Crystal Structure of Paprika Ferredoxin-NADP⁺ Reductase

IMPLICATIONS FOR THE ELECTRON TRANSFER PATHWAY*

Received for publication, May 26, 2000, and in revised form, October 24, 2000
Published, JBC Papers in Press, October 26, 2000, DOI 10.1074/jbc.M004576200

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cDNA of Capsicum annuum Yolo Wonder (paprika) has been prepared from total cellular RNA, and the complete gene encoding paprika ferredoxin-NADP⁺ reductase (pFNR) precursor was sequenced and cloned from this cDNA. Fusion to a T7 promoter allowed expression in Escherichia coli. Both native and recombinant pFNR were purified to homogeneity and crystallized. The crystal structure of pFNR has been solved by Patterson search techniques using the structure of spinach ferredoxin-NADP⁺ reductase as search model. The structure was refined at 2.5-Å resolution to a crystallographic R-factor of 19.8% (Rfree = 26.5%). The overall structure of pFNR is similar to other members of the ferredoxin-NAD⁺ reductase family, the major differences concern a long loop (residues 167–177) that forms part of the FAD binding site and some of the variable loops in surface regions. The different orientation of the FAD binding loop leads to a tighter interaction between pFNR and the adenine moiety of FAD. The physiological redox partners [2Fe-2S]-ferredoxin I and NADP⁺ were modeled into the native structure of pFNR. The complexes reveal a protein-protein interaction site that is consistent with existing biochemical data and imply possible orientations for the side chain of tyrosine 362, which has to be displaced by the nicotinamide moiety of NADP⁺ upon binding. A reasonable electron transfer pathway could be deduced from the modeled structures of the complexes.

Ferredoxin-NAD⁺ reductase (FNR)³ belongs to a family of flavoproteins found in higher plants, eukaryotic algae, and photosynthetic bacteria (1). This flavoprotein is the last enzyme in the electron transport chain of linear photosynthesis, where electrons are transferred through a series of electron carriers and finally produce NADPH. FNRs have also been identified in various tissues and organisms not capable of photosynthesis, where FNR is involved in nitrogen fixation and steroid hydroxylation (2). In photosynthesis, FNR catalyzes the reduction of NADP⁺ to NADPH according to the reaction,

\[ 2 \text{Fd}_{\text{red}} + \text{NADP}^+ + H^+ \rightarrow 2 \text{Fd}_{\text{ox}} + \text{NADPH} \]

which can be divided into two half-reactions. For catalyzing this reaction, FNR utilizes the noncovalently but strongly bound prosthetic group FAD as the only redox center. In the first step, FNR catalyzes the successive transfer of two electrons from each molecule of the reduced one-electron carrier [2Fe-2S]-ferredoxin I (Fd) to a single molecule of FAD. In the second step, FNR utilizes these two electrons to convert NADP⁺ into NADPH via hydride (H⁻) transfer from N5 of FAD (1).

The three-dimensional structures of the oxidized and reduced form of native spinach FNR and of the complexes with the competitive inhibitor 2'-phospho-5'-AMP have been determined by Bruns and Karplus (3, 4) as first structures for this family of flavoproteins. In 1996, Serre et al. (5) reported the structure of Anabaena PCC 7119 FNR and that of a complex with NADP⁺. None of these studies showed a complex characteristic for productive electron transfer between FNR and NADP⁺. More recently, crystal structures of the complexes between two mutants of pea FNR (Y308S and Y308W) and NADP⁺ have been solved that reveal a single productive NADP⁺ binding mode (6). FNR consists of two distinct domains, one responsible for binding the prosthetic group FAD and the other for NADP⁺ binding. The FAD-binding domain is a six-stranded antiparallel β-barrel, which is capped at one side by an α-helix, while the NADP⁺ binding domain consists of a five-stranded parallel β-sheet flanked by α-helices. This unique structural two-domain motif has been proposed to be a prototype for a large family of flavoproteins (7). Structurally characterized members of this family involve phthalate dioxygenase reductase (PDR)(8), the FAD-containing fragment of NADPH-dependent nitrate reductase (9), cytochrome b₅ reductase (10), NADP-cytochrome P₄₅₀ reductase (11), and flavodoxin reductase (12). While all members show the characteristic two-domain FNR motif, some members like PDR also contain extra domain(s) to extend their catalytic capability.

