Purification and Characterization of a Ferredoxin-NADP⁺ Oxidoreductase-Like Enzyme from Radish Root Tissues

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ABSTRACT

An enzyme able to reduce cytochrome c via ferredoxin in the presence of NADPH, was isolated, purified from radish (Raphanus sativus var acanthuriforis cultivar miyashige) roots and characterized. The enzyme was purified by DEAE-cellulose, Blue-Cellulofine, Ferredoxin-Sepharose 4B, and Sephadex G-100 column chromatography. Molecular mass of the enzyme was estimated to be 33,000 and 35,000 daltons by Sephadex G-100 gel filtration and SDS-PAGE, respectively. Its absorption spectrum suggested that the enzyme contains flavin as a prosthetic group. The Km values for NADPH and ferredoxin were calculated to be 9.2 and 1.2 micromolar, respectively. The enzyme required NADPH and did not use NADH as an electron donor. The optimal pH was 8.4. The enzyme also catalyzed the photoreduction of NADP⁺ in the spinach leaf thylakoid membranes depleted of ferredoxin and ferredoxin-NADP⁺ oxidoreductase. The effect of NaCl and MgCl₂ concentration on the activity and amino acid composition of the enzyme were demonstrated. The results suggest that the enzyme is similar to ferredoxin-NADP⁺ oxidoreductase from chloroplasts and cyanobacteria and is the key enzyme catalyzing the electron transport between NADPH, generated by the pentose phosphate pathway, and ferredoxin in plastids of plant heterotrophic tissues.

In photosynthetic tissues the enzymes involved in nitrogen metabolism, Fd-GOGAT², and Fd-NiR are located in chloroplasts. The reduced ferredoxin required by these enzymes is generated by PSI.

In heterotrophic tissues, on the other hand, ferredoxin-dependent enzymes of nitrogen assimilation have also been demonstrated by using an in vitro system supplemented with leaf ferredoxin (11, 19, 26). Several investigations have shown that Fd-NiR in roots is located in plastids (4, 24). Using methyl viologen as an electron donor, Washtianati and Sato (37, 38) found that Fd-NiR and Fd-GOGAT are present in proplastids from tobacco cultured cells. There are a number of reports indicating that nitrite reduction in both root plastids and cultured tobacco cell proplastids depends on the oxidation of Glc-6-P and/or 6-PG by Glc-6-P dehydrogenase and/or 6-PG dehydrogenase (5, 7, 18, 39). Emes and Fowler (6) indicated that all enzymes involved in the pentose-phosphate pathway are in plastids from pea roots. The close relationship between nitrite reduction and the pentose-phosphate pathway in pea root plastids was confirmed (3). Oji et al. (25) reported that an electron carrier and a diaphorase, which has FNR activity, are involved in electron transport from NADPH (generated by Glc-6-P dehydrogenase and 6-PG dehydrogenase) to nitrite in plastids from barley roots. A ferredoxin-like electron carrier in dark-grown cultured tobacco cells was investigated by Ninomiya and Sato (23) and Suzuki et al. (31) isolated a nonheme iron protein electron carrier and a pyridine nucleotide reductase from maize roots. They also indicated that the ferredoxin-like electron carrier was able to be reduced with either NADPH or NADH via the root pyridine nucleotide reductase and to donate the electrons to Fd-GOGAT. Recently, Wada et al. (34, 35) showed that unique ferredoxins are present in radish roots and that they are distinct from the chloroplast ones on the basis of amino acid sequence.

In the present paper, we describe purification and characterization of an enzyme having FNR activity in plant heterotrophic tissues.

MATERIALS AND METHODS

Radish (Raphanus sativus var acanthuriforis cultivar miyashige) and spinach (Spinacia oleracea L.) were purchased from a local market. Ferredoxin from Spirulina sp. was prepared as described by Wada et al. (32, 33). Bovine heart Cyt c was prepared as described by Hagihara et al. (10). NADPH and NADH were purchased from Oriental Yeast Co. Ltd. (Osaka) and Sigma Chemical Co., respectively. DEAE-cellulose was obtained from Nacalai Tesque, Inc. (Kyoto), Blue-Cellulofine from Seikagaku-Kogyo Co. Ltd. (Tokyo), and Sephadex G-100 (superfine) from Pharmacia Fine Chemicals. The molecular weight markers were purchased from Sigma Chemical Co.

