

Differential Interaction of Maize Root Ferredoxin:NADP⁺ Oxidoreductase with Photosynthetic and Non-Photosynthetic Ferredoxin Isoforms¹

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In higher plants ferredoxin (Fd):NADP⁺ oxidoreductase (FNR) and Fd are each distributed in photosynthetic and non-photosynthetic organs as distinct isoproteins. We have cloned cDNAs for leaf FNR (L-FNR I and L-FNR II) and root FNR (R-FNR) from maize (*Zea mays* L.), and produced recombinant L-FNR I and R-FNR to study their enzymatic functions through kinetic and Fd-binding analyses. The K_m value obtained by assay for a diaphorase activity indicated that R-FNR had a 10-fold higher affinity for NADPH than L-FNR I. When we assayed for NADPH-cytochrome *c* reductase activity using maize photosynthetic Fd (Fd I) and non-photosynthetic Fd (Fd III), the R-FNR showed a marked difference in affinity between these two Fd isoproteins; the K_m for Fd III was 3.0 μM and that for Fd I was 29 μM . Consistent with this, the dissociation constant for the R-FNR:Fd III complex was 10-fold smaller than that of the R-FNR:Fd I complex. This differential binding capacity was confirmed by an affinity chromatography of R-FNR on Fd-sepharose with stronger binding to Fd III. L-FNR I showed no such differential interaction with Fd I and Fd III. These data demonstrated that R-FNR has the ability to discriminate between these two types of Fds. We propose that the stronger interaction of R-FNR with Fd III is crucial for an efficient electron flux of NADPH-FNR-Fd cascade, thus supporting Fd-dependent metabolism in non-photosynthetic organs.

Non-photosynthetic plastids are important subcellular compartments for the reductive assimilation of nitrate and sulfate. Some of the key enzymes in this non-photosynthetic assimilatory process, such as nitrite reductase, sulfite reductase, and Glu synthase, are dependent on reduced ferredoxin (Fd) for their activity, as is the case in chloroplasts (Knaff, 1996). Previous studies have indicated that, in non-photosynthetic plastids, the oxidation of Glc-6-P via the oxidative pentose phosphate pathway (OPPP) provides reducing power for supporting the activities of nitrite reductase and Glu synthase (Oji et al., 1985; Bowsher et al., 1989, 1992). As NADPH cannot be utilized as an immediate electron donor for these Fd-dependent enzymes, an electron transfer system, in which Fd is reduced using the NADPH generated by OPPP, is needed in non-photosynthetic plastids.

The presence of an Fd:NADP⁺ oxidoreductase (FNR)-like protein in non-photosynthetic organs was

first reported in maize (*Zea mays* L.) roots (Suzuki et al., 1985). Later, several groups purified FNR from roots of spinach (*Spinacia oleracea*; Morigasaki et al., 1990a), radish (Morigasaki et al., 1990b), tomato (Green et al., 1991) and pea (Bowsher et al., 1993), and showed that the root FNRs were able to mediate the reduction of Fd with NADPH. cDNAs encoding the precursor of root FNR have been cloned from rice (Aoki and Ida, 1994), maize (Ritchie et al., 1994), and pea (Bowsher and Knight, 1996), and the root FNRs have been found to be distinct from their leaf counterparts in their primary structure. Fd is also known to be distributed as distinct isoproteins in photosynthetic and non-photosynthetic organs in several plants (Wada et al., 1986; Kimata and Hase, 1989; Green et al., 1991; Hase et al., 1991a; Alonso et al., 1995). This implies that the root isoproteins of FNR and Fd are in situ partners in facilitating the electron transfer from NADPH to Fd in the direction opposite to that occurring in the photosynthetic process. To date, however, little data is available for determining the kinetic and regulatory properties of the enzymatic reactions conducted by root isoproteins of FNR and Fd, and our knowledge of their molecular characteristics supporting Fd-linked metabolism in non-photosynthetic plastids remains incomplete.

Fd and FNR form a 1:1 protein:protein complex, and this specific interaction is considered to be important for efficient electron transfer between the

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two. The focus of our work has been to compare the abilities of root and leaf FNRs from maize that interact with Fd isoproteins distributed differentially in photosynthetic and non-photosynthetic organs. Previous works on maize Fd have shown that at least six isoproteins (Fd I–Fd VI) are present; these are divided into two major groups, photosynthetic and non-photosynthetic Fds, based on the characteristics of organ distribution and light-inducibility in their gene expression (Hase et al., 1991a; Matsumura et al., 1997). Fd I and Fd III are the major and representative Fd isoproteins in leaves and roots, respectively.

In this study, we cloned new cDNAs encoding maize leaf FNR and the same previously isolated cDNA encoding maize root FNR (Ritchie et al., 1994); we then produced their recombinant enzymes for use in detailed biochemical analyses. We have found that the leaf and root FNRs differ in their abilities in electron transfer and protein-protein interactions with Fd I and Fd III. Herein, we report the advantageous properties of root FNR and Fd III over other combinations of the FNRs and Fds, which may contribute to efficient electron allocations from NADPH to Fd-dependent metabolism in root plastids.

