Short Communication

Purification of NADH-Nitrate Reductase by Affinity Chromatography

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ABSTRACT

Assimilatory nitrate reductase (NADH:nitrate oxidoreductase, EC 1.6.6.1) from Chlorella vulgaris has been purified to electrophoretic homogeneity with an overall yield of 60% by a procedure that utilizes blue dextran-agarose as an affinity column. Nitrate reductase binds to blue dextran and is not eluted in the presence of high ionic strength buffer, but is rapidly eluted in the presence of molar concentrations of NADH.

NADH-nitrate reductase catalyzes the reduction of nitrate to nitrite by NADH. This reaction is the first step in the reduction of nitrate to ammonia and is considered to be a major control point in the regulation of nitrogen metabolism (2). Recent evidence suggests that nitrate reductase activity in Chlorella may be regulated, in vivo, by a reversible conversion of the enzyme to an inactive form that contains bound cyanide (7).

We have recently purified the nitrate reductase of Chlorella to electrophoretic homogeneity and have found that it contains 2 moles each of flavin adenine nucleotide, heme (Cyt b557), molybdenum, and "cyanide binding sites" per mole of native enzyme (13). The native enzyme is composed of at least 3 subunits of equal mol wt (about 100,000 daltons). These are the only quantitative data obtained to date on the prosthetic group and subunit composition of an assimilatory nitrate reductase, perhaps owing to apparent difficulties in obtaining the enzyme in a homogeneous state.

The purification procedure previously described (13) was a multistep procedure requiring about 10 days to complete, and the yield of pure enzyme was about 25%. I describe here a rapid purification procedure for nitrate reductase that can be completed in 1 day with a 60% yield of pure enzyme.

A preliminary account of this research has been presented (11).

MATERIALS AND METHODS

Growth of Cells and Preparation of Cell Extracts. Chlorella vulgaris cells were grown under continuous illumination with CO2 as the carbon source and nitrate as the nitrogen source, as previously described (13). Cells were harvested with a Sharples Model T-1P Presuritate Super-Centrifuge, resuspended to about 0.1 the original volume with double-distilled H2O, centrifuged, and resuspended to a final density of 250 μl of packed cells/ml with 10 mm potassium phosphate, pH 7.6, 5 mm KNO3. The cell suspension was disrupted by sonication with a Raytheon sonic oscillator Model DF 101. Aliquots of not more than 50 ml were sonicated for three 3-min periods, interrupted by 5 min of cooling. Temperature was maintained at 10 to 15 C during sonication. The extracts were freed of whole cells and larger particles by centrifugation for 10 min at 5000g and were stored frozen at −20 C.

Measurement of Enzyme Activities. NADH-nitrate reductase and NADH-Cyt c reductase activities were measured at 20 C as previously described (13). A spot test specific for NADH dehydrogenase activity, a partial activity of nitrate reductase, was used occasionally to locate the peak of nitrate reductase activity in column fractions. A stock solution for this test was prepared fresh and contained 10 mg of nitroblue tetrazolium and 10 mg of NADH in 50 ml of 0.08 M potassium phosphate, pH 7.6. To 200 μl of the stock solution, in a well of the spot plate, was added 20 μl of a column fraction. The formation of a blue formazan derivative indicates the presence of NADH dehydrogenase or nitrate reductase.

Electrophoresis. Procedures and conditions for analytical disc gel electrophoresis of the native enzyme, SDS gel electrophoresis, and protein staining were the same as previously described (13).

Preparation of Blue Dextran-Sepharose. Coupling of blue dextran to Sepharose 4B was done using the procedure described by Ryan and Vestling (10) except that during cyanoargent bromide activation of the Sepharose, pH was maintained with an alkaline phosphate buffer (pH 12) as described by Porath et al. (9).

PURIFICATION OF NITRATE REDUCTASE

Protamine Sulfate Treatment. The temperature for this and subsequent steps was maintained at 0 to 4 C. Frozen cell extract was thawed and centrifuged for 30 min at 10,000g. To the supernatant solution was added, with stirring, 0.1 volume of 2% (w/v) protamine sulfate (adjusted to pH 7.2 with 0.1 M NaOH). The green sediment was removed by centrifugation for 15 min at 10,000g.

Ammonium Sulfate Fractionation. To the supernatant from the previous step was added, with stirring, 0.25 volume of saturated (NH4)2SO4 which had been adjusted to pH 7.3 with NaOH. The sediment was removed by centrifugation and an additional 0.57 volume of saturated (NH4)2SO4 was added to the supernatant (45% (NH4)2SO4 saturation final). The sediment was collected by centrifugation and was dissolved in 0.08 M potassium phosphate, pH 7.3, 0.15 mM dithioerythritol, 0.1 mM EDTA (buffer A) to give a final volume of about 0.2 the original volume of cell extract.