Preparation of FNR-Like Enzyme from Radish Roots

All preparation steps were carried out at 4°C or on ice and all centrifugations were performed with a Hitachi HIMAC SCR 20B. About 8 kg of lower white roots were sliced into about 1 mm thickness with a slicer. The sliced roots were homogenized in 1 kg lots with 1 L of buffer A (5 mm Tris, 1 mm EDTA, 0.5 mm PMSF, and 0.03% [w/v] Triton X-100, the pH of the solution was not adjusted) with a Waring
Blender at high speed for 30 s. The combined homogenates were squeezed through a bleached cotton bag. About 14 L of the crude extract were obtained and diluted with water to final volume of 32 L. DEAE-cellulose (800 mL), equilibrated with 10 mM Tris-HCl buffer (pH 7.5), was added to the extract. The suspension was stirred gently for 5 min. After allowing the DEAE-cellulose to settle for 30 min, the supernatant was gently decanted. Another batch of DEAE-cellulose (800 mL) was added to the decanted solution and the supernatant from the second DEAE-cellulose treatment was discarded. The combined DEAE-cellulose matrices were packed into six columns (5 × 20 cm) and were washed with about 500 mL for each column of buffer B (10 mM Tris-HCl buffer [pH 7.8] and 0.4 mM EDTA). The protein was eluted from the columns with buffer B containing 1.0 M NaCl. The eluate was filtered through glass wool to remove lipids. The delipidated eluate (760 mL) was fractionated with ammonium sulfate between 30 and 80% saturation. The precipitate was dissolved in 100 mL of buffer B and dialyzed against 4.0 L of buffer B for 24 h with a change of the buffer solution. The dialysate was centrifuged for 20 min at 27,000g. The supernatant (122 mL) was applied to a DEAE-cellulose column (2.7 × 35 cm) equilibrated with buffer B. After washing with about 500 mL of buffer B, the column was developed with a 600 mL linear gradient system of 0 to 0.5 M NaCl in buffer B. The fractions showing FNR activity were combined and concentrated by ultrafiltration (Advantec ultrafiltr W-10).

The concentrated eluate (35 mL) was applied to a Blue- Cellulofine column (1.5 × 30 cm) equilibrated with buffer C (10 mM Tris-HCl buffer [pH 7.8] and 0.1 mM EDTA). After washing with 200 mL of buffer C, the column was eluted with a 500 ML linear gradient of 0.1 to 1.0 M NaCl in buffer C. The active fractions were combined, concentrated as above (15 mL) and applied to a Ferrodoxin-Sepharose 4B column (1.5 × 30 cm) equilibrated with buffer C. After washing with 200 mL of buffer C, the enzyme was eluted with a 500 ML linear gradient of 0 to 0.5 M NaCl in buffer C. After concentration by ultrafiltration, the enzyme fractions were further purified on a Sephadex G-100 column (2.5 × 72 cm) equilibrated with buffer C containing 0.1 M NaCl. The fractions showing FNR activity were pooled, concentrated, and used as the enzyme preparation.

### Assay for FNR Activity

FNR activity was assayed by measuring ferredoxin-dependent Cyt c reduction in the presence of NADPH. The reaction mixture contained 10 mM Tris-HCl buffer (pH 7.8), 50 μM Cyt c, 100 μM NADPH, and 10 μM ferredoxin, and the enzyme in total volume of 1.0 mL. NADPH was added (without ferredoxin) and the reaction was monitored at 350 nm for 1 min. Ferredoxin was then added and rate of reaction was measured. In Table I, the reaction was started by addition of NADPH solution into the reaction mixture with or without ferredoxin. The differences in the reaction rates observed with and without ferredoxin was used to calculate activity.

### Analysis of Molecular Mass

The molecular mass of the enzyme was analyzed with Sephadex G-100 (superfine) gel filtration, SDS-PAGE and amino acid composition. SDS-PAGE (12.5% gel concentration) was carried out according to Laemmli (15) and the gel stained with Coomassie brilliant blue R-250. BSA (66,000 D), egg albumin (45,000 D), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36,000 D), bovine erythrocyte carbonic anhydrase (29,000 D), bovine pancreas trypsinogen (24,000 D), soybean trypsin inhibitor (20,100 D), and bovine milk α-lactalbumin (14,200 D) were used as molecular mass markers. Gel filtration was carried out with a Sephadex G-100 column (2.5 × 72 cm) equilibrated with buffer C containing 0.1 M NaCl. The column was developed with the same buffer solution at a flow rate of 10 mL/h. BSA, bovine erythrocyte carbonic anhydrase, horse heart Cyt c (12,400 D), and bovine lung aprotinin (6,500 D) were used as molecular mass markers.