RESULTS

Cloning of cDNAs Encoding Leaf and Root FNRs

A cDNA library prepared from maize leaves was screened with antibodies against spinach leaf FNR. One confirmed positive clone with a 1.2-kb insert was isolated. An open reading frame encoding a 346-amino acid polypeptide without the initiation Met was contained in the insert. The deduced amino acid sequence was homologous to other known FNRs in the corresponding regions. To isolate a full-length cDNA, the same library was screened by nucleic acid hybridization with the 1.2-kb insert as a probe. Thirty clones hybridizing to the probe were isolated and divided into two groups by mapping the insert DNAs with various restriction enzymes; one corresponded to the same clone as described above, and the other was a group of new clones. Two clones with the longest insert, pL-FNR1 (1,389 bp) in the former group and pL-FNR2 (1,628 bp) in the novel group, were sequenced. pL-FNR1 and pL-FNR2 encoded precursors of FNR, designated L-FNR I (355 amino acid residues) and L-FNR II (368 amino acid residues), respectively. The deduced amino acid sequences are compared with those of other FNRs from maize root FNR and rice leaf and root FNRs (Fig. 1). As the N terminus of the mature protein of maize FNR is unknown, we tentatively assigned the processing site in comparison with the chemically determined N-terminal sequence of spinach FNR (Karplus et al., 1984). L-FNR I and L-FNR II are 84% identical with each other in the mature region, and their homologies to rice leaf FNR (L-FNR I, 87%; L-FNR II, 86%) are higher than those to root FNRs from maize

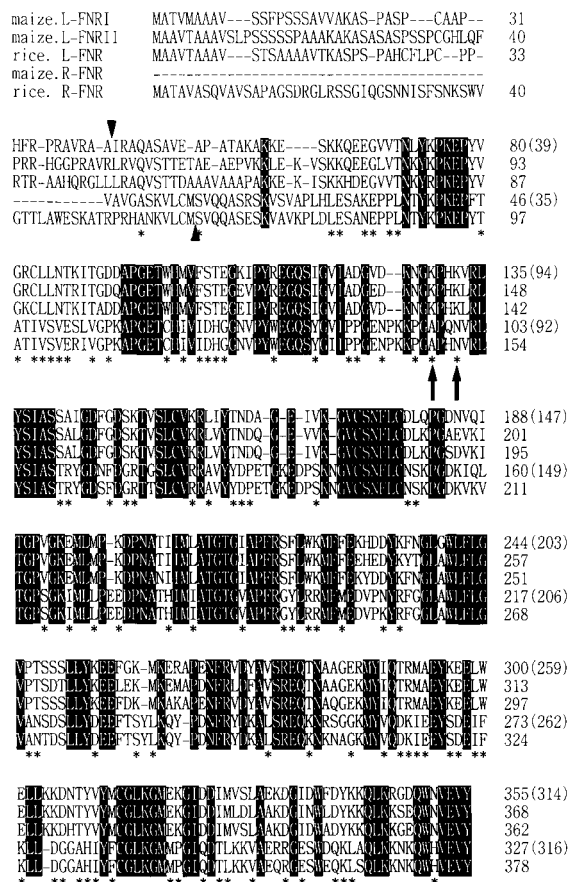


Figure 1. Comparison of amino acid sequences of L- and R-FNRs from maize and rice. Sequences of maize L-FNR I (GenBank accession no. AB035644) and L-FNR II (GenBank accession no. AB035645) are from this study, and those of rice L-FNR (Aoki et al., 1994), rice R-FNR (Aoki and Ida, 1994), and maize R-FNR (Ritchie et al., 1994) have been previously reported. Identical amino acid residues among all FNRs are indicated by white letters on a black background, and the residues differentially conserved between L- and R-FNRs are marked with stars. Putative processing sites for maize L-FNR I and R-FNR are indicated by arrowheads, and the numbering from the N terminus of the mature protein is shown in the parentheses. Potential Fd-binding residues in leaf FNRs, Lys-88 and Lys-91 (numbering of maize mature L-FNR I), are changed to Ala-86 and Asn-89 in root FNRs (numbering of maize mature R-FNR) as marked with arrows. For details, see "Discussion."

(L-FNR I, 51%; L-FNR II, 52%) and rice (L-FNR I, 51%; L-FNR II, 53%), indicating that plant FNRs can be divided into two subgroups, leaf and root types. We used L-FNR I as a representative molecular species in the following studies. At the moment, we do not know the physiological meaning of the presence of two leaf FNRs in maize.

Zmrprn 1, a nearly full-length cDNA encoding maize root FNR, has been reported (Ritchie et al., 1994), and we obtained a cDNA corresponding to this cDNA from our maize root cDNA library (Matsumura et al., 1997) by PCR as described in "Materials and Methods" (Fig. 2). We confirmed that the

nucleotide sequence of the amplified cDNA was identical with that of Zmrprn 1.

