata*, are described with emphasis on differences of the properties from those of the membrane-bound NADH dehydrogenase.

Materials and Methods

Materials

NADH, NADPH and cytochrome c (horse heart) were purchased from Sigma Chemical, USA; Sephadex G-150, AH-Sepharose 4B and Sepharose 6B from Pharmacia, Sweden; Cellulofine GC-200 m from Seikagaku Kogyo, Tokyo; and DCIP and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide from E. Merck, Darmstadt, West Germany. Other materials were of the highest purity commercially available.

Culture condition

*Rps. capsulata*, strain 37b4 (German Collection of Microorganisms, Göttingen, strain number DSM938),
was grown aerobically in the dark in the yeast extract-malate medium RÅH as described previously [3]. The cells were harvested, washed and stored as described previously [3].

**Enzyme and protein assays**

Determinations of activities of soluble NADH and NADPH dehydrogenases, and protein assays were carried out as described previously [3].

**Preparation of FMN-Sepharose 6B**

The procedure of Waters et al. [4] and Michaliszyn et al. [5] for coupling 6-aminohexanoic acid and FMN to Sepharose 6B was slightly modified. Sepharose 6B (30 ml) washed with water was suspended in 0.2 M Na$_2$CO$_3$ (30 ml) and activated with 2 g of finely ground CNBr at 20°C for 10 min. The suspension was filtered and the activated gel was washed with cold 0.1 M NaHCO$_3$ (300 ml). The gel resuspended in 30 ml of 0.2 M NaHCO$_3$-NaOH buffer (pH 8.5) containing 2 g of 6-aminohexanoic acid was stirred slowly for about 3 h at room temperature. The gel was washed sequentially with 300 ml each of 0.1 M NaHCO$_3$, 0.01 M HCl, 0.5 M NaCl and H$_2$O, and then suspended in 30 ml of 0.2 M glycine (pH 8.0). After stirring gently for 3 h at room temperature, the gel was again washed sequentially as described above. The resulting aminohexanoyl-Sepharase 6B was suspended in 30 ml of water and reacted in the dark with 2 g of FMN by addition of 1-ethyl-3-(3-dimethyl amino-propyl)carbodiimide hydrochloride (0.3 g) according to the procedure used by Robinson et al. [6]. The reaction mixture was adjusted and maintained at pH 6 with 0.1 N HCl, and stirred gently at room temperature for 5 h. The mixture was successively washed with 0.05 M NaHCO$_3$ and water, and then the gel was stored in the dark at 5 °C until used.

**Electrophoresis**

Gels were prepared in absence (7.5% polyacrylamide) and presence of 0.1% SDS (12% polyacrylamide) by the method of Laemmli [7], (slightly modified). The gel polymerized in the absence of SDS was subjected to pre-electrophoresis with 50 mM potassium phosphate buffer containing 10 µM FMN for about 3 h, and then electrophoresis was carried out with the same buffer system. Slab gel (0.1 x 10 x 11.5 cm) electrophoresis in the presence of SDS was performed according to Laemmli [7]. The marker proteins used were bovine serum albumin, catalase, ovalbumin, heart lactate dehydrogenase, myoglobin and lysozyme. Protein and NADH dehydrogenase-activity bands were visualized as described previously [3].

**Preparation of soluble fraction**

The soluble enzyme fraction from frozen cells of Rps. capsulata was prepared as reported previously [3].

**Purification**

All operations were carried out at 0-6 °C. Unless otherwise stated, 50 mM Tris-HCl (pH 7.5) supplemented with 0.2 mM DTE was basically used as a buffer. The soluble NADH dehydrogenase was purified by the following procedure:

1. **(NH$_4$)$_2$SO$_4$ fractionation**

Solid (NH$_4$)$_2$SO$_4$ (25% saturation) was added to the soluble fraction (66 ml, supernatant at 144000 x g, for 90 min) obtained from 45.6 g wet weight cells. The precipitate was removed by centrifugation (24,400 x g, 20 min) and discarded. The pH was kept at about 7.5 with 10% NH$_4$OH. The supernatant solution was brought to 50% saturation with (NH$_4$)$_2$SO$_4$, and the precipitate obtained by centrifugation was dissolved in a minimum volume of the basic buffer.

2. **Sephadex G-150 column chromatography**

The enzyme solution (14 ml) was applied on a Sephadex G-150 column (2.2 x 91 cm), previously equilibrated with the basic buffer. Main active fractions were collected.