### Amino Acid Analysis

About 5 μg of enzyme was hydrolyzed in an evacuated, sealed tube with 5.7 N HCl for 24 or 72 h at 110°C. Amino acid composition was analyzed with Hitachi L-8500 automatic amino acid analyzer according to the method of Spackman et al. (30).

### Reconstitution with Spinach Thylakoids

Spinach thylakoid membranes were obtained from the pellet of the first centrifugation of spinach leaf homogenate for

| Table I. Preparation of the FNR-Like Enzyme from Radish Roots |
|---------------|-----|-----|----------------|
| Preparation Steps | Protein* | Total Activity | Specific Activity |
| mg | units | % | units/mg | protein | ratio |
| Crude extract | 3,350 | 489 | 100 | 0.13 | 1.0 |
| First DEAE-cellulose | 4,100 | 472 | 96.5 | 0.12 | 0.9 |
| (NH₄)₂SO₄ treatment | 1,770 | 356 | 72.8 | 0.20 | 1.5 |
| Second DEAE-cellulose | 131 | 137 | 28.7 | 1.05 | 8.1 |
| Blue-Cellulofine | 1.0 | 86.8 | 17.8 | 86.8 | 670 |
| Fd-Sepharose 4B | 0.16* | 30.0 | 6.1 | 188 | 1,450 |
| Sephadex G-100 | 0.054* | 10.5 | 2.1 | 194 | 1,490 |

* Protein contents were determined by the dye binding method, except for the preparation steps after Fd-Sepharose 4B. ** The UV absorption method.
10 min at 4200g, as described by Kuwabara and Murata (14). The thylakoid membranes after heat treatment at 50°C for 5 min, were centrifuged down and resuspended to give 1 mg/mL of Chl concentration. These thylakoid membranes did not show any NADP⁺ photoreduction without supplements of FNR. After fractionation with ammonium sulfate (80% saturation) of the first supernatant, ferredoxin and FNR were obtained from the supernatant and precipitate, respectively. Ferredoxin and FNR were purified by DEAE-cellulose and Ferredoxin-Sepharose 4B column chromatography, respectively (29). The reaction mixture contained 40 mM Tris-HCl buffer (pH 7.8), 80 μM DCPIP, 4 mM ascorbate, 160 μM NADP⁺, 14 μg Chl/mL spinach thylakoids, 4 μM spinach ferredoxin, and the enzyme from radish roots or spinach FNR. The reaction was started by irradiance through a To-shiba VB46 filter and monitored at 340 nm. Chl concentration was determined according to Arnon (1).

**Preparation of Ferredoxin-Sepharose 4B**

Ferredoxin-Sepharose 4B column was made of Spilurina ferredoxin and BrCN-activated Sepharose 4B as recommended in the supplier’s manual. The Ferredoxin-Sepharose 4B column was stored in a column (1.5 x 30 cm) at 4°C in buffer C containing 1.0 M NaCl.

**Determination of Protein**

Protein was determined by the dye binding method of Bradford (2) by using BSA as the standard or the UV absorption method of Waddell (36).
RESULTS AND DISCUSSION

Figure 1 shows a DEAE-cellulose chromatographic profile of the FNR-like enzyme following fractionation with ammonium sulfate. FNR activity, which was eluted at 100 mM NaCl concentration, was contaminated with a red protein with absorption maximum at 400 nm and blue protein with absorption maximum at 620 nm. It was considered that the former was peroxidase which had been characterized by Morita et al. (20) and the latter, a copper protein. Both were completely removed by Blue-Cellulofine affinity column chromatography. FNR activity was found in the second protein peak which eluted at 510 mM NaCl concentration. The active fractions pooled were subjected to another affinity chromatography step (Ferredoxin-Sepharose 4B column). FNR activity was found as a single peak at 250 mM NaCl concentration. The FNR-like enzyme was further purified with Sephadex G-100 gel filtration (Fig. 2). Purification is summarized in Table I. Substantial activity was lost at the second DEAE-cellulose chromatography. On the other hand, the affinity chromatography with Blue-Cellulofine was very effective, increasing specific activity by 80-fold. The final yield of the enzyme was about 2.1%. The specific activity for the enzyme resembled that for spinach and Nostoc FNRs (137 and 100 units/mg protein, respectively [9, 12]).

From the absorption spectrum (Fig. 3) the FNR-like enzyme was a typical flavoprotein, showing peaks at 456, and 388 nm and a shoulder at 483 nm. The prosthetic group(s) (FAD or FMN) could not be determined due to the shortage of sample. The ratio of absorbance at 280 nm to at 456 nm was about 7.4. This value implies that the enzyme preparation is essentially pure as compared with other of FNRs studied so far (28).

