Expression, Assembly, and Processing of an Active Plant Ferredoxin-NADP⁺ Oxidoreductase and Its Precursor Protein in *Escherichia coli*\

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The flavoprotein ferredoxin-NADP⁺ reductase (FNR) catalyzes the final step of the photosynthetic electron transport chain, i.e., the reduction of NADP⁺ by ferredoxin. A cloned FNR cDNA from a pea library (Newman, B., and Gray, J. (1988) *Plant Mol. Biol.* 10, 511–520) was used to construct plasmids which express the apoenzyme in *Escherichia coli*. Two recombinant vectors were prepared, one containing the sequence corresponding to the mature enzyme and another including, in addition, the sequence of the transit peptide that directs FNR to the chloroplast. These proteins were expressed as fusion products to the NH₃-terminal portion of ß-galactosidase. In both cases, a 35-kDa immunoreactive polypeptide was the major product, suggesting that the proteins were processed in *vivo*. NH₂-terminal sequence determination of the purified recombinant proteins indicate cleavage at positions -1/-2 with respect to the normal processing site in chloroplasts. The processed enzymes showed enzymatic activities and spectral properties that were similar or identical to those of native plant FNR. When a La protease-deficient *E. coli* strain was used as a host, the expressed FNR precursor was found to be poorly processed, associated to bacterial pellets, and showed no detectable FNR activity. The overall results indicate that the sequence of the native enzyme conformation and assembly of the prosthetic group takes place in the bacterial host, generating an enzyme that is, as far as studied, indistinguishable from plant FNR.

Ferredoxin-NADP⁺ oxidoreductase (FNR)\textsuperscript{1} (EC 1.18.1.2) is a FAD-containing protein present in chloroplasts and cyanobacteria (1). It catalyzes the last step of the photosynthetic electron transport chain, the electron transfer between reduced ferredoxin and NADP⁺, generating the reducing power that will ultimately lead to carbon fixation through the Benson-Calvin cycle (1). The mature enzyme is a monomer of 35 kDa, firmly attached to the stromal surface of the thylakoid membrane (2, 3). It has been purified from higher plants, eucaryotic algae, and cyanobacteria, and extensively studied with respect to both structural and kinetic properties (1, 4–6).

The reductase is synthesized in the cytoplasm of eucaryotic organisms as a precursor whose amino-terminal transit peptide is proteolytically removed upon chloroplast uptake (7). The compartment in which FAD is incorporated into the apoenzyme has not been elucidated yet (1), although newly synthesized precursor can incorporate the prosthetic group in *vivo* to produce an active preholoenzyme (8). Even when several CDNA clones coding for this enzyme have been isolated and sequenced (9–11), only limited information concerning chloroplast import, posttranslational processing, and *in vivo* assembly is at present available.

Several authors have studied the involvement of amino acid side chains in enzyme catalysis and interaction with NADP⁺, ferredoxin, and FAD, using group-specific modifiers and analogs (12–18). These methods have some limitations that could be overcome by site-directed mutagenesis. Thus, expression of FNR in bacteria is highly desirable and, in this sense, Aliverti et al. (19) reported the expression of an active, mature FNR in *Escherichia coli*. They constructed a modified gene that contained, besides the full sequence for the spinach flavoprotein, an additional methionine codon at the 5' end to provide for translation initiation. However, a truncated protein was the major expression product, resulting from alternative initiation at the GTG codon for Val-29 (19). It was necessary to mutate the GTG triplet to obtain the full-length protein (19).

In this work, we report the expression of pea FNR in *E. coli*. We have constructed expression plasmids in which the mature enzyme or its precursor were produced as fusion proteins to the NH₂-terminal region of ß-galactosidase. The recombinant proteins incorporated FAD in *vivo* and were fully active. Remarkably, a portion of their NH₂-termini was removed after synthesis, resulting in peptides that were one to two amino acids longer than mature pea FNR. This system should be useful to study the mechanism(s) of FAD assembly and constitutes a tool to elucidate the relationship between FNR structure and function.

**MATERIALS AND METHODS**

Plasmid Construction—An EcoRI cDNA fragment (1.4 kilobase pairs) containing the coding sequence for pea FNR precursor and flanking regions (kindly provided by Dr. J. C. Gray, University of Cambridge, United Kingdom (9)) was inserted into the EcoRI site of pUC19 (20). A plasmid which had the same orientation between the lacZ' gene of the vector and the FNR gene was selected by restriction analysis and named pCV101 (Fig. 1A). A 1,303-bp fragment lacking the untranslated 5' region of FNR cDNA, obtained by digestion of pCV101 with PstI and EcoRI, was ligated to compatible sites of pUC9...
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(20) and transformed into E. coli cells. The recombinant plasmid (pCV102, Fig. 1A) contains the first six nucleotide codons of β-galactosidase fused in frame to the third codon of pre-FNR apoenzyme (Fig. 1B).

