**Note**

Isolation and Partial Characterization of Homogeneous Nitrite Reductase from a Cyanobacterium, *Aphanotoceae sacrum*

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The biosynthesis of amino acids in chlorophyllous plants using inorganic nitrate involves the reduction of nitrite to ammonia as an obligatory step. The enzymes catalyzing this reaction, nitrite reductases (NiRs) have so far been studied mainly in a variety of higher plants 2) and algae. 3) Ferredoxin (Fd) can act as an effective electron donor for these NiR (Fd-NiRs)[ammonia: ferredoxin oxidoreductase, EC 1.7.7.1] and the reaction products have been shown to be ammonia in all cases. Extensive purifications of Fd-NiRs have been accomplished with *Spinacia oleracea*, 4) *Phaseolus vulgaris*, 5) and *Porphyra yezoensis*. 6) However, such data from cyanobacteria are very scanty. Cyanobacterial NiRs have not yet been widely reported compared with NiRs from other sources. So, we previously reported the purification and the properties of cyanobacteria *Spirulina platensis*. 7)

In this line of work, we want to report our procedure for the purification of Fd-NiR from another cyanobacterium, *Aphanotoceae sacrum*, by characterizing it. Fd was purified from *Chlorella vulgaris* and *A. sacrum* cells by the method of Tagawa and Arnon 8) (A422/A660=0.45) and *Chlorella* Fd immobilized on Sepharose 4B was prepared by the method of Shin and Oshino. 9) The preparation of specific antiserum against *S. platensis* strain OU-1 NiR was done with homogeneous *Spirulina* NiR purified as described in our previous report 7) in a New Zealand white rabbit. When an Ouchterlony immunodiffusion analysis 10) was done to examine the immunospecificity of the antiserum, only one precipitin band was observed between the antiserum and a crude enzyme preparation from *S. platensis*, and two precipitin bands were completely fused between homogeneous *S. platensis* NiR and the crude one. These results suggested that the antiserum is specific against the enzyme. The assay method for Fd-NiR was essentially the same as described in our previous paper. 7) Fresh cells of *A. sacrum*, commonly called *Suzenji-Nori*, were purchased from Endo-Kanagawa-Do Company, Fukuoka. These cells were washed several times with tap water to free them from contaminants, and then stored frozen. The frozen cells were autolyzed in two volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 200 mM sodium chloride for 12 hr at 4°C. The autolysate thus obtained was filtered and the filtrate was used as the cell-free extract. Six kilograms (wet weight) of the cells was used in this experiment. *Aphanotoceae sacrum* NiR was purified 1000-fold by the procedures, similar to that previously described for *Spirulina* enzyme. 11) ammonium sulfate fractionation (0—75% saturation), 1st anion-exchange chromatography on DEAE-cellulose DE-53 with linear gradient elution (0—300 mM sodium chloride), 2nd anion-exchange chromatography on DEAE-cellulose DE-53 with the same conditions, hydrophobic chromatography on Butyl-Toyopearl 650S with linear gradient elution (40—0% saturation of ammonium sulfate), followed by gel filtration on Sephadex G-75 and affinity chromatography on Fd-Sepharose 4B. In this affinity column, the enzyme was eluted in 400 mM sodium chloride.

The purified enzyme was almost homogeneous, as judged by polycrylamide gel electrophoresis (PAGE) 11) (Fig. 1a) and SDS-PAGE 12) (Fig. 1b), and final specific activities of 186 µmol of nitrite reduced/min per mg of protein were measured with reduced methyl viologen as electron donor (Table I). Enzymatic activity was followed by assays using methyl

**Table I. Summary of The Purification of Nitrite Reductase from *Aphanotoceae sacrum***

<table>
<thead>
<tr>
<th>Component</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>440</td>
<td>2450</td>
<td>0.180</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 0—75% sat.</td>
<td>392</td>
<td>2490</td>
<td>0.157</td>
<td>89</td>
</tr>
<tr>
<td>1st DEAE-cellulose</td>
<td>254</td>
<td>218</td>
<td>1.17</td>
<td>58</td>
</tr>
<tr>
<td>2nd DEAE-cellulose</td>
<td>177</td>
<td>67.0</td>
<td>2.64</td>
<td>40</td>
</tr>
<tr>
<td>Butyl Toyopearl</td>
<td>114</td>
<td>9.40</td>
<td>12.1</td>
<td>26</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>95.1</td>
<td>6.64</td>
<td>14.3</td>
<td>22</td>
</tr>
<tr>
<td>Fd-Sepharose 4B</td>
<td>22.5</td>
<td>0.121</td>
<td>186.0</td>
<td>5</td>
</tr>
</tbody>
</table>

(a) About 10 µg of the enzyme, measured by the method of Bradford, 12) was electrophoresed on a 7% polyacrylamide gel slab. 11) A constant electric current (15 mA) was applied for 3 hr. The protein was stained with 0.025% Coomassie brilliant blue in 7% acetic acid. The direction of electrophoresis was from top (—) to bottom (+).

(b) About 5 µg of the enzyme was incubated at 100°C for 3 min with 0.1% SDS and 1% β-mercaptoethanol. The treated protein was electrophoresed on a 7% SDS-polyacrylamide gel slab. 12) A constant electric current (15 mA) was applied for 3 hr.

The marker proteins have the following molecular weights: carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), phosphorylase b (94,000). Proteins were detected by the same methods as in (a).

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**Abbreviations:** NiR, nitrite reductase; Fd, ferredoxin; PAGE, polyacrylamide gel electrophoresis.
viologen and dithionite during the purification as described in the table. Comparable Fd-dependent activity was observed when the purified enzyme preparation was used in the presence of 2 mg of Aphanothece Fd instead of the methyl viologen. Molecular weights of about 52,000 and 56,000 were obtained by gel filtration on a Sephadex G-75 column and by SDS-PAGE (Fig. 1b). It appears that this enzyme consists of a single polypeptide like the enzymes from other plants. This molecular weight is about the same as that of Spirulina NiR. 7)

The absorption spectrum (Fig. 2) showed three peaks at 280, 404 (Soret band), and 570 (α band) nm. The ratio of absorption intensity at 404 nm to that at 280 nm was 0.37, and that at 570 nm to that at 404 nm was 0.36. The spectrum is very similar to that shown by Spirulina and other plant NiRs. The nature of the cited spectrum suggests the presence of a siro-heme component in the enzyme molecule.

The pH-activity curve obtained with Tris-HCl buffer had a rather broad optimum around 7.5 like other plant NiRs.

The $K_m$ values calculated from Lineweaver-Burk plots of the data were $2.3 \times 10^{-4}$ M (nitrite), $6.5 \times 10^{-4}$ M (Aphanothece Fd). So, both Spirulina and Aphanothece NiRs have $K_m$ values of the same order of magnitude.

When the examinations of the cross-reactivity between anti S. platensis NiR serum and homogeneous A. sacrum NiR were done by using Ouchterlony double diffusion analysis and immunoprecipitation test, no cross-reactions were observed. These results suggest that NiRs from A. sacrum and S. platensis have no identical antigenic determinant.

From these results, we conclude that this enzyme is similar to NiRs from S. platensis and other plants, with the exception of its immunological properties.

References