FAD assembly and thylakoid membrane binding of ferredoxin:NADP$^+$ oxidoreductase in chloroplasts

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Abstract We investigated the process of flavin adenine dinucleotide (FAD) incorporation into the ferredoxin (Fd):NADP$^+$ oxidoreductase (FNR) polypeptide during FNR biosynthesis, using pull-down assay with resin-immobilized Fd which bound strongly to FAD-assembled holo-FNR, but hardly to FAD-deficient apo-FNR. After FNR precursor was imported into isolated chloroplasts and processed to the mature size, the molecular form pulled down by Fd-resin increasingly appeared. The mature-sized FNR (mFNR) accumulated transiently in the stroma as the apo-form, and subsequently bound on the thylakoid membranes as the holo-form. Thus, FAD is incorporated into the mFNR inside chloroplasts, and this assembly process is followed by the thylakoid membrane localization of FNR.
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Key words: Ferredoxin:NADP$^+$ oxidoreductase; Ferredoxin; Flavin adenine dinucleotide assembly; Thylakoid membrane binding; Chloroplast import

1. Introduction

Ferredoxin (Fd):NADP$^+$ oxidoreductase (FNR) (EC 1.18.1.2), a flavoenzyme containing one non-covalently bound flavin adenine dinucleotide (FAD) moiety, acts as the last enzyme in the photosynthetic linear electron transfer chain and catalyzes photoreduction of NADP$^+$ using photosystem I-reduced Fd as an electron donor. FNR is localized peripherally at the stromal side of thylakoid membranes through association with an intrinsic membrane protein [1,2].

FNR is one of the nuclear-encoded chloroplast proteins and synthesized on cytosolic ribosomes as a larger precursor (pFNR) with an N-terminal transit peptide, which is cleaved off by a stromal protease [3] to generate a mature-sized molecule (mFNR) [4,5]. At some stage of this biosynthetic process, FNR polypeptide needs to assemble with FAD by a mechanism that is still not well understood. So far, it has been reported that pFNR forms a catalytically active struc-ture assembled with FAD when it is expressed in *Escherichia coli* cells [6] or synthesized in vitro in the presence of FAD [7]. The binding of FAD, however, causes a remarkable decrease in the efficiency of pFNR translocation across the envelope membranes in comparison to an unfolded pFNR lacking FAD [8], implying that FAD assembly occurs inside chloroplasts under physiological conditions.

The flavoprotein subunit of yeast succinate dehydrogenase contains an FAD moiety covalently bound via a histidine residue. Covalent attachment of FAD can be analyzed using an FAD-specific antibody because FAD is not released from the polypeptide during an immunodetection procedure [9]. As no such simple and efficient method is applicable to the analysis of FAD assembly into FNR, we developed a method which selectively detected holo-FNR using Fd-immobilized resin. Here we examined whether newly imported FNR was converted to an Fd-interactive form inside chloroplasts.

2. Materials and methods

2.1. Preparation of apo- and holo-FNRs

Recombinant maize (*Zea mays* L.) leaf FNR I was prepared as described previously [10]. FNR (6 mg) was denatured in 50 mM Tris [tris(hydroxymethyl) aminomethane]–HCl, pH 7.5, at 95°C for 10 min. The resulting precipitant was dissolved in 8 M urea, and then refolded by dilution in 50 volumes of 50 mM Tris–HCl, pH 7.5, 0.1% (v/v) 2-mercaptoethanol at 4°C, in the presence or absence of 1 mM FAD, for holo-FNR or apo-FNR, respectively. After incubation at 4°C for 30 min, the resulting solution was extensively dialyzed against dilution buffer at 4°C for 2 days.

2.2. Plant materials and intact chloroplast isolation

Fresh spinach (*Spinacia oleracea*) leaves were purchased and intact chloroplasts were isolated by Percoll gradient centrifugation [11]. The isolated chloroplasts were suspended in 50 mM HEPES–KOH, pH 7.7, 330 mM sorbitol, at a final concentration of 1 mg chlorophyll ml^{-1}. The concentration of chlorophyll was measured by the method of Arnon [12].