Electron transfer reactions involving protein-protein interactions require the formation of a transient complex that brings together the two redox centers exchanging electrons. Extensive biochemical studies revealed the involvement of elec-
trostatic interactions in complex formation between FNR and its redox partner Fd (13) and implied a model for complex formation (14). In the proposed complex, FNR is predominantly positively and Fd is predominantly negatively charged. It was suggested that FNR and Fd are initially steered toward each other via complementary charge interactions of the molecular dipoles. Subsequently, interaction of basic residues on the surface of FNR and acidic residues on the surface of Fd are thought to help to attain an optimal orientation. Finally, short range forces, such as hydrophobic packing, van der Waals contacts, and hydrogen bonding should also contribute to fine structural rearrangements of the two redox partners, which optimize the interprotein electron transfer. Attempts to solve the three-dimensional structure of an efficient electron transfer complex between FNR and Fd (and NADP+) remained unsuccessful, despite extensive efforts and different crystallographic approaches (4).

To gain detailed insight into the catalytic reaction mechanism of FNR, we have undertook to sequence the gene of Capsicum annuum FNR precursor and determined the crystal structure of mature pFNR at 2.5-Å resolution. Moreover, comparison of mature FNRs from different species on the amino acid level as well as on the structural level reveals structurally and functionally conserved features. The crystal structure of pFNR enabled us to model complexes between FNR and its physiological redox partner Fd, resulting in a complex consistent with known biochemical data. This model suggests a mechanism for complex formation between FNR and Fd. Furthermore, we were able to derive a potential electron transfer pathway for successive electron transfer between FNR and Fd. This should help us to gain further understanding regarding the mechanisms of intramolecular and intermolecular electron transfer processes, which are still debated.

EXPERIMENTAL PROCEDURES

Extraction of RNA, cDNA Synthesis, and DNA Sequence Analysis— Fruit leaves of C. annuum Yolo Wonder were harvested, frozen in liquid nitrogen, and stored at –70 °C until use. With a mortar and pestle, 3.5 g of frozen plant material was ground to a fine powder under liquid nitrogen. The powdered leaves were directly added to 10 ml of a buffer composed of 4 m sodium hydrochloride, 25 mM sodium citrate, 10 mM EDTA (pH 8.0), 0.1 M mercaptoethanol, and 0.5% (v/v) N-lauroylsarcosine and homogenized with an Ultraturrax, followed by the addition of 1 ml of 2 M sodium acetate buffer (pH 4.2). Nucleic acids were then isolated by phenol-chloroform and chloroform extractions. Total RNA concentration was determined by UV absorption at 260 nm, while RNA integrity was analyzed by electrophoresis using formaldehyde-agarose gels and ethidium bromide staining. Poly(A) mRNA was isolated from total RNA by chromatography on oligo(dT)-cellulose (Quia- gen). 2 μg of the obtained mRNA was used to synthesize cDNA with the reverse transcriptase from avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) according to the manufacturer’s instructions (15). Oligo(dT) primer and random primer (hexanucleotides of random sequence) were used in this reaction to prime synthesis of the first strand of cDNA. cDNA thus synthesized was isolated by phenol-chloroform and chloroform extractions, followed by precipitation with 0.1 volumes of 3 M sodium acetate (pH 6.0) and 3 volumes of ethyl alcohol. The precipitated total RNA was dissolved in 100 μl of water and stored at –20 °C. The RNA concentration was determined by UV absorption at 260 nm, while the intactness was analyzed by electrophoresis using formaldehyde-agarose gels and ethidium bromide staining. Poly(A)+ mRNA was isolated from total RNA by chromatography on oligo(dT)-cellulose (Qua- gen). 2 μg of the obtained mRNA was used to synthesize cDNA with the reverse transcriptase from avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) according to the manufacturer’s instructions (15). Oligo(dT) primer and random primer (hexanucleotides of random sequence) were used in this reaction to prime synthesis of the first strand of cDNA. cDNA thus synthesized was isolated by phenol-chloroform and chloroform extractions, followed by precipitation with 0.1 volumes of 3 M sodium acetate (pH 9.0), and 3 volumes of ethyl alcohol. The precipitated cDNA was dissolved in 20 μl of water and stored at –20 °C.