Blue Dextran-Agarose Chromatography. The 20 to 45%

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(NH₄)₂SO₄ fraction was fully activated (4, 12) by the addition of 50 mM K₄[Fe(CN)₆] to a final concentration of 2 mM ferricyanide and was then applied to a 1.5 × 9 cm column of blue dextran-Sepharose equilibrated with buffer A. Fractions of about 10 ml were collected. After application of the sample, the column was washed with 20 ml of buffer A. A large proportion of the protein does not bind to the column and is removed in the first wash. Other proteins are removed by elution with 50 ml of 0.4 M potassium phosphate, pH 7.3, 0.15 mM dithioerythritol, 0.1 mM EDTA. After elution with this high ionic strength buffer, the absorbance at 280 nm was <0.01. The column was re-equilibrated with buffer A. NADH. 0.1 mM dextran-Sepharose equilibrated with 20 ml of 0.1 M potassium phosphate, pH 6.9, 0.15 mM dithioerythritol, and 50% (v/v) glycerol at a concentration of about 1 mg of protein/ml and was stored at −20 °C. The pure enzyme is stable indefinitely when stored under these conditions (13).

RESULTS

As shown in Figure 1, nitrate reductase was eluted by very low concentrations of NADH. The non-linearity of the NADH gradient at lower NADH concentrations is consistently observed and is possibly due to a low level of NADH oxidase activity associated with nitrate reductase. Table I summarizes the results of a purification. The overall recovery of purified nitrate reductase was about 60%. The specific activity of the purified enzyme was slightly higher than that reported previously (13) and is several times higher than reported values for partially purified nitrate reductase from several other sources (1, 13). Pan et al., however, recently reported a specific activity of about 124 units/mg protein.

![ELUTION VOLUME, ml](image)

Fig. 1. Blue dextran affinity chromatography of NADH-nitrate reductase. One unit of nitrate reductase is 1 μmole of nitrate reduced by NADH/min at 20 C. Nitrate reductase was measured after full activation with ferricyanide (4, 12).

Table I. Purification of NADH-Nitrate Reductase from Chlorella

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mol</td>
<td>units/mg</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract</td>
<td>106</td>
<td>0.08</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Protamime sulfate</td>
<td>102</td>
<td>0.24</td>
<td>3</td>
<td>96</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>102</td>
<td>0.58</td>
<td>7.3</td>
<td>96</td>
</tr>
<tr>
<td>Blue dextran</td>
<td>63</td>
<td>86.2</td>
<td>930</td>
<td>60</td>
</tr>
</tbody>
</table>

1 One unit is 1 μmole of nitrate reduced/min at 20 C. Nitrate reductase was measured after full activation with ferricyanide (4, 12).

2 Protein was measured by a modified method of Lowry as previously described (12).

for an electrophoretically pure NADPH-nitrate reductase from Neurospora crassa in a paper delivered at the 1975 Federation of American Societies for Experimental Biology Meeting (8). If the differences in the apparent mol wt of the Neurospora nitrate reductase (228,000, ref. 3) and the Chlorella nitrate reductase (356,000, ref. 13) are taken into account, the turnover numbers for the two enzymes appear to be similar.

The purified NADH-nitrate reductase from Chlorella was electrophoretically homogeneous. One protein-staining band was observed after SDS gel electrophoresis corresponding to a subunit mol wt of about 100,000. The ratio of NADH-Cyt c reductase to NADH-nitrate reductase activity was about 3. These results are in agreement with the results we reported previously (13).

Crude cell extracts can also be applied directly to the blue dextran-Sepharose column with comparable results, but longer washing times were required and lower overall yields were achieved. When crude cell extracts were applied directly to the blue dextran-Sepharose, it was necessary to oxidize all of the NADH present in the extract since even very low concentrations of NADH will prevent the binding of nitrate reductase to blue dextran. NADH is conveniently oxidized by adding a slight excess of ferricyanide to the cell extract. In addition to serving as an electron acceptor for the NADH dehydrogenase activity associated with nitrate reductase (14), ferricyanide also converts the inactive form of nitrate reductase to the active form (4, 12).

DISCUSSION

The major advantages of the purification procedure described herein over the purification procedure previously reported by us (13) are in speed, simplicity, efficiency, and economy. Slight modifications may have to be made in the procedure for the purification of NAD(P)H-nitrate reductase from other sources but I anticipate that this procedure will facilitate the purification of nitrate reductase from other sources as well.

The method takes advantage of the high affinity of nitrate reductase for blue dextran and the specific elution of the enzyme by low concentrations of NADH. Blue dextran affinity columns have been used for the rapid purification of phosphofructokinase (5, 15), lactate dehydrogenase (10), and malate dehydrogenase (6). Thompson et al. (15) have proposed recently that the blue dextran chromatophore is specific for a supersecondary structure called the dinucleotide fold. After examining the interaction of blue dextran-Sepharose affinity columns with a wide range of proteins known to possess the dinucleotide fold as well as several proteins which do not possess the fold, they concluded that blue dextran affinity chromatography can be used to identify readily proteins that possess the dinucleotide fold. The results presented in this communication indicate that NADH-nitrate reductase
also probably possesses a dinucleotide fold. In contrast to other proteins that bind specifically to blue dextran, NADH-nitrate reductase is not readily dissociated from blue dextran by buffer of high ionic strength. This unique property of nitrate reductase makes blue dextran affinity chromatography an especially effective technique for the purification of nitrate reductase.

LITERATURE CITED