2.3. In vitro chloroplast import

The cDNA of maize leaf pFNR I [10] was inserted into pGEM4Z vector (Promega). The transcripts were synthesized from pFNR I/pGEM4Z using SP6 RNA polymerase (Promega), and translated using wheat germ extracts (Promega) in the presence of [^{3}H]leucine (Amersham Pharmacia Biotech). The supernatant derived from the pFNR translation mixture was incubated with isolated chloroplasts at a chlorophyll concentration of 0.5 mg ml^{-1} in HS buffer at 27°C under continuous light. HS buffer contained 10 mM dithiothreitol, 10 mM MgCl$_2$, 2.5 mM leucine, 50 mM HEPES–KOH, pH 7.7, 330 mM sorbitol. To remove pFNR polypeptides not sequestered by chloroplasts, the import mixture was treated with trypsin (30 μg ml^{-1}) on ice, followed by the addition of trypsin inhibitor (120 μg ml^{-1}). For the chase experiment, the trypsinized chloroplasts were subsequently incubated in 50 mM HEPES–KOH, pH 7.5, 330 mM sorbitol under

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Abbreviations: Fd, ferredoxin; FNR, ferredoxin:NADP$^+$ oxidoreductase; pFNR, precursor FNR; mFNR, mature-sized FNR

light at 27°C, and aliquots were taken up at the indicated times during the chase reaction.

For subchloroplast fractionation the intact chloroplasts were ruptured in 50 mM Tris–HCl, pH 7.5, and separated into supernatants and pellets by centrifuging at 15 000 rpm for 5 min at 4°C. Thylakoid membrane-bound proteins were solubilized in 50 mM Tris–HCl, pH 7.5, containing 0.1% (v/v) Triton X-100, and the supernatant was collected by centrifugation at 15 000 rpm for 10 min. To obtain total chloroplast lysates, chloroplasts were directly suspended in 50 mM Tris–HCl, pH 7.5, 0.1% (v/v) Triton X-100.

2.4. Pull-down assay of FNR with Fd-resin and Fd affinity chromatography

Recombinant maize Fd I [13] or D65N/D66N Fd I [14] was prepared and immobilized onto CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech), as described previously [10]. After import reaction, total chloroplast lysates (300 μl at a chlorophyll concentration of 0.5 mg ml^{-1}) were incubated with Fd-immobilized resin (100 μl) at 4°C for 15 min, followed by a wash step in 50 mM Tris–HCl, pH 7.5, 10 mM NaCl. The proteins bound to Fd-resin were eluted with 300 mM NaCl in 50 mM Tris–HCl, pH 7.5. Small-scale Fd affinity chromatography was performed with Fd I-immobilized resin (200 μl) as described previously [10].

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting and fluorography

SDS–PAGE was carried out, followed by Western blotting with rabbit antibodies raised against spinach leaf FNR, as described previously [10]. For fluorography, SDS–PAGE gel was fixed in EN^{3}HANCE (DuPont-New England Nuclear) and exposed to X-ray film (Kodak) at −80°C for an appropriate period. All bands were quantified using BAS5000 Bio-Imaging analyzer (Fuji).

3. Results

3.1. Binding of holo- and apo-FNRs to Fd-immobilized resin

The reconstituted holo-FNR showed the absorption spectrum and the enzymatic activity identical to those of native FNR, while the reconstituted apo-FNR, obtained in a soluble form, showed neither an absorption spectrum derived from an FAD moiety nor any enzymatic activity (data not shown). A direct addition of FAD to this apo-FNR did not completely restore the enzymatic activity (data not shown).

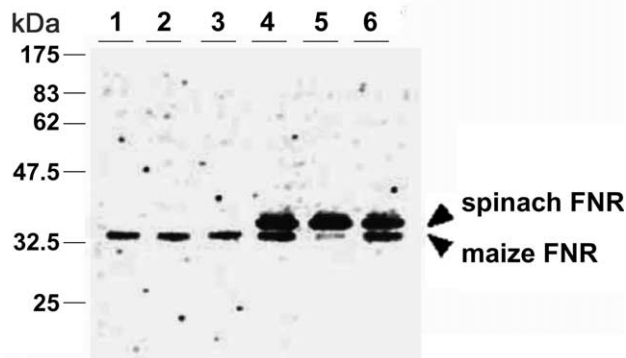Using Fd-immobilized resin, Fd affinity assay showed that