For the preparation of pCV106, pCV101 was digested with SacI, self-ligated, and transformed into E. coli cells. A plasmid containing the desired SacI deletion (pCV104) was further digested with EcoRI and BamHI. The resulting 1.160-bp fragment was isolated, cloned into compatible sites of pUC9, and transformed into E. coli cells. Plasmid pCV105 contains the first 16 nucleotide codons of β-galactosidase fused in frame ~2 codons from the mature FNR apoenzyme (Fig. 1B).

DNA treatments with restriction or modification enzymes, agarose gel electrophoresis, transformation and growth of bacterial cells, selection of recombinants, and plasmid preparations were carried out following conventional procedures (21). E. coli JM109 (22) and Y1069 (23) strains were used throughout the experiments. Y1089 cells were cured from plasmid pMC9 by growing in Luria-Bertani (LB, Ref. 21) liquid medium supplemented with 0.3 mg/ml ethidium bromide (24).

Detection of Expressed Recombinant Proteins—E. coli cells bearing plasmids pCV102 or pCV105 were grown overnight with gentle shaking at 37 °C in 5 ml of LB medium containing 0.1 mg/ml ampicillin. A 100 µl aliquot of this culture was added to 10 ml of fresh medium, and cells were grown in the same conditions until the suspension reached an absorbance of 0.2 at 550 nm. IPTG (0.5 mM final concentration) was added and incubation continued for an additional 4 h period. All subsequent steps were carried out at 4 °C. Cells were harvested by centrifugation (5,000 × g for 5 min), washed once with 5 ml of 20 mM Tris-HCl, pH 7.6, 0.1 M NaCl, resuspended in 1 ml of 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (buffer A), and disrupted by sonic oscillation (30 s at maximal intensity in a MSE-PG-830 sonicator). Lysates were clarified by centrifugation (12,000 × g for 15 min) and the pellets washed once in buffer A. The two supernatants were combined and the washed pellet resuspended in the same buffer.

Lysates and pellets were analyzed by SDS-PAGE followed by immunoblotting. Samples (50-90 µg of protein) were denatured by incubation in 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol, 2% SDS for 5 min in a boiling water bath, and separated by SDS-PAGE using 12 or 15% gels (25). After electrophoresis, the samples were transferred to a nitrocellulose membrane (0.2-µm pore, Schleicher & Schuell), and immunoblot analysis was carried out (26) using rabbit anti sera raised against spinach FNR (27).

Quantitation of Recombinant FNR—A slot-blot apparatus and Z-probe membranes (Bio-Rad) were used to quantify the amount of recombinant FNR produced in E. coli. As a standard we used purified FNR from pea (9), mixed with a soluble E. coli extract from nontransformed cells in order to apply to each slot a constant amount (15 µg) of FNR per slot.

RESULTS

Construction of pCV102 and pCV105—The strategy followed to construct vectors for the expression of mature FNR and its precursor protein is summarized in Fig. 1A. The regions fused to the 5′ end of the lacZ′ gene are indicated in

FIG. 1. Construction of pea FNR expression plasmids pCV102 and pCV105. The general strategy followed is outlined in panel A. pCV101 is a pUC19 plasmid with an EcoRI 1.4-kilobase pair insert that contains cDNA sequences of pea FNR (9). The hatched areas within recombinant plasmids represent DNA coding for mature pea FNR; the solid areas, sequences coding for the transit peptide; the stippled areas, untranslated flanking regions of the FNR cDNA; and the open areas, plasmid DNA. The boxed regions in pCV102 and pCV105 are magnified in panel B to show the nucleotide and amino acid sequences at the junction between β-galactosidase and FNR. True FNR sequences are underlined, with the numerals below indicating their position relative to the precursor (pCV102) or mature (pCV105) FNR NH2 termini. Numerals above the amino acids indicate their position with respect to the β-galactosidase initial methionine.
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Expression of the Recombinant Proteins—The presence of FNR polypeptides in transformed bacteria was studied by PAGE and immunoblot analysis. The results are shown in Fig. 2. In both cases, a major immunoreactive peptide with an apparent molecular mass of 35 kDa was detected in the soluble fraction of disrupted cells (Fig. 2B, lanes 6 and 8). The appearance of this polypeptide was strictly dependent on the addition of IPTG (B, lanes 5–8), indicating that the transcribed genes were under the control of the lac promoter. pUC9-transformed cells showed no immunoreactive peptide (B, lanes 3 and 4).