Preparation of Recombinant FNR Isoenzymes

We constructed a large-scale expression system of the FNR cDNAs encoding L-FNR I and R-FNR in *Escherichia coli* cells. As shown in Figure 2A, the codon usages near the translation start site were altered in such a way that the third nucleotides (G or C) were substituted synonymously with T or A. The *E. coli* cells transformed with the resulting plasmid,

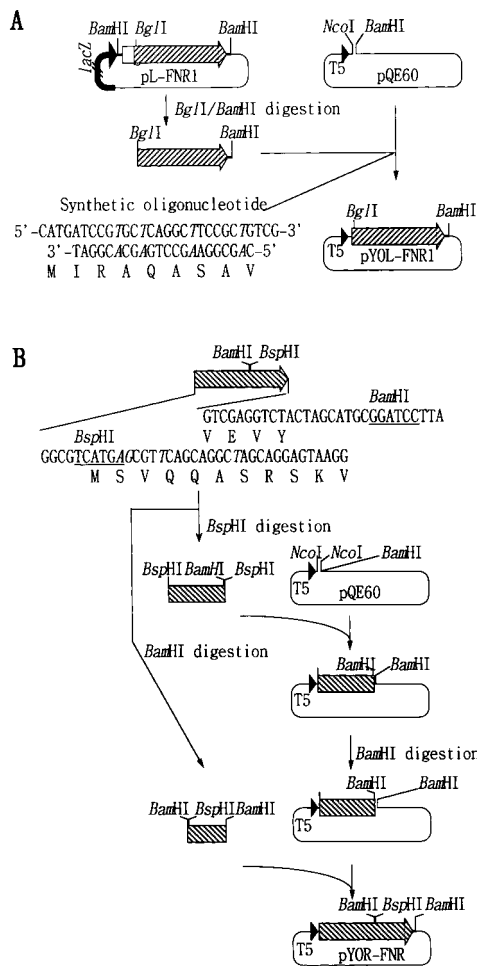


Figure 2. Construction of plasmids for expression of maize leaf and root FNR cDNAs in *Escherichia coli*. A, The leaf FNR expression plasmid, pYOL-FNR1, was created, inserting the two fragments into pQE60 vector under control of T5 promoter. One is the fragment from the ninth residue to the C terminus of the mature region of L-FNR I, which was excised from pL-FNR I with *Bgl*II and *Bam*HI. The other is a double strand of oligonucleotides encoding the initiation Met and the eight N-terminal amino acid residues. B, A cDNA encoding maize root FNR was amplified by PCR as described in "Materials and Methods," and the resulting fragment with *Bsp*HI and *Bam*HI sites at each end was inserted into pQE60 vector with a two-step ligation procedure, as shown in this figure, to create the root FNR expression plasmid, pYOR-FNR. Nucleotides altered from the original codons are denoted by italic letters.

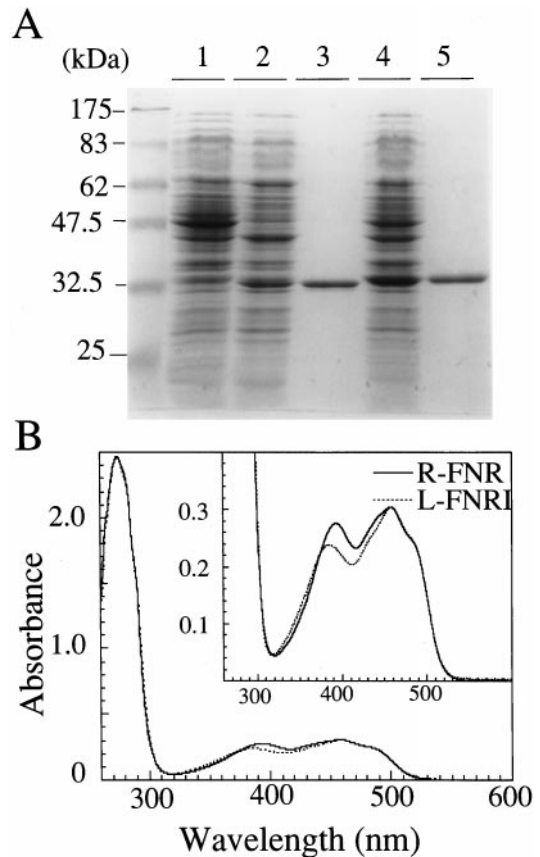


Figure 3. SDS-PAGE (A) and absorption spectra (B) of recombinant maize FNRs. Crude extracts of *E. coli* cells (equivalent to 0.1 A₅₅₀ units each) transformed with control vector (lane 1), pYOL-FNR1 (lane 2), and pYOR-FNR (lane 4), and purified enzymes (2 μg each) of L-FNR I (lane 3) and R-FNR (lane 5) were separated by SDS-PAGE with 10% (w/v) acrylamide and stained with Coomassie Brilliant Blue.

pYOL-FNR1, had a yellow-green color and overexpressed L-FNR I with a yield of about 10 mg/L bacterial culture. The changes in the codon usage, probably reducing the secondary structure within mRNA around the translation start site, seemed to be very effective for increasing translation efficiency (De Boer and Hui, 1990), because little accumulation of the FNR protein was observed in the bacterial cells transformed with the original cDNA without the codon changes. An expression plasmid for R-FNR, pYOR-FNR, was also constructed in a similar way to obtain a higher expression (Fig. 2B).