Fig. 1. Pull-down assay of holo- and apo-FNRs with Fd-immobilized resin. Authentic maize FNR (lanes 1 and 4), reconstituted apo-FNR (lanes 2 and 5), and holo-FNR (lanes 3 and 6) (120 ng each) were separately incubated with Fd-resin together with spinach chloroplast lysates (equivalent to 75 μg chlorophyll). The original FNRs (25 ng each) (lanes 1–3) and the fractions bound to Fd-resin (lanes 4–6) were subjected to SDS–PAGE, followed by Western blot analysis with anti-FNR antibody. The upper and lower arrowheads indicate the positions of spinach and maize FNRs, respectively. The positions of molecular mass markers (in kDa) are indicated on the left.
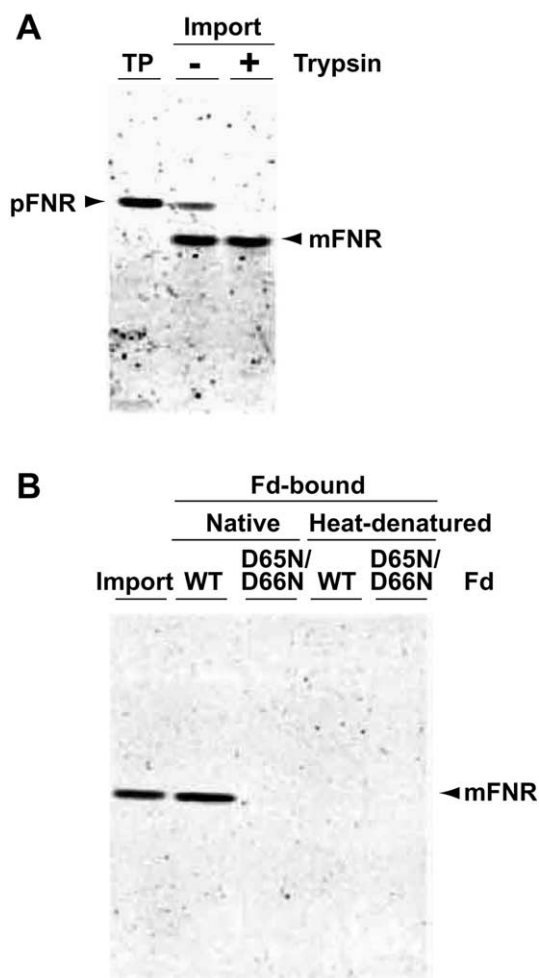

Fig. 2. Chloroplast import of pFNR and Fd affinity assay of imported FNR. A: After pFNR was incubated with isolated chloroplasts, an aliquot of the chloroplast suspension was subsequently treated with trypsin. In vitro synthesized pFNR (lane TP: 10% of the total input for the import reaction), and the lysates of non-trypsinized (−Trypsin) and trypsinized (+Trypsin) chloroplasts were subjected to SDS–PAGE, followed by fluorography. B: The chloroplast lysates (lane Import) corresponding to lane +Trypsin of A were subjected to Fd affinity assay using resin immobilized with native or heat-denatured Fds of wild type or with those of D65N/D66N mutant (lanes Fd-bound). The Fd-resin-bound fractions were analyzed as in A.

the reconstituted holo-FNR was recovered in the Fd-bound fraction in a comparable amount to authentic FNR, while the apo-FNR was recovered at a negligible level (Fig. 1). In all the cases, spinach chloroplast-derived endogenous FNR with a slower electrophoretic migration was recovered together in similar amounts. A Y95A mutant FNR, which is deficient in FAD binding ability but still retains the native-like folded structure in the NADP$^+$ binding domain [15], hardly showed affinity for Fd (Onda and Hase, unpublished data). Thus, the pull-down assay was applicable to specific detection of holo-FNR. However, recovery of holo-FNR in this pull-down assay stayed at about 30–40%. For the complete recovery, the amount of the Fd-resin used for the assay was needed to increase up to orders of ml. As such scale of assay was not suitable for chloroplast import assay, all the pull-down assays shown below were carried out under a semiquantitative condition.