The apparent molecular masses of the major bands were similar to that of pea FNR (Fig. 2B, lane 1) and different from the ones expected for the calculated fused proteins. These results suggest that the recombinant proteins were processed in the bacterial cells. Faint reactive bands of 33 kDa in lanes 6 and 8 of Fig. 2B resemble the alternative translation initiation products observed by Allerti et al. (19) for the spinach gene. They were estimated to represent 1–5% in amount of the 35-kDa FNR. In addition to these two species, cells transformed with pCV102 showed a 40-kDa polypeptide which accounted for 10–20% of the total immunoreactive product (Fig. 2B, lane 6). This molecular mass corresponds to the one expected for fused preFNR (9).

The amount of recombinant enzyme produced in JM109 E. coli cells, measured by slot-blot and immunoreaction, ranged between 2–4% (pCV102) and 3–7% (pCV105) of the total soluble protein in different experiments (data not shown). Following disruption of induced JM109 cells and fractionation of their contents, more than 90% of the immunoreactive material present in the cells was recovered in the soluble fraction (see below). The remainder was associated with bacterial pellets and showed no detectable FNR-dependent activities (not shown).

Purification of Recombinant FNR—Besides its physiological role in NADP+ photoreduction, FNR is able to catalyze in vitro a reverse electron flow from NADPH to suitable electron acceptors like ferricyanide (diaphorase activity) or oxidized ferredoxin (cytochrome c reductase). Lysates from transformed E. coli cells showed significant diaphorase activity, amounting to about 2–3 (pCV102) and 4–7 (pCV105) units/mg of total soluble protein, more than 30 times higher than cells containing only pUC9. These values were reduced to constitutive levels when the lysates were preincubated with anti-FNR antibodies (not shown). The overall results indicate that the recombinant enzymes were able to bind the prosthetic group and to acquire an active conformation in E. coli.

We used a protocol described for the purification of pea FNR (9) to isolate the active fractions from transformed bacteria. The elution profiles of the recombinant enzymes in DEAE-cellulose and Blue Sepharose chromatographies were very similar to those of pea FNR, irrespective of which plasmid, pCV102 or pCV105, directed the expression (r t shown). Yields for transgenic reducates ranged between 15–30% of the activity found in crude lysates. These enzymes were more than 90% pure, as judged by SDS-PAGE, and showed a major band corresponding to an apparent molecular mass of 35 kDa (not shown). The main contaminant was a 33-kDa protein which accounted for about 5% of the protein detected in the gel. This band reacted with an FNR antiserum. The 40-kDa immunoreactive band observed in crude extracts of pCV102-transformed cells (Fig. 2B, lane 6) was lost in the course of the purification. Diaphorase specific activities were almost identical for the purified enzymes (see Table I).

The recombinant flavoproteins were further purified for sequencing studies by preparative electrophoresis. The NH2-terminal sequences of the 35 kDa species were determined to investigate the processing site in E. coli. The resulting sequences are shown in Fig. 3, indicating NH2 termini at positions −2 (pCV102) and −1 (pCV105) with respect to the amino terminus of the mature pea enzyme.

In an attempt to characterize the processing that occurs in both recombinant enzymes (Figs. 2 and 3), we expressed these proteins in an E. coli lon− strain, which lacks the ATP-dependent La protease. We observed that when expression was directed by pCV105, the patterns obtained with either the JM109 or the lon− strains were very similar (Fig. 4, lanes 1 and 2), although the proportion of reductase in the insoluble fraction increased in the protease-deficient cells (Fig. 4, lane 2). Since the difference in molecular mass (≈ 2 kDa) between the unprocessed fusion protein and the 35-kDa processed

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Expression of FNR and its precursor protein in JM109 E. coli cells. Cell lysates (corresponding to 50 μg of total soluble protein) without (lanes 3, 5, and 7) or with 0.5 mM IPTG (lanes 4, 6, and 8) induction for 4 h were analyzed by 15% SDS-PAGE and immunoblots. A, Coomassie Brilliant Blue staining; B, immunoblots. Lane 1, purified FNR from pea; lane 2, molecular weight standards (Sigma); lanes 3–8, lysates from E. coli cells transformed with pUC9 (lanes 3 and 4), pCV102 (lanes 5 and 6), and pCV105 (lanes 7 and 8). Experimental conditions are described under "Materials and Methods."
FNRI should have been resolved in our gels, the closely migrating bands indicate similar or identical processing in both strains (Fig. 4, lane 2). On the other hand, pCV102-transformed lon- cells showed a complex expression pattern (Fig. 4B, lanes 3 and 4). In the soluble fraction, the 33-kDa FNR level increased to an almost 1:1 ratio with respect to full-length reductase (Fig. 4B, lane 3), whereas bacterial pellets contained large amounts of unprocessed preFNR as compared to JM109 cells (Fig. 4, lane 4). The insoluble material also contained variable amounts of a 15-kDa reactive product that was not characterized (Fig. 4, lanes 2 and 4). Pellets showed no detectable diaphorase activity despite of being enriched in both precursor and processed FNR.