L-FNR I and R-FNR were purified from the recombinant bacterial cells (Fig. 3A). Both FNRs showed the absorption spectra typical of flavin-containing enzymes (Fig. 3B). Note the distinctive spectra derived from the FAD moiety; L-FNR I shows two peaks at 459 and 385 nm, whereas R-FNR shows those at 457 and 393 nm. Such subtle differences have also been reported for photosynthetic and non-photosynthetic FNRs from mung bean seedlings (Jin et al., 1994).

Organ Distribution of FNR Isoenzymes

Total extracts of the first leaves and roots from 5-d-old seedlings were separated by SDS-PAGE, followed by western blotting with affinity-purified antibodies against L-FNR I and R-FNR. The two antibodies showed little cross-reactivity in the recognition of each FNR. The polypeptide detected by the anti-R-FNR antibodies was distributed only in roots as a single band, whereas two major and one minor polypeptides, differing in their sizes, were detected only in leaves by the anti-L-FNR I antibodies (Fig. 4). Results obtained from the third leaves of 14-d-old seedlings were essentially the same (data not shown). Thus, we confirmed the organ-specific distribution of leaf and root FNRs. The origin of the multiple FNR polypeptides in leaves seems to be due to the presence of two leaf FNRs, L-FNR I and L-FNR II, and/or due to their N-terminal heterogeneities, as reported in other plants (Karplus et al., 1984), although study of these FNR species on a chemical basis is needed to draw a definitive conclusion.

Catalytic Activities of FNR Isoenzymes

Diaphorase activity of two FNR isoproteins was measured using 2,6-dichlorophenol indophenol (DCPIP) as an electron acceptor (Table I). R-FNR showed a k_{cat}/K_m value for NADPH higher than L-FNR I and this was attributed mainly to a large difference in their K_m values. At the moment, it remains unknown whether such a difference in K_m values under the range of sub-micromolar order is of physiological significance. No activity was measurable in an assay using NADH at concentrations up to 500 μM as an electron donor, although higher concentration of NADH might have shown activity.

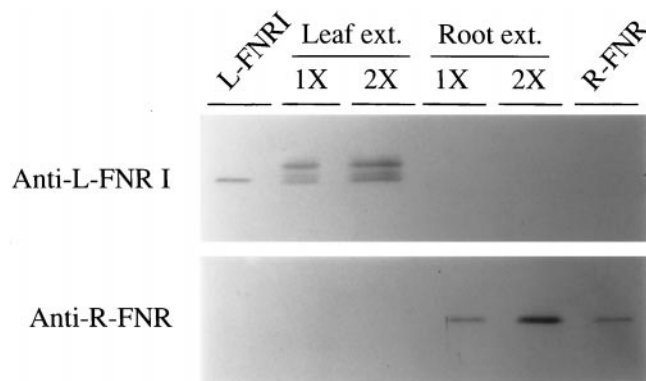


Figure 4. Western-blot analysis of FNR isoenzymes in leaf and root extracts of maize seedlings. The total extracts of leaves and roots were subjected to SDS-PAGE, followed by western blotting with rabbit antibodies against L-FNR I and R-FNR, which had been affinity purified with the original antigen. Lane 1, Purified L-FNR I (20 ng); lanes 2 and 3, leaf extracts (10 and 20 μg total protein, respectively); lanes 4 and 5, root extracts (5 and 10 μg total protein, respectively); lane 6, purified R-FNR (20 ng).

Table I. Kinetic parameters of L-FNR I and R-FNR in NADPH-dependent diaphorase activity with DCPIP

K_m and k_{cat} for NADPH were determined from a double-reciprocal plot. The values are means \pm SD of three independent determinations.

FNR Species	K_m μM	k_{cat} s^{-1}	k_{cat}/K_m $\mu\text{M}^{-1} \text{s}^{-1}$
L-FNR I	4.4 ± 0.1	44 ± 1	9.9 ± 0.1
R-FNR	0.39 ± 0.01	28 ± 1	71 ± 1

NADPH-dependent cytochrome c (cyt c) reductase activity was measured using maize Fd I and Fd III, two major isoproteins distributed in leaves and roots, respectively. R-FNR showed higher activity with Fd III than with Fd I at all Fd concentrations examined. The activity of L-FNR I was similar with both Fds. Kinetic parameters obtained from the measurements with all combinations of FNRs and Fds are listed in Table II. R-FNR had a much larger K_m value for Fd I than that for Fd III, whereas L-FNR I had similar K_m values for both Fds. The k_{cat} values of R-FNR were about 3-fold larger than those of L-FNR I. Thus, the catalytic efficiency of R-FNR with Fd III, which corresponded to the pairing in root plastids, was found to be the highest among all combinations.