3.2. Fd binding ability of imported FNR

This Fd affinity assay was applied to analysis of FAD incorporation into the FNR polypeptide during chloroplast import. When maize leaf pFNR I was imported into isolated chloroplasts, pFNR was processed to an mFNR with a molecular mass of approximately 35 kDa, which was not accessible to trypsin externally added (Fig. 2A), being consistent with the previous report [4]. A part of this mFNR (about 30% of the input) was recovered in the Fd-resin binding fraction (Fig. 2B). The validity of the pull-down assay was confirmed by the absence of significant binding when heat-denatured Fd-resin or resin immobilized with a mutant Fd (D65N/D66N) was used. The aspartic acids of Fd at positions 65 and 66 are responsible for its electrostatic interaction with FNR [16] and the affinity of D65N/D66N Fd mutant toward FNR is much lower than that of wild-type Fd [14]. These data suggested that the newly imported and processed mFNR was converted to holo-FNR.

We monitored the kinetics of this holo-FNR formation. After pFNR was imported into chloroplasts for an initial 5-min period, import was stopped by digesting pFNR outside chloroplasts with trypsin prior to further incubation. Fd affinity assays showed that holo-FNR increased time dependently during the chase incubation, whereas the total amount of mFNR was slightly degraded (Fig. 3). This result clearly demonstrated that after pFNR was processed to the mFNR,
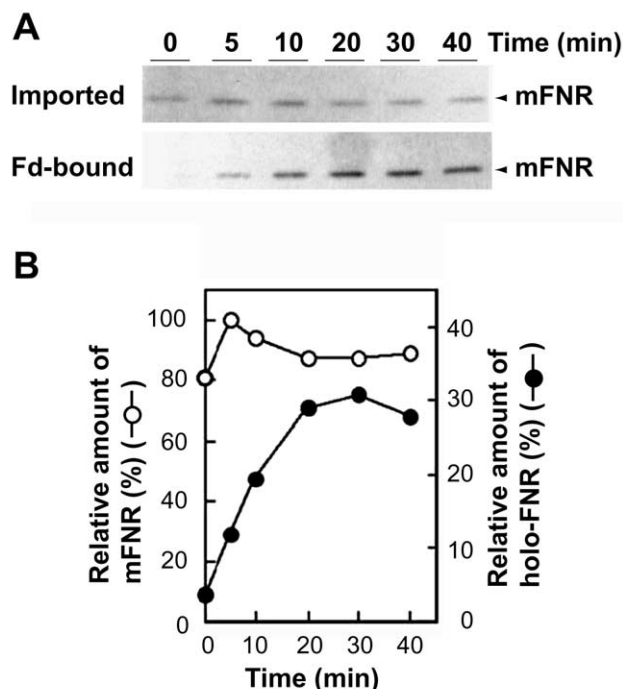

Fig. 3. Time course of holo-FNR formation after completion of pFNR translocation across chloroplast envelopes. A: After pFNR was preincubated with chloroplasts for 5 min, the trypsinized chloroplasts were further incubated and aliquots were taken up at the indicated times. The chloroplast lysates (upper panel) and corresponding fractions bound to the Fd-resin (lower panel) were analyzed by fluorography. 12.5% of each sample in the lower panel was subjected to the electrophoresis shown in the upper panel. B: mFNR bands in the upper (open circles) and lower panels (closed circles) of A were quantified, and the values were converted to a percentage of the highest mFNR level.
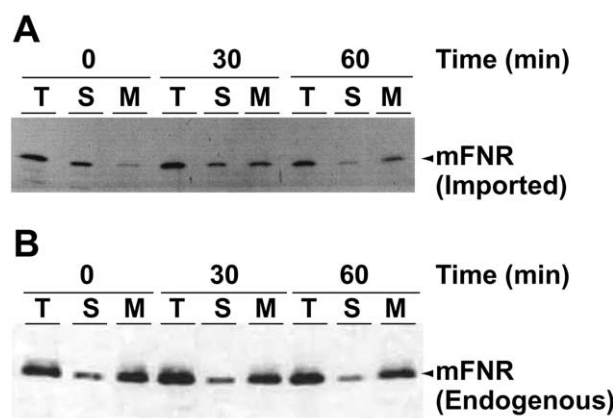

Fig. 4. Subchloroplast distribution of imported FNR. After pFNR was preincubated with intact chloroplasts for an initial 10-min period, the trypsinized chloroplasts were further incubated for 0, 30 and 60 min. The chloroplast lysates (T) were fractionated into the stromal (S) and the thylakoid membrane-bound (M) samples. Each fraction was analyzed by fluorography (A) and immunoblotting with anti-FNR antibody (B), to detect the newly imported and the chloroplast-derived endogenous FNRs, respectively.

the resulting mFNR was gradually converted to the holo-form inside chloroplasts.