DNA sequencing was performed by PCR using degenerate oligonucleotides designed against the 3′-end and the 5′-end of the coding region of the FNR precursor gene. Sequence comparisons of known nuclear acid sequences from plant species that were sequenced showed that degenerate oligonucleotides. PCR was performed with Deep Vent, DNA Polymer- ase (New England BioLabs Inc.), screening the different degenerate oligonucleotides against each other at different annealing temperatures. PCR fragments of appropriate size were sequenced by automated Sanger dideoxynucleotide sequencing.

Sequence—The complete coding sequence of C. annuum Yolo Wonder ferredoxin-NADP+ reductase precursor has been deposited with the EMBL Nucleotide Sequence Data base (accession code AJ250378).

Sequence Comparison—Best fit alignments of the amino acid sequence of pFNR with known amino acid sequences of other plant FNRs as well as alignments of nucleic acid sequences were calculated with the Pileup program of the GCG package (Genetics Computer Group, Madison, WI).

Cloning and Expression of the fnr Gene—To obtain the mature form of pFNR, the proposed processing site of the chloroplast transit peptide was deduced by sequence alignment. PCR using Deep Vent, DNA Polymerase was used to add flanking BamHI (3′ site) and Ndel (5′ site) restriction sites. To prevent degeneration of different pFNR-polyclones in Escherichia coli, as reported for recombinant spinach FNR (16), we used the codons for residues Val79 and Val107 from GTG to the synonymous GTT by site-directed mutagenesis. The resulting construct was cloned into the Ndel and BamHI sites of expression vector pET22b (+ (Novagen) to yield pET22b (+)+fnr. E. coli strain BL21 (DE3) was transformed with pET22b (+)+fnr. The bacteria were grown in 12 liters of LB medium containing 100 μg/ml ampicillin to an A600 of 0.8, and expression of mature pFNR was induced with 1 mM isopropyl-β-thiogalactopyranosid for 5 h.

Purification of Native and Recombinant FNR—For preparation of the crude extract, the washed and precooled paprika leaves (~1 kg) were homogenized in an appropriate amount of buffer A (25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 2 mM DTT, 1 mM phenylmethanesulfonfluoride, 5 mM benzamidinedihydrochloride) using a Waring Blender with glass pearls. The homogenate was mixed with DNase and RNase, filtered through a nylon net and one layer of glass wool, and centrifuged at 10,000 × g (60 min, 4 °C) to remove the remaining solid components. The supernatant was applied to a Reactive Red 120 affinity column (50 ml; Sigma) equilibrated with buffer B (25 mM Tris-HCl (pH 8.0), 50 mM NaCl). After washing off the unabsorbed material with an excess of the same buffer, bound proteins were eluted using a linear NaCl gradient from 0.05 to 1.0 M in buffer B. The fractions containing pFNR were identified by SDS-polyacrylamide gel electrophoresis, diazed against buffer B, and applied to a DEAE-Sepharose column (100 ml; Sigma) equilibrated in buffer B. After extensive washing with buffer B, a gradient from 0.05 to 0.5 M NaCl in the same buffer was started. The pFNR-containing fractions were dialyzed and diazed against buffer C (25 mM Tris-HCl (pH 8.0), 1.5 M (NH4)2SO4) and then applied to a phenyl-Sepharose column (50 ml; Sigma) and eluted in a linear decreasing gradient from 1.5 to 0 M (NH4)2SO4 in 25 mM Tris-HCl (pH 8.0). The resulting pFNR was concentrated and desalted by dialysis against buffer B. Polyacylamide gel electrophoresis and absorption spectrum were used as a criterion for purity of the protein. Protein concentrations were measured by the method of Bradford (17) with a bovine serum albumin standard curve. For purification of the recombinant pFNR, the E. coli cells that overexpressed pFNR were harvested and resuspended in buffer A. Cells were broken by sonification, and cell debris was removed by centrifugation at 40,000 × g (60 min, 4 °C). The purification procedure was the same as described above.

