Note

Isolation and Partial Characterization of Homogeneous Nitrite Reductase from a Red Alga, *Porphyra yezoensis*

Teruhiko IDE and Goro TAMURA*

Department of Agricultural Chemistry,
Faculty of Horticulture, Chiba University,
Matsudo, Chiba 271, Japan

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Assimilatory reduction of inorganic nitrate to the $-NH_2$ level in amino acids has been confirmed to proceed through nitrite reduction to ammonia as an intermediate step. The enzymes catalyzing this reaction, NiRs, have so far been studied mainly with a variety of higher plants^{1~10)} and algae.^{11~13)} Ferredoxin can act as the effective electron donor for these NiRs (Fd-NiRs) [ammonia: ferredoxin oxidoreductase, EC 1.7.7.1] and the reaction product has been shown to be ammonia in all cases. Extensive purification of Fd-NiRs have been accomplished with *Spinacia oleracea*³⁾ and the cyanobacterium *Spirulina platensis*.¹³⁾ However, such data from red alga are very scanty.¹¹⁾ We intended to purify a red-algal Fd-NiR. This paper communicates the purification and some enzymatic properties of this purified Fd-NiR from a red alga, *Porphyra yezoensis*.

Ferredoxin was obtained from spinach leaves as described by Tagawa and Arnon¹⁴⁾ ($A_{422}:A_{277}=0.48$). Ferredoxin-Sepharose was prepared by the method of Shin and Oshino.¹⁵⁾ All the purification procedures were at 4°C.

The fresh thalli of *Porphyra yezoensis*, grown in Tokyo Bay in winter, were kindly supplied by Mr. Araki of Yamamoto Nori Research Laboratory. These thalli were washed several times with tap water to free them from salts, and then, with the addition of several drops of toluol, autolyzed in two volumes of 0.2 M Tris-HCl buffer, pH 7.7, for 96 hr at 4°C. The autolysate thus obtained was filtered through cheesecloth with the aid of a hand press and the filtrate was used as the cell-free extract. One kilogram (wet weight) of the thalli were used in this experiment. To the cell-free extract described above, 1% acrinolum solution was added slowly with stirring to 0.04%. The mixture was stirred for an additional 10 min and the precipitate was discarded by filtration through an absorbent cotton layer. The purification procedure used

was very similar to that described for the isolation of methyl viologen-NiR from etiolated bean shoot;⁵⁾ however, some differences in results and in the procedure may be noted: 1. In the hydrophobic chromatography, Toyopearl HW-60S was used instead of Butyl-Toyopearl. 2. Ultrogel AcA44 gel filtration was used before the ferredoxin-Sepharose affinity chromatography. To the filtrate, obtained above, were added 2-mercaptoethanol, PMSF, and Polyclar AT to final concentration of 10 mM, 0.5 mM, and 5%, respectively. The resulting solution was filtered through cheesecloth. Solid ammonium sulfate was slowly added to the filtrate to 30% saturation. After removing the resulting precipitate by centrifugation ($10,000\times g$ for 20 min), ammonium sulfate was further added to the supernatant to a concentration of 85%. The precipitate obtained was collected by centrifugation ($10,000\times g$ for 30 min) and suspended in buffer A (50 mM Tris-HCl buffer, pH 7.7, containing 100 mM NaCl and 10 mM 2-mercaptoethanol) and dialyzed against the same buffer. The enzyme solution (120 ml) was put on a column of DEAE-cellulose (4×35 cm), previously equilibrated with buffer A. After the column was washed with buffer A, a linear concentration gradient of eluent was established with 500 ml of buffer A in the mixing vessel and 500 ml of 300 mM NaCl (in buffer A) in the reservoir. The active fractions from the column chromatography on DEAE-cellulose were pooled. The pooled fraction (150 ml), described above, was supplemented with ammonium sulfate to bring it to 40% saturation. The resulting solution was put on a Toyopearl HW-60S column (2.5×12 cm) previously equilibrated with buffer B (50 mM Tris-HCl buffer, pH 7.7, containing 40% ammonium sulfate and 10 mM 2-mercaptoethanol). After the column was washed with buffer B, a linear concentration gradient of eluent was established with 300 ml of 40% of saturation ammonium sulfate in buffer B in the mixing vessel and 300 ml of buffer B in the reservoir. The active fractions were combined and concentrated in a dialysis sac against buffer C (50 mM Tris-HCl buffer, pH 7.7, containing 10 mM 2-mercaptoethanol and 80% ammonium sulfate) and were then dialyzed against buffer D (30 mM Tris-HCl buffer, pH 7.7, containing 150 mM NaCl, 10 mM 2-mercaptoethanol, and 10% glycerol). The dialyzed enzyme solution was filtered through an Ultrogel AcA44 column (1×90 cm) equilibrated with buffer D. The active fractions were pooled and were concentrated in a dialysis sac against buffer E (30 mM Tris-HCl buffer, pH 7.7, containing 20% glycerol and 10 mM 2-mercaptoethanol). The dialyzed solution (9 ml) was chromatographed on a column of ferredoxin-Sepharose (1.5×2 cm) previously equilibrated with buffer E. Undesired proteins were removed from the column by washing with buffer E and Fd-NiR was then eluted with buffer F (30 mM Tris-HCl buffer, pH 7.7, containing 200 mM NaCl, 10% glycerol, and 10 mM 2-mercapto-