3.3. Subchloroplast localization of imported FNR

We examined the subchloroplast distribution of the newly imported FNR during the chase experiment described above. The imported mFNR was initially found in the stromal fraction and subsequently moved to the thylakoid membranes (Fig. 4A). This translocation process of mFNR was significantly retarded when the chloroplasts were placed on ice during the chase incubation (Fig. 5A). The endogenous FNR contained in chloroplasts was recovered mainly in the thylakoid membranes in both experiments (Figs. 4B and 5B), indicating subchloroplast fractionation was effective. The membrane-associated form of imported mFNR was solubilized with increasing concentrations of Triton X-100 in a similar manner to the endogenous FNR (data not shown).
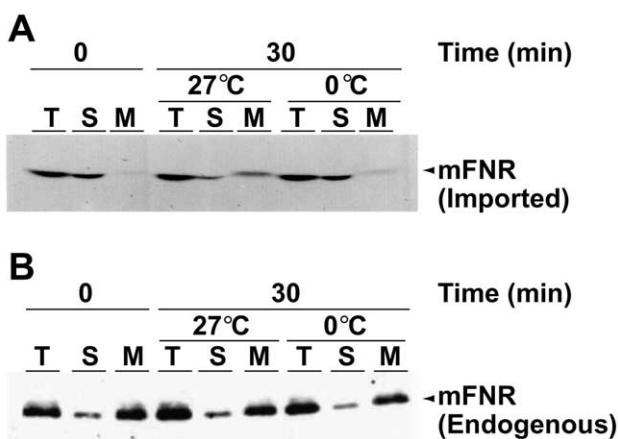

Fig. 5. Effect of temperature on subchloroplast distribution of imported FNR. After pFNR was preincubated with intact chloroplasts for 10 min at 27°C, the trypsinized chloroplasts were further incubated for 30 min at 27 or 0°C. Total chloroplast lysates (T), and their stromal (S) and thylakoid membrane-bound (M) samples were analyzed by fluorography (A) and immunoblotting (B) as in Fig. 4.
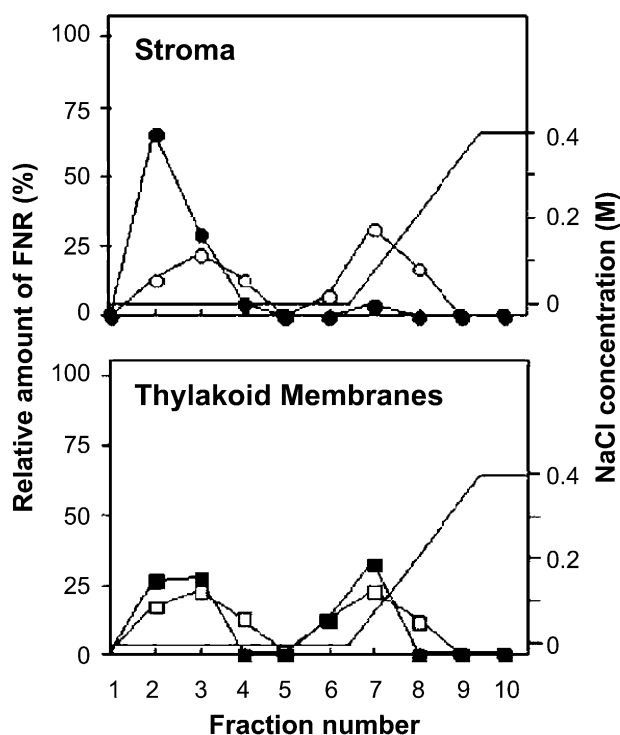
Fig. 6. Fd affinity chromatography of stroma and thylakoid membrane fractions of FNR-imported chloroplasts. Stroma (upper panel) and solubilized thylakoid membrane (lower panel) samples, corresponding to lanes S and M at 30 min in Fig. 4, respectively, were analyzed by Fd affinity chromatography with a linear NaCl gradient from 0 to 0.4 M in 50 mM Tris–HCl, pH 7.5. Each fraction was analyzed by fluorography as in Fig. 4A and immunoblotting as in Fig. 4B, to quantify the newly imported (closed symbols) and the endogenous (open symbols) FNRs, respectively. The relative levels are presented by taking the sum of the signals as 100%.