Static Interaction of FNR Isoenzymes with Fd Isoproteins

We examined the protein-protein interaction between FNR and Fd isoproteins. As shown in Figure 5, the formation of complexes between R-FNR and Fd I or Fd III was analyzed by difference absorption spectroscopy as described in "Materials and Methods." Dissociation constant (K_d) values of R-FNR to Fd I and Fd III were determined to be 33 and 2.5 μM , respectively. This large affinity difference was in good agreement with the results obtained by the kinetical analysis described in the above section. The interaction of FNR and Fd was further examined using Fd I- or Fd III-immobilized Sepharose columns. As shown in Figure 6, R-FNR bound to both affinity columns, but the elution profiles obtained by developing them with a linear gradient of NaCl indicated that R-FNR interacted with Fd III more strongly than with Fd I. No such marked difference was seen when using L-FNR.

DISCUSSION

To our knowledge, this is the first report characterizing root and leaf FNRs in detail in terms of protein-protein interactions with Fd I and Fd III. Our results demonstrate that the two FNRs differ significantly in their catalytic ability to transfer electrons from NADPH to Fd as follows: (a) Root FNR has a K_m value for Fd III 10-fold smaller than that for Fd I, whereas the leaf enzyme shows no such discrimination (Table

Table II. Kinetic parameters of L-FNR I and R-FNR in NADPH-dependent *cyt c* reduction

These data were extracted from the Fd saturation curves. K_m and k_{cat} for Fds were determined from a double-reciprocal plot. The values are means \pm SD of three independent determinations.

FNR Species	K_m		k_{cat}		k_{cat}/K_m	
	Fd I	Fd III	Fd I	Fd III	Fd I	Fd III
	μM		s^{-1}		$\mu M^{-1} s^{-1}$	
L-FNR I	3.3 \pm 0.1	3.4 \pm 0.1	120 \pm 10	130 \pm 10	37 \pm 2	39 \pm 3
R-FNR	29 \pm 3	3.0 \pm 0.3	380 \pm 20	380 \pm 30	13 \pm 1	130 \pm 3

II), and (b) root FNR exhibits more rapid catalytic turnover than leaf FNR (Table II). In addition to these kinetic data, the differential affinity of root FNR for the two Fd isoproteins was also demonstrated by the electrostatic binding assays (Figs. 5 and 6). Several groups have assayed other plant FNRs using either a non-physiological compound such as ferricyanide and DCPIP or Fd I only as an electron transfer partner; they have reported that they found no remarkable differences between root and leaf FNRs (Morigasaki et al., 1990b; Green et al., 1991; Bowsher et al., 1993). We observed that maize root FNR showed a weak affinity to spinach leaf Fd as well as maize Fd I (data not

shown). Thus, the high efficiency of root FNR with root Fd may be the general phenomenon.

As noted in the introductory section, the supply of reductant for nitrogen assimilation correlates with the activity of the NADPH-generating OPPP in root plastids, and the reducing equivalent from OPPP needs to be transferred to Fd by a redox pathway consisting of NADPH, FNR, and Fd to become available for Fd-dependent enzymes such as nitrite reductase, Glu synthase, and sulfite reductase (for review, see Emes and Neuhaus, 1997). The ability of FNR and Fd to facilitate the transfer of reducing power

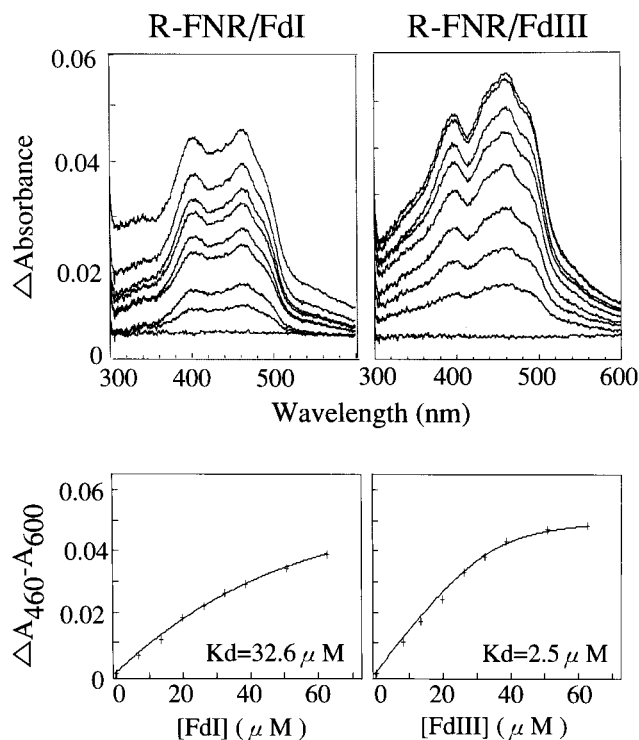


Figure 5. Spectrometric analysis of the complex of R-FNR with Fd I or Fd III. The complex formation was titrated with Fd I or Fd III. The amounts of Fd added to R-FNR ($34 \mu M$) were increased up to $68 \mu M$. Difference spectra (the mixed versus the sum of spectra of FNR and Fd) obtained by each addition of Fd are shown, and difference values ($A_{460} - A_{600}$) are plotted as the function of Fd concentrations. The best fitting curve obtained by a weighted least-squares error minimization procedure (Smith et al., 1992) is shown, giving K_d values of R-FNR for Fd I and Fd III at 32.6 and $2.5 \mu M$, respectively.