3. **AH-Sepharose 6B column chromatography**

The enzyme solution (19 ml) was applied to a AH-Sepharose 6B column (1.8 x 11 cm) previously equilibrated with the basic buffer. The enzyme was eluted by a NaCl gradient in the basic buffer. The active fractions collected were dialyzed against the basic buffer and concentrated by ultrafiltration.

4. **FMN-Sepharose 6B column chromatography**

The concentrated enzyme solution (19 ml) was further applied to a FMN-Sepharose column (2 x 11 cm) previously equilibrated with the basic buffer.
buffer. The column was washed with the same buffer, the enzyme was eluted by a linear-salt gradient.

**Estimation of molecular weight**

The molecular weight of soluble NADH dehydrogenase was estimated by Cellulofine GC-200-m gel filtration. A Cellulofine GC-200-m column (1.5 cm x 68 cm) was equilibrated with the basic buffer containing 10 μM FMN. The standard proteins (1 - 3 mg) used were bovine serum albumin, ovalbumin, chymotrypsinogen A and myoglobin.

**Results and Discussion**

**Purification**

The soluble fraction of cell extracts of *Rps. capsulata* contains both NADH and NADPH dehydrogenases [3]. Both dehydrogenases were precipitated with (NH₄)₂SO₄ (25 – 50% saturation). The soluble NADH dehydrogenase was eluted from the Sephadex G-150 column as one major peak at a position similar to that of low molecular weight NADPH dehydrogenase (Fig. 1A). Two peaks of NADPH dehydrogenase activity were observed, suggesting the presence of two NADPH dehydrogenases. The activity of soluble NADH dehydrogenase, but not of NADPH dehydrogenase was lost when FMN was removed from the reaction mixture. The soluble NADH dehydrogenase was eluted with the basic buffer containing 0.2 M NaCl as a broad peak and separated from NADPH dehydrogenase by the AH-Sepharose 6B column (Fig. 1B). The NADH dehydrogenase was concentrated by ultrafiltration and further purified by FMN-Sepharose 6B affinity chromatography. When the column was washed with the basic buffer, the enzyme was retained, but most of contaminating protein containing a small amount of NADPH dehydrogenase was passed through. NADH dehydrogenase was eluted with a linear gradient established between 120 ml of the basic buffer and 120 ml of the same buffer supplemented with 15 μM FMN (Fig. 1C). Fractions of high activity were pooled and concentrated by ultrafiltration. Polyacrylamide gel electrophoresis of the enzyme in the presence of FMN showed only one major band, which coincided with the NADH-DCIP-MTT activity. Finally, the pooled fraction gave a specific activity of 6.2 units/mg protein. The enzyme was about 110-fold purified, and the yield obtained from the soluble fraction was 5% (Table I). The specific activity of the purified soluble NADH dehydrogenase was relatively low compared with that of the membrane-bound NADH dehydrogenase [3]. The Mr of the soluble NADH dehydrogenase was determined to be about 37 000 by molecular sieve chromatography on Cellulose GC-200-m. When the enzyme was incubated with 1.0% SDS and 1% 2-mercaptoethanol at 65°C for 45 min and subjected to slab gel electrophoresis in the presence of 0.1% SDS, it migrated as a single band. The Mr of the subunit
was estimated to be 38,000 from semilogarithmic plots of $M_r$ versus relative electrophoretic mobility. This shows that the enzyme consists of a single polypeptide chain. In contrast, the $M_r$ of the membrane-bound NADH dehydrogenase was about 97,000 consisting of 6 identical subunits of $M_r$ of 15,500 [3]. Thus, subunit structures of both enzymes are largely different from each other.

When FMN was removed from the purified enzyme solution by gel filtration on Sephadex G-25, the enzyme lost its activity completely. Full activity of the flavin-free enzyme was restored by addition of FMN or riboflavin to the assay mixture, and 67% of the full activity was restored by addition of 25 $\mu$M FAD. The addition of FMN or FAD but not riboflavin to the flavin-free enzyme solution increased the stability of the enzyme heat (Fig. 2). We interpret these data in that FMN appears to be a prosthetic group of the enzyme. The membrane-bound NADH dehydrogenase of *Rps. capsulata* neither loses the activity by gel filtration nor is influenced by addition of flavin compounds to the assay mixture [3]. This shows that the membrane-bound enzyme, but not the soluble one, contains covalently bound flavin. Moreover, unlike the membrane-bound enzyme [3], the soluble enzyme was not inactivated by incubation at 25 °C for 5 min with FMN, riboflavin and NADH (Fig. 2).