3.4. Differential Fd binding ability between FNRs present in the stroma and the thylakoid membranes during chloroplast import

Fd affinity chromatography was performed in order to analyze whether the two molecular forms of stromal and thylakoid membrane-associated imported FNRs were the holo- or apo-forms. The elution profiles of labeled FNR showed a sharp contrast between stromal and thylakoidal fractions (Fig. 6): the labeled FNR from the thylakoid membranes was absorbed on the Fd-resin and eluted with a profile similar to endogenous FNR, while most of the labeled FNR in the stroma was not retained in the column, indicating that the thylakoidal FNR was mainly of the holo-form while the stromal one was of the apo-form. A portion of endogenous FNR from the stroma bound to Fd-resin with a profile similar to that of the thylakoid membranes, implying the presence of FNR isoprotein in spinach leaves which was distributed, in contrast to maize leaf FNR I, in the stroma, as reported previously [2].

4. Discussion

Using Fd-resin with discriminative binding to holo- and apo-FNRs, the present study provides the first evidence that FAD incorporation occurs after pFNR is processed to mFNR in the stroma, and that FNR associates with the thylakoid membranes shortly after being correctly folded with FAD.

The Fd affinity assay provides a simple but effective tool to monitor the process of FNR assembly with FAD during chloroplast import. 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 [14,17,18]. The crystal structure of the Fd–FNR complex reveals that five pairs of charged residues between FNR and Fd (Lys304, Glu154, Lys33, Lys91 and Lys88 in FNR, and Glu29, Arg40, Asp61, Asp65 and Asp66 in Fd, respectively) form intermolecular salt bridges, establishing a topology for efficient electron transfer from the [2Fe–2S] cluster to FAD [16]. The imported FNR hardly interacted with a D65N/D66N Fd mutant when this was used for Fd affinity assay (Fig. 2B), being consistent with these crystallographic data.

pFNR is shown to form a folded conformation with an assembled FAD [6,7], and bind to chloroplasts as a folded molecule [19]. We have also observed that pFNR synthesized in wheat germ extracts can interact with Fd-resin (data not shown). Unfolding of chloroplast-targeting precursors is widely considered to be necessary for membrane translocation, and there occurs a strong unfolding activity, such as the intermembrane space Hsp70, on the outer envelope membranes of chloroplasts [20–22]. It is very likely that even if pFNR might be able to assemble with FAD in the cytosol, such a molecule would readily unfold during translocation across the envelope membranes, resulting in the release of FAD from the pFNR polypeptide. The present study shows that holo-FNR increases time dependently after pFNR is processed to mFNR (Fig. 3), and subsequently localized from the stroma to the thylakoid membranes (Figs. 4 and 6), demonstrating that FAD assembly is an event following the transit peptide cleavage from pFNR inside chloroplasts.

mFNR has been shown to interact transiently with the stromal homolog of Hsp70 and subsequently with that of Cpn60 in chloroplasts [23]. Acyl-coenzyme A (CoA) dehydrogenase, with FAD bound non-covalently, is folded Hsp60 dependently in the mitochondrial matrix, requiring FAD to be released from the Hsp60 and assembled as a homotetrameric form [24]. When FAD is gently removed, the apo-mFNR forms an intermediate conformational state with partially folded secondary and tertiary structure, being similar to the Y95A mFNR mutant which lacks hydrogen bonds and π–π stacking to interact with the FAD isoalloxazine ring through [15]. The apo-mFNR transiently accumulates in the stroma before the holo-mFNR subsequently transfers to the thylakoid membranes (Figs. 4 and 6) time and temperature dependently (Fig. 5), implying some protein factor involvement in the FAD assembly process. These findings suggest that FAD assembly plays an important role in folding mFNR into a proper native conformation and then localizing mFNR on the surface of the thylakoid membranes. The thylakoid membrane binding site of FNR remains a subject for further study.

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