Crystallization and X-ray Data Collection—Crystals were grown by sitting drop vapor diffusion against a reservoir containing 0.1 M Tris-HCl (pH 8.5) and 1.9 M (NH4)2SO4. The droplets consisted of 4.5 μl of protein solution (15.0 mg/ml, buffer with 20 mM Tris-HCl (pH 8.0)) and 4.5 μl of reservoir solution and were equilibrated against 5 ml of reservoir solution at 20 °C. Under these conditions, rectangular crystals grew to an approximate size of 0.3 × 0.3 × 0.25 mm3 within 5 days. The pFNR crystals belong to space group P21 with unit cell parameters a = 44.72 Å, b = 108.98 Å, c = 90.36 Å, α = 90.0°, β = 95.57°. X-ray diffraction data to 2.5-Å resolution were collected on a MAR-Research image plate (Hamburg, Germany) equipped with a Rigaku (Tokyo, Japan) RU 200 rotating anode x-ray generator. Data were collected with a single pFNR crystal at room temperature in 1° frames with exposure times of 1000 s/frame. The data were indexed with MOSFLM (18) and scaled and merged using programs of the CCP4 suite (19). Statistics for the data are shown in Table I.

Molecular Replacement and Structure Refinement—Due to the 77% identity between the amino acid sequences of spinach FNR and pFNR, it was expected that their overall three-dimensional structures would be similar. Therefore, the electrons structure was solved by the molecular replacement technique using the program XPLOR (20). A poly(A)-model of the 1.7-Å resolution crystal structure of spinach FNR (4) was used as search model. The FAD prosthetic group was omitted. A native Patterson map was calculated using programs of the CCP4 suite (19). A pseudo-origin peak could be detected in the Harker section at y = 0.5. Self-rotation functions were calculated with the program GRASP (21)
determine the noncrystallographic symmetry. In the α, β, and 90° section, no peaks could be detected. These calculations indicate that the asymmetric unit contains two molecules of pFNR related by a translation. Therefore, Patterson search techniques were applied for two pFNR molecules. Patterson rotation searches were calculated with resolution limits of 0.0 to 2.5 Å and an angular step size of 2.5°. Translation functions corresponding to the highest rotation peaks resulted in a promising solution with two monomers in the asymmetric unit. After a rigid body refinement, correlation coefficient and R_cryst factor were 56.4 and 37.3%, respectively.

Structure refinement of the model was done with X-PLOR (22) using the parameters of Englar and Huber (23). Model building into 2Fo − Fc and Fc − Fo maps calculated with XPLOR was done using the program FRODO (24). The electron density maps could not be improved by 2-fold real space averaging using MAIN (25) with noncrystallographic symmetry operators determined with LSQMAN (26). The electron density maps showed well defined density into which we fitted the FAD molecule, using the FAD coordinates of spinach FNR as starting coordinates. By contrast, the N-terminal residues 55–66 (first 12 N-terminal residues of mature pFNR) and the surface loop comprising residues 171–175 remained poorly defined in the electron density map. In further rounds of refinement, an overall anisotropic B-factor was included, and in later rounds individual isotropic B-factors were refined. Refinement parameters for FAD were calculated with the program XPLOR2D (27). To check the different stages of the refinement, the restraint R values (28) were calculated from 5% of the diffraction data. When the refinement nearly converged, one phosphate ion and 219 water molecules per pFNR molecule were progressively added to the molecular model. Finally, rebuilding and refinement cycles converged at R_cryst and R_free values of 19.8 and 26.5%, respectively. The polypeptide chain could be traced from residue 66 to 362. The final model includes 592 residues, the two FAD cofactors, two phosphate ions located at the NADP+−binding sites, and 438 solvent molecules in the asymmetric unit. The stereochemistry of the model was analyzed using X-PLOR and PROCHECK (29). Details of the refinement are given in Table I.

Molecular Model Building of Substrate Complexes—Molecular modeling to dock two macromolecules implementing the geometric surface recognition principle of Katchalski-Katzir (30) was done using the program FTDOCK (31). The refined structure of pFNR and the structure of the mutant E92K of [2Fe-2S]-ferredoxin I from Spinacia oleracea (32) were chosen for calculating the structure of an efficient electron transfer complex. During docking calculations, pFNR represented the fixed molecule and Fd the mobile molecule, respectively. The electrostatic function of FTDOCK was used to improve the final rank of the docking solutions. The 15 solutions with the highest scores for surface complementarity and favorable electrostatic interactions were used for refinement and energy minimization with MULTIDOCK (33). One final solution showing a large decrease in total energy during refinement resulted in a promising complex of the two molecules with favorable electrostatic interactions and high surface complementarity. Moreover, NADP+, which was generated by superimposing the structure of the NADP+−FNR-Fd complex (30) on the structure of pFNR, was positioned into the active site of the modeled pFNR-Fd complex, following the binding mode described by Deng et al. (6). Using the model building program O (34), new possible orientations were built for the C-terminal pFNR tyrosine residue 362, which has to be displaced to permit stacking interactions between the nicotinamide moiety of NADP+ and the isoalloxazine ring of FAD. Energy minimization of the pFNR-Fd−NADP+ complexes with different orientations of the C-terminal tyrosine residue 362 was then carried out using the program CNS (35). pFNR and both ligands were minimized simultaneously.