Properties of the Recombinant Enzymes—The diaphorase activity of ferredoxin-NADP+ reductase expressed in JM109 cells was determined in both crude extracts and purified preparations (Table I). In crude bacterial lysates the amount of FNR was estimated by the slot-blot procedure. The specific activities of purified reductase agreed well with that of plant pea FNR (Table I). Similar results were obtained in crude lysates from pCV105-transformed cells. At variance, soluble extracts from JM109 cells that carried plasmid pCV102 showed specific activities that were 20-30% lower than the previous values (Table I), suggesting that the soluble precursor was not active in the diaphorase assay. The kinetic values of the ferredoxin-dependent cytochrome c reductase activity of purified recombinant FNRIs were essentially the same to those of the enzyme isolated from pea (Table I).

Absorption as well as fluorescence excitation and emission spectra of purified recombinant reductases were also very similar to those reported for the plant flavoenzymes (not shown). Characteristic absorption maxima at 385 and 456 nm, with shoulders at 430 and 470 nm, were found for all enzymes (from pCV102, pCV105, and pea). Similar 275:456 spectral ratios (Table I) indicate that in E. coli one FAD is incorporated/molecule of apoprotein, as it occurs in plants (1).

DISCUSSION

We have constructed several recombinant plasmids in order to express the plant flavoprotein ferredoxin-NADP+ reductase in E. coli under control of the lac promoter. Fusion proteins were produced which contained, in addition to short stretches corresponding to the β-galactosidase NH2-terminus, the sequences of mature pea FNR or its precursor protein (Fig. 1). In both cases, the major recombinant protein produced in strain JM109 was a 35-kDa, soluble reductase (Fig. 1, lanes 3 and 4). Assembly of the holoenzyme was found to occur in the bacterial host, as indicated by (i) diaphorase and cytochrome c reductase activities (Table I) and (ii) spectroscopic and fluorometric properties that were very similar or identical to those of chloroplast FNR.

Both recombinant proteins were processed in vivo at their NH2-termini, yielding products that were one (pCV105) or two (pCV102) residues longer than pea mature FNR. The recovery of unprocessed preFNR was 10-20% in E. coli JM109 but could be increased by expressing pCV102 in a La protease-deficient strain (Fig. 4B). Several lines of evidence suggest that this precursor is not active. (i) In JM109 crude lysates, in which preFNR accounts for 10-20% of total soluble reductase, the specific activity based on quantitation of all immunoreactive species was lower than that of purified reductase from any source (Table I). (ii) The precursor was lost in the course of FNR purification, (monitored by measuring diaphorase activity). (iii) In lon- cells, in which preFNR processing is impaired (Fig. 4B), the precursor is associated with bacterial pellets and showed no detectable FNR-dependent activities.

In a recent paper, Aliverti et al. (19) reported expression of an active mature FNR from spinach in E. coli. They introduced an additional methionine codon at position −1 to provide for translation initiation. However, the major FNR species expressed in vivo was a truncated product resulting from alternative initiation at the GTG triplet that encodes Valine 29 (19). This difficulty could only be overcome by in vitro mutagenesis of GTG to the synonymous GTT, which resulted in a sharp decrease of the truncated protein. In our case, the major product was the 35 kDa species (Figs. 2 and 4A), although a smaller FNR (≈33 kDa), representing less than 5% of the total recombinant protein, was also observed. It copurified along with the 35 kDa species. Interestingly, the DNA sequences around the Val-29 codon are highly conserved between spinach and pea FNR genes (9, 10). Since in our system FNR was synthesized from a true bacterial translation origin, the initial ATG is located at an optimal position with respect to ribosomal binding sites of the lacZ' transcript and could compete with advantage against any alternative translation origin.

Aliverti et al. (19) also observed that FNR expression patterns directed by their recombinant plasmids did not change when lon- E. coli strains were used. This result is not unexpected since proteolysis seems to be limited to amino acids −1/−2 preceding the mature protein sequence, and those residues were absent in their constructs (19).

La protease is thought to degrade mostly unfolded or misfolded proteins in a non-specific way (23). The results reported here suggest that in vivo, mature FNR contains a core that is poorly accessible to proteolytic degradation. It is worth men-
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Incorporation of FAD by the precursor or the mature reductase has been demonstrated in vitro (8, 15). However, further work will be necessary to elucidate whether FAD assembly occurs spontaneously in vivo or it requires cellular protein factors. The bacterial expression system described here might be useful to address some of these questions. It also provides a tool to study FNR structure/function relationships as well as the process of chloroplast recognition, uptake, and thylakoid attachment.

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