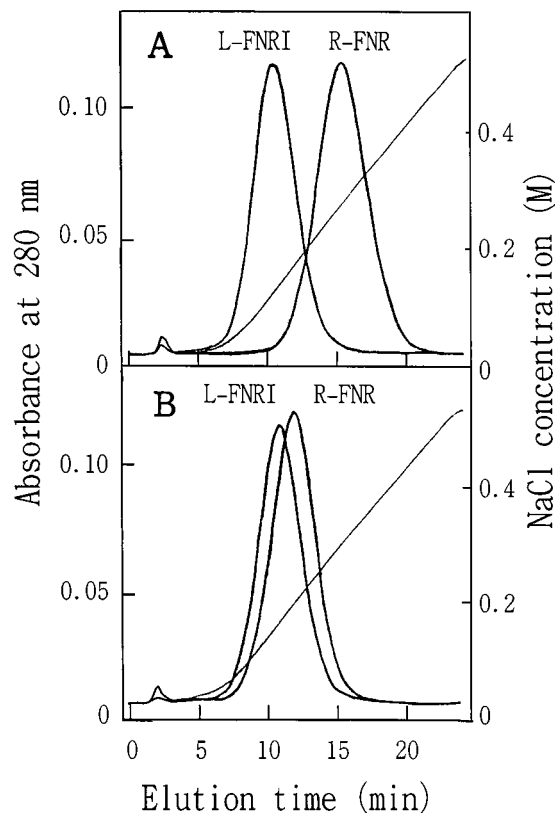


Figure 6. Affinity chromatography of L-FNR I and R-FNR on Fd III-immobilized (A) and Fd I-immobilized (B) Sepharose columns. L-FNR I and R-FNR were applied on each column and eluted with a linear NaCl gradient. From the Fd III affinity column, L-FNR I and R-FNR were eluted at 0.10 and $0.24 M$ NaCl, respectively, and from the Fd I affinity column, L-FNR I and R-FNR at 0.12 and $0.15 M$ NaCl, respectively.

for nitrite reduction was nicely demonstrated by the reconstitution of an NADPH-dependent nitrite-reducing system in vitro using FNR, Fd, and nitrite reductase purified from the unicellular alga *Chlamydomonas reinhardtii*. (Jin et al., 1998). We have reconstituted a similar electron transfer system for NADPH-dependent sulfite reduction using maize sulfite reductase, either root or leaf FNRs, and either Fd I or Fd III, and found that the pairing of root FNR and Fd III is the most efficient for supporting the sulfite reduction (Yonekura-Sakakibara et al., 2000). The redox potential of Fd III (−345 mV) is significantly higher than that of Fd I (−423 mV; Akashi et al., 1997), indicating that the electron transfer from NADPH to Fd III is thermodynamically more favorable than that to Fd I. From the kinetic and physicochemical viewpoints, the high catalytic efficiency and the strong static interaction of root FNR and Fd III are advantageous properties for converting the reducing power of NADPH into a form available for Fd-dependent enzymes. Thus, these combined data provide a molecular basis for understanding the physiological significance of the root isoproteins of FNR and Fd participating in the assimilations of nitrogen and sulfur and other Fd-linked reductive metabolism reactions such as lipid desaturation (Shanklin and Cahoon, 1998) in non-photosynthetic plastids.

FNR and Fd form a tight 1:1 complex stabilized by electrostatic forces through the positive charges of FNR and the negative charges of Fd (Knaff, 1996); this complex formation has been extensively studied using leaf isoproteins of FNR and Fd at the atomic structure level (Zanetti et al., 1988; Karplus et al., 1991; De Pascalis et al., 1993). Lys-85 and Lys-88 of spinach leaf FNR were suggested to be basic residues contributing to the complex formation by a cross-linking study (Zanetti et al., 1988). Recently, Lys-72 and Lys-75 in *Anabaena* sp. PCC 7119 FNR (equivalent to Lys-88 and Lys-91 in the spinach FNR) were identified as critical basic residues for the interaction and electron transfer to *Anabaena* Fd by site-directed mutagenesis (Martinez-Julvez et al., 1998; Hurley et al., 1999). These data suggest that the three basic residues conserved among leaf and cyanobacterial FNRs, Lys-85, Lys-88, and Lys-91 (for numbering for maize L-FNR I, see Fig. 1) are potential interaction sites for Fd I. Note that two of these three basic residues are not conserved in the primary structure of root FNRs; Ala-86 and Asn-89 of maize R-FNR are found at the positions corresponding to Lys-88 and Lys-91 of maize L-FNR I. We postulate that these amino acid changes in root FNRs are the major reasons for the inability to form the tight complex with Fd I. Our preliminary experiments show that substitution of Lys for Ala-86 and Asn-89 of maize R-FNR significantly increases its affinity with Fd I (Y. Onda and T. Hase, unpublished results).