Coordinates—The refined model of pFNR has been deposited with the Research Collaboratory for Structural Bioinformatics Protein Data Bank (accession code 1PB9).

RESULTS AND DISCUSSION

Sequencing and Sequence Analysis of pFNR—We identified the gene encoding the FNR precursor of C. annuum Yolo Wonder by PCR using a set of degenerate oligonucleotides targeted to the 3′ and 5′-ends of the coding region. A PCR fragment of 1089 base pairs was obtained, which showed high sequence identities when compared with FNR nucleic acid sequences of other plant species. The complete nucleotide sequence of the gene was determined from the isolated cDNA. The fnr gene of pFNR precursor codes for a polypeptide of 362 amino acids with a calculated molecular mass of 40,407 Da and a pI of 8.57. pFNR precursor bears an amino-terminal transit peptide consisting of residues 1–54, which allows the protein to pass the chloroplast membrane, where it is proteolytically processed to the mature form, which contains residues 55–362. Mature pFNR has a molecular mass of 33,177 Da and a pl of 6.33. pFNR precursor can be aligned significantly over its entire length with proteins of the ferredoxin-NADP+ reductase family (Fig. 1). It is about 75% identical to spinach FNR precursor, ice plant FNR precursor, pea FNR precursor, bean FNR precursor, and tobacco FNR precursor. The sequence similarity for the FAD-binding domains and the NADP+−binding domains of different members of the ferredoxin-NADP+ reductase family is extremely high, but it is even considerable for the different transit peptides containing many serine, threonine, and small hydrophobic residues but few acidic residues. Regarding the degree of similarity of the mature proteins, the first N-terminal amino acids share only a few common features in contrast to the rest of the protein. In the molecular structures of pFNR and spinach FNR, this N-terminal part of the protein is even invisible in the electron density map, indicating high flexibility and lower importance for functional and structural integrity. The high degree of similarity between the different mature FNRs on the amino acid level as well as on a structural level (see below) indicate that both the overall fold and the common amino acid features are important factors for electron transfer.

Cloning, Recombinant Expression, and Purification of pFNR—To have at hand large amounts of native pFNR for detailed mechanistic and structural studies, the Ndel-BamHI mature pFNR fragment was isolated by PCR from paprika cDNA and cloned in the expression vector pET22b (+). E. coli BL21(DE3) transformed with pET22b (+)−fnr, where fnr is under the control of a T7 promoter, produced the mature flavoprotein in a completely active form and yielded higher levels of soluble protein when E. coli cells were induced at temperatures lower than 37 °C. Furthermore, high levels of pFNR production were still obtained when cultures were induced in the late logarithmic rather than in earlier phases of growth, thus allowing a higher biomass yield.

We developed a purification procedure for the isolation of pFNR from paprika fruit leaves and from bacterial lysates, respectively. The main purification step of this procedure was the affinity chromatography to Reactive Red 120 Sepharose followed by ion exchange chromatography and hydrophobic interaction chromatography. Purified pFNR was more than 90% pure, as judged from Coomassie-stained SDS-polyacrylamide gels (not shown) and showed a major band at an apparent molecular mass of 33 kDa. The integrity of the purified samples was tested by UV-visible spectra showing the expected maxima at 280 nm (protein) and 385 and 458 nm (protein-bound FAD) (1).