The purified soluble enzyme exhibited an optimum activity at pH 7.5 (Fig. 3) similar to the addition of FMN or riboflavin to the assay mixture, and 67% of the full activity was restored by addition of 25 $\mu$M FAD. The addition of FMN or FAD but not riboflavin to the flavin-free enzyme solution increased the stability of the enzyme heat (Fig. 2). We interpret these data in that FMN appears to be a prosthetic group of the enzyme. The membrane-bound NADH dehydrogenase of *Rps. capsulata* neither loses the activity by gel filtration nor is influenced by addition of flavin compounds to the assay mixture [3]. This shows that the membrane-bound enzyme, but not the soluble one, contains covalently bound flavin. Moreover, unlike the membrane-bound enzyme [3], the soluble enzyme was not inactivated by incubation at 25 °C for 5 min with FMN, riboflavin and NADH (Fig. 2).

The purified soluble enzyme exhibited an optimum activity at pH 7.5 (Fig. 3) similar to the

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**Table I. Purification scheme of soluble NADH dehydrogenase from *Rps. capsulata.*

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<tr>
<td>Soluble fraction</td>
<td>66</td>
<td>1197</td>
<td>68.4</td>
<td>0.057</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ (25–50%) fraction</td>
<td>14</td>
<td>693</td>
<td>65.0</td>
<td>0.094</td>
<td>95</td>
<td>1.7</td>
</tr>
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<td>Sephadex G-150</td>
<td>84</td>
<td>434</td>
<td>52.7</td>
<td>0.121</td>
<td>77</td>
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<tr>
<td>AH-Sepharose 4B</td>
<td>181</td>
<td>60.8</td>
<td>16.0</td>
<td>0.263</td>
<td>23</td>
<td>4.6</td>
</tr>
<tr>
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<td>27</td>
<td>0.596</td>
<td>3.72</td>
<td>6.24</td>
<td>5.4</td>
<td>109</td>
</tr>
</tbody>
</table>

* About 45.6 g of wet weight cells were used.
membrane-bound one, but the pH optimum for the soluble enzyme was narrower than that of the membrane-bound enzyme [3]. The soluble NADH dehydrogenase was specific for NADH as an electron donor. With NADPH less than 0.01% of NADH-linked activity was measured. Boll [8] reported that the soluble enzyme of phototrophically grown \textit{R. rubrum} can utilize NADPH at about 3-5% of the rate of NADH. This may be due to the contamination of the partially purified enzyme preparation with NADPH dehydrogenase.

The soluble NADH dehydrogenase oxidized NADH with DCIP, menadione, ferricyanide or cytochrome \textit{c} as an electron acceptor. The relative activities of the enzyme with DCIP (70 \textmu M), menadione (150 \textmu M), ferricyanide (1.5 mM) and cytochrome \textit{c} (81 \textmu M) were 100, 135, 176 and 150, respectively, compared to 100, 123, 181 and 2.8 for the membrane-bound NADH dehydrogenase of \textit{Rps. capsulata}. Thus, like the enzymes from phototrophically grown cells of \textit{R. rubrum}, [9], the two soluble NADH dehydrogenases of \textit{Rps. capsulata} have different reactivities for cytochrome \textit{c}.

Michaelis constants (determined by the method of Velick and Vavra [10]) for NADH, FMN, DCIP and cytochrome \textit{c} of the soluble enzyme were 45, 2.9, 7.9 and 15 \textmu M, respectively. The value of the Michaelis constant for NADH of the soluble enzyme is large compared with that of the membrane-bound enzyme, but the value for DCIP of the soluble enzyme is smaller than that of membrane-bound one [3].

The results shown above indicate the presence of two different kinds of NADH dehydrogenase in chemotrophically grown cells of \textit{Rps. capsulata}. In photosynthetically grown cells of \textit{R. rubrum}, both soluble and membrane-bound NADH dehydrogenases are known to be present [8, 11, 12]. While the membrane-bound enzyme is clearly functional with the respiratory chain [1], the function of the soluble enzyme remains to be determined.

\textit{Acknowledgements}

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