Leaf FNR is able to interact with Fd III with an affinity comparable to that of Fd I. By site directed

mutagenesis of conserved acidic residues in both types of Fd, Asp-65 of Fd I (Matsumura et al., 1999) and Asp-66/Asp-67 of Fd III (Akashi et al., 1999) have been found to be the major electrostatic binding sites for leaf FNR. These data suggest that leaf FNR interacts with both types of Fd in a similar way. More interestingly, our data also indicate that root FNR and Fd III may have distinctive interaction modes leading to the formation of a tight complex. Evidence for this is: (a) root FNR has the capability to discriminate between Fd I and Fd III as described above, and (b) root FNR retains the affinity to D66N/D67N Fd III mutant (Y. Kimata and T. Hase, unpublished results), to which leaf FNR significantly decreases the affinity (Akashi et al., 1999). The three-dimensional structure of maize R-FNR has only recently been reported (Aliverti et al., 1999) and we are currently trying to determine the three-dimensional structure of the complex of maize R-FNR and Fd III to clarify this unique protein-protein interaction.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L. cv Golden Cross Bantam T51) seedlings were grown on vermiculite with Hoagland nutrients (Arnon and Hoagland, 1940) at 28°C during the day and 20°C at night with a 14-h daylength and a photosynthetic photon flux density of 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The first leaves and the primary roots from 5-d-old seedlings and the third fully expanded leaves from 14-d-old seedlings were harvested, frozen in liquid nitrogen, and stored at −80°C for subsequent protein analysis.

Screening of a cDNA Library, PCR Amplification, Subcloning, and Sequencing

A cDNA library, constructed in pUEX1 vector (Amersham, Buckinghamshire, UK) with poly(A⁺) RNA from maize leaves (Sakakibara et al., 1991), was screened by immunological detection methods using antibodies raised against spinach (*Spinacia oleracea*) leaf FNR. Further screening was carried out by a nucleic acid hybridization method using a cDNA from the first screening as a probe. Insert DNAs were excised from the recombinant plasmids by *Bam*HI digestion and subcloned into M13 mp19 or pUC19 for sequencing. A pair of primers, 5'-GGCGTCATGAGC GTTCAGCAGGCTAGCAGGAGTAAGG-3', and 5'-TAAG GATCCGCATGCTAGTAGACCTCGAC-3', was used for amplification of maize root FNR cDNA corresponding to Zmrprn 1 (Ritchie et al., 1994) using cDNAs synthesized with poly(A⁺) RNA from nitrate-induced roots (Sakakibara et al., 1995) as templates. PCR was carried out with a program of 25 cycles of 30 s at 98°C for denaturation and 45 s at 72°C for annealing and extension using KOD DNA Polymerase (TOYOBO, Osaka). The amplified fragment was directly cloned into pQE60 vector (Qiagen, Valencia, CA) as described below. DNA sequencing was carried out with a dideoxynucleotide-sequencing kit (Prism with

TaqFS, Applied Biosystems, Foster City, CA) and an automated DNA sequencer (model 370A, Applied Biosystems).

Construction of Expression Plasmids of FNR Isoforms

A double strand of oligonucleotides encoding the initiation Met and the eight N-terminal amino acids of L-FNR I was synthesized; it contained compatible sites for *Nco*I and *Bgl*II at each end (Fig. 2A). This synthetic oligonucleotide and the *Bgl*II/*Bam*HI fragment from pL-FNR1 containing a coding region from the ninth residue to the C terminus of L-FNR I were inserted into *Nco*I/*Bam*HI sites of pQE60 vector to construct a plasmid, pYOL-FNR1. The cDNA cloning of R-FNR had been designed to introduce new *Bsp*HI and *Bam*HI sites at each end. The same restriction sites were present within the coding region. The *Bsp*HI fragment corresponding to the N-terminal one-half of R-FNR was first inserted into *Nco*I site of pQE60. The resulting plasmid was digested with *Bam*HI to create new *Bam*HI site ends derived from the coding region and polylinker of pQE60, and then the *Bam*HI fragment containing the C-terminal one-half of R-FNR was inserted into the *Bam*HI ends to obtain pYOR-FNR (Fig. 2B).