* To whom reprint requests should be addressed. *Abbreviations:* NiR, nitrite reductase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

TABLE I. SUMMARY OF PURIFICATION

The assay method for nitrite reductase was as described elsewhere¹⁾ with a slight modification. The reaction mixture contained, in a total volume of 1 ml, 75 μ mol of Tris-HCl buffer, pH 7.7, 2 μ mol of sodium nitrite, 0.5 mg of ferredoxin, 0.036 μ mol of sodium dithionite, and the enzyme preparation. The reaction was at 30°C. One unit of nitrite reductase is the amount of enzyme that reduces 1 μ mol of nitrite per min under these conditions.

Step in procedure	T.A. (units)	T.P. (mg)	S.A. (units/mg protein)	Yield (%)
(NH ₄) ₂ SO ₄ (30 ~ 85% sat.)	508	1570	0.32	100
DEAE-cellulose	375.3	85.6	4.36	74
Toyopearl HW-60S	257	8.8	29.2	51
Ultrogel AcA44	195	4.75	41	38
Fd-Sepharose 4B	81	0.24	337	16

ethanol). The main Fd-NiR fractions were pooled and were used as the purified enzyme in the subsequent experiments. Table I shows a summary of the purification starting from 1 kg of fresh thalli of *Porphyra yezoensis*.

When the purified enzyme was electrophoresed on a 7.5% polyacrylamide gel disc, a single intensely stained protein band was obtained.

Here, the final preparation was 1050-fold purified compared to the ammonium sulfate step preparations. A final specific activity of 337 μ mol of nitrite reduced per min per milligram of protein was measured with reduced ferredoxin as an electron donor.

Enzymatic activity was followed by assays using spinach ferredoxin-dithionite during the purification as described above. Comparable methyl viologen-linked activity was observed when the purified enzyme preparation was used in the presence of 0.75 μ mol of methyl viologen and 95 μ g of bovine serum albumin: however, in the absence of bovine serum albumin, methyl viologen was less effective as the electron donor (about 50%).

The absorption spectrum of the enzyme in the oxidized form is shown in Fig. 1. Maxima were found at 278, 385, and 580 nm, being indicative of the presence of a siroheme, which has been detected in enterobacterial NADPH-sulfite reductase¹⁶⁾ and also in spinach nitrite³⁾ and sulfite¹⁷⁾ reductases. The ratios of the absorbance at ($A_{385}:A_{278}$) and ($A_{580}:A_{278}$) were 0.46 and 0.16, respectively. This spectrum is rather different from that of Spinach Fd-NiR³⁾ and also that of cyanobacterial (*Spirulina platensis*) Fd-NiR¹³⁾ reported previously.

When estimated by SDS-PAGE, the molecular weight of Fd-NiR from *Porphyra yezoensis* was 59,000, about the

FIG. 1. Absorption Spectra Were Made on a Hitachi 150-20 Spectrophotometer.

The concentration of purified enzyme was 0.14 mg of protein per milliliter. The protein was in 30 mM Tris-HCl buffer, pH 7.7, containing 200 mM NaCl, 10 mM 2-mercaptoethanol, and 10% glycerol.

same size as that from higher plants,¹⁾ and some 11% larger than that from cyanobacteria.¹³⁾

The K_m values calculated from Lineweaver-Burk plots of the data were 2.8×10^{-4} M (nitrite) and 2.5×10^{-5} M (spinach ferredoxin). So, both algal and higher plant¹⁾ Fd-NiRs have K_m values of the same order of magnitude.

The pH-activity curve obtained with Tris-HCl buffer had an optimum at around 7.7.

Specific antiserum against NiR homogeneously purified from green shoots of the red bean (*Phaseolus angularis*)⁶⁾ gives a negative Ouchterlony result with *Porphyra* Fd-NiR.

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