As shown in Figure 4, the purified enzyme, migrated on SDS-PAGE as a single band. The accompanying lower molecular mass compound which was not removed by Sephadex G-100 gel filtration, appeared to be another form of FNR on the basis of its amino acid composition (Table II). The molecular mass of the main band was calculated to be 33,000 D, consistent with the value (35,000 D) which was obtained by the gel filtration (Fig. 2).

Amino acid composition of the enzyme was analyzed and compared with that of the FNRs from Spirulina sp., spinach and pea (whose sequences have been defined from protein and cDNA methods) (Table II). It was found that the content of Thr, Ser, and Ala were higher and that of Gin, Met, and Lys, lower than that with the other FNRs. If the contents of Cys and Trp were assumed to be 5 and 6 residues, respectively, as well as those of FNRs from spinach and pea, the molecular mass of the FNR-like enzyme from radish root was calculated to be 33,100 D, excluding the prosthetic group. This value is very close to that of the FNRs from Spinulina sp. (33,350 D), spinach (35,317 D), pea (34,760 D) and Mesembryanthemum crystallinum (35,713 D from cDNA [17]).

Some investigators have reported the effect of NaCl on Cyt c reduction activity of FNRs (16, 21). Using spinach FNR, Nakamura and Kimura (21) reported that activity was highest at 120 mM NaCl concentration and decreased at higher concentrations of NaCl. With regard to the root FNR-like enzyme, the effect of NaCl concentration on activity was consistent with that observed for other FNRs. When MgCl2 was used instead of NaCl, the decrease of activity was observed at a lower salt concentration (80 versus 380 mM at 50%). The
maximal activity of FNR-like enzyme followed a similar pattern, being highest at 150 mM NaCl and 30 mM MgCl₂.

The affinities of NADPH and ferredoxin to FNR-like enzyme from roots were determined by Lineweaver-Burk plots. The concentrations of NADPH and ferredoxin which yielded half-maximal activity were 9.2 and 1.2 μM, respectively. These values were of good agreement with Kₐ values for NADPH and ferredoxin of FNRs from Spirulina sp. and spinach (8, 16). As described in purification steps, this enzyme had a stronger affinity to cibacron-blue dye than to ferredoxin, judging from the NaCl concentration required for elution from Blue-Cellulofine and Ferredoxin-Sepharose 4B.

When NADH, was used as an electron donor instead of NADPH, ferredoxin-dependent Cyt c reduction by the FNR-like enzyme was not observed even when NADH concentration was increased to 200 μM (Table III). At an extremely high concentration of NADH (2 mM) the activity obtained was only 4.8% of the maximal activity with NADPH (Table III). Therefore, it was concluded that radish root FNR-like enzyme is NADPH specific. This conclusion contradicts that of Suzuki et al. who found that the pyridine nucleotide reductase from maize roots was able to use NADH as well as NADPH (31).

The effect of pH on ferredoxin-dependent Cyt c reduction of FNR-like enzyme from radish roots was demonstrated using 100 mM phosphate and Tris-HCl buffers (data not shown). The optimal pH was determined to be pH 8.4 in Tris-HCl buffer (100 mM). The activity in phosphate buffer (100 mM) was lower than that in Tris-HCl buffer. The optimal pH for FNRs was usually 7 to 8.5 (9, 12, 16, 27). The value obtained in the present experiment was reasonable and close to the value for spinach FNR.

NADP⁺ photoreduction activity with the FNR-like enzyme from radish roots was similar to or somewhat higher than that with spinach leaf FNR (data not shown).

The results described above suggest that the FNR-like enzyme from radish roots is functionally very similar to FNR from leaf tissues. We propose that this enzyme is called root FNR. The root FNR is considered to be different from leaf FNR at the structural level. We conclude that root FNR may be an important enzyme in roots, functioning in the electron transport system from NADPH, generated by the pentose-phosphate pathway, to ferredoxin, thereby donating electrons to some ferredoxin-dependent enzymes such as nitrite reductase and glutamate synthase.

<table>
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<th>Amino Acid</th>
<th>Radish¹ Roots</th>
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* Average values were estimated from samples of 24 and 72 h hydrolysis, except that * values were extrapolated to zero time of hydrolysis and † higher values of 24 and 72 h hydrolysis were adopted. Values were calculated from amino acid sequences obtained by ² Yao et al. (40) and ³ Karplus et al. (13) and from cDNA sequence by ⁴ Newman and Gray (22). ², ³, and ⁴ not determined.
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LITERATURE CITED