Expression and Purification of Recombinant FNR Isoforms

Escherichia coli TG1 cells were transformed with pYOL-FNR1 or pYOR-FNR. Seed cultures were inoculated into 8 L of Luria-Bertani medium containing ampicillin (50 µg/mL). The cells were grown for 2 h at 37°C under vigorous aeration; isopropyl-β-D(-)-thiogalactopyranoside was then added to a final concentration of 1 mM. After further propagation overnight, the cells were harvested by centrifugation at 5,000g for 5 min and stored at -30°C. The frozen cells were suspended in 3 volumes of 50 mM Tris [tris(hydroxymethyl) aminomethane]-Cl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride. NaCl was added to the suspension at final concentrations of 200 and 50 mM for preparations of L-FNR I and R-FNR, respectively. The bacterial cells were disrupted by ultrasonic irradiation on ice and centrifuged at 10,000g for 10 min. The supernatant was applied to a column of DE52 (Whatman, Maidstone, UK). Neither FNR isoform bound to the resin under the concentrations of NaCl described above. The flow-through fraction was fractionated with (NH₄)₂SO₄ between 40% and 70% saturation. The resulting precipitate was dissolved, desalted with 50 mM Tris-Cl (pH 7.5), and loaded on a column of DEAE-Toyopearl (Tosoh, Tokyo) equilibrated with the same buffer. The column was eluted with a linear gradient of 0 to 300 mM NaCl. L-FNR I and R-FNR were further purified by affinity chromatography using Fd I- and Fd III-immobilized Sepharose, respectively. The FNR-loaded affinity column was washed with 50 mM Tris-Cl (pH 7.5) to separate unbound proteins and was developed with a linear gradient of 0 to 500 mM NaCl in the same buffer. Both

FNR isoforms were eluted as a single peak in different NaCl concentrations.

Recombinant Fd I and Fd III were prepared as described previously (Hase et al., 1991b; Matsumura et al., 1999). For preparation of Fd-affinity resin, 50 mg each of Fd I and Fd III were immobilized on about 35 mL of swollen cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech, Uppsala) according to the method recommended by the supplier.

Protein Extraction from Maize Leaves and Roots, and Western Blotting

About 1 g of the frozen leaves and roots of maize seedlings were ground with a mortar and pestle with 5% (w/w) Polyclar AT (Wako Pure Chemical Industries, Osaka) and a small amount of quartz sand in two volumes of 50 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 3,000g for 3 min at 4°C, and the resulting crude supernatant was denatured with 1% (w/v) SDS for 5 min at 100°C. The total extracts of leaf (equivalent to 10 and 20 µg of total protein) and root (equivalent to 5 and 10 µg total of protein) were separated by SDS-PAGE, electroblotted to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA), and decorated either with rabbit antibodies raised against L-FNR I or with those raised against R-FNR. The antigen-antibody complex was visualized by reaction with alkaline phosphatase conjugated with goat antibodies against rabbit IgG (Bio-Rad, Hercules, CA).

Enzyme Assays

Diaphorase activity with DCPIP as an electron acceptor and NADPH-cyt *c* reductase activity with Fd as an electron carrier were measured in 50 mM Tris-Cl (pH 7.5) and 1 mM MgCl₂ at 25°C. The former reaction was carried out in a 1-mL reaction mixture containing 550 µM DCPIP, 100 nM FNR, and various concentrations of NADPH in the presence of an NADPH-generating system, 3 mM sodium Glc-6-P, and 50 µg of Glc-6-P dehydrogenase. The reduction of DCPIP was monitored by A₆₀₀. The FNR-dependent NADPH-cyt *c* reduction assay was carried out essentially as described previously (Hase et al., 1991b). The reaction mixture contained, in a total volume of 0.6 mL, 100 mM NaCl, 200 µM cyt *c*, 40 nM FNR, and 50 µM NADPH in the presence of the NADPH-generating system. The reaction was initiated by the addition of Fd at final concentrations from 0.4 to 80 µM. The reduction of cyt *c* was monitored by the increase at A₅₅₀.

Analysis of Complex Formation of FNR and Fd

To determine the K_d of the FNR-Fd complexes, binding spectra (the mixed versus the sum of absorbance of FNR and Fd) were measured according to the method described previously (Batie and Kamin, 1981) using a spectrophotometer (model UV-2500 PC, Shimadzu, Kyoto). Binding spectra were measured by titrating Fd from 0 to 68 µM into

FNR with a fixed concentration of 34 μM in 50 mM Tris-Cl (pH 7.5). The K_d values were obtained by fitting the data for a 1:1 binding stoichiometry of Fd and FNR using a weighted least-squares error minimization procedure (Smith et al., 1992).

Small-Scale Affinity Chromatography

The Fd-immobilized resin (1 mL) was packed into a small column (5 \times 50 mm) and equilibrated with 50 mM Tris-Cl (pH 7.5) using the SMART system (Pharmacia Biotech). FNR isoproteins (100 μg each) were loaded on the column, and a linear gradient of NaCl from 0 to 500 mM in the same buffer was applied as eluent at a flow rate of 200 $\mu\text{L}/\text{min}$. Elution of FNR was monitored by A_{280} .

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