Crystallization and Quality of the Final Model—pFNR crystals were grown by sitting drop vapor diffusion to an approximate size of 0.3 × 0.3 × 0.25 mm3, and x-ray diffraction data were collected to 2.5-Å resolution. The structure of pFNR has been solved by Patterson search techniques using spinach poly(A)-FNR as search model (4). The final model includes two pFNR monomers in the asymmetric unit, but the noncrystallographic symmetry between them was restrained only in the earlier stages of refinement. Real space 2-fold averaging did not improve the electron density maps. Unambiguous parts and side chains could be added during refinement. In the final electron density map, all residues are well defined except the N-terminal segment, including residues 55–66. Moreover, residues 171–175 located in an external loop on the FAD-binding site of pFNR are poorly defined in the electron density map.

Crystal Structure of Paprika Ferredoxin-NADP+ Reductase
This is comparable with the structures of spinach FNR (3, 4) and Anabaena FNR (5) and presumably reflects the flexibility of this part of the protein. For the reflection data between 2.5- and 8.0-Å resolution, the crystallographic $R$-factor for the final model is 19.8%, and the $R_{\text{free}}$ is 26.5%. The mean error of model coordinates as estimated by a Luzzatti plot (37) as well as by the $s$A method (38) is 0.27 and 0.28 Å, respectively. The stereochemistry of the model is well defined, since deviations from ideal values are 0.007 Å for bond length and 1.6° for bond angles (Table I). All but three nonglycine main-chain dihedral angles are within energetically favorable regions of the Ramachandran plot (39), with 86.7% of the angles in the most favorable area. For the three residues in disallowed regions, Lys69, Lys70, and Asn 170, the electron density is well defined. Asn170 is located at the FAD-binding site and is hydrogen-bonded to AN1 of the adenine moiety of FAD. Its unfavorable main chain conformation may be required for tight binding of the adenine portion of FAD to pFNR, whereas Lys69 and Lys70 are in the N-terminal region located at the protein surface with apparently higher flexibility.

Overall Structure of pFNR—In the crystal structure of pFNR, the molecule exists as a monomer like other members of the protein family (3–5). pFNR is divided into two distinct domains, the FAD-binding domain comprising residues 67–201 and the NADP$^+$-binding domain containing residues 202–362, which are connected by a long loop (Fig. 2, a and b). The secondary structure elements assigned to the two domains of FNR and their nomenclature are shown in Fig. 2a.

The FAD-binding domain is made up of a barrel built of six antiparallel $\beta$-strands, which are arranged in two perpendicular $\beta$-sheets (Table). All but three nonglycine main-chain dihedral angles are within energetically favorable regions of the Ramachandran plot (39), with 86.7% of the angles in the most favorable area. For the three residues in disallowed regions, Lys69, Lys70, and Asn170, the electron density is well defined. Asn170 is located at the FAD-binding site and is hydrogen-bonded to AN1 of the adenine moiety of FAD. Its unfavorable main chain conformation may be required for tight binding of the adenine portion of FAD to pFNR, whereas Lys69 and Lys70 are in the N-terminal region located at the protein surface with apparently higher flexibility.
Crystal Structure of Paprika Ferredoxin-NADP⁺ Reductase

**Table I**

| Data collection and refinement statistics |
|------------------------------------------|
| Space group                              | P₂₁                                |
| Unit cell dimensions                      | a = 44.72 Å, b = 129.45 Å          |
|                                           | c = 90.36 Å                        |
| Resolution limit (Å)                      | 20.0–2.5                           |
| Reflections                              |                                     |
| Observed                                  | 61,664                              |
| Unique                                    | 26,895                              |
| Completeness (%), overall                 | 83.9                                |
| Completeness (%), (2.58–2.5 Å)            | 83.5                                |
| Rmerge (%), overall                       | 9.4                                 |
| Rmerge (%), (2.58–2.5 Å)                 | 31.9                                |
| Amino acid residues                       | 592                                 |
| Solvent molecules                         | 438                                 |
| FAD molecules                             | 2                                  |
| Phosphate ions                            | 2                                  |
| Resolution range (Å)                      | 8.0–2.5                             |
| Reflections used for refinement           | 24,312                              |
| R value (%)                               | 19.8                                |
| Rfree (%)                                 | 26.5                                |
| Root mean square S.D.                     |                                     |
| Bond lengths (Å)                          | 0.007                               |
| Bond angles (°)                           | 1.6                                 |
| Dihedral angles (°)                       | 23.7                                |
| Improper angles (°)                       | 1.2                                 |
| Bonded B factors (Å²)                     | 2.9                                 |
| B-factors (Å² / B)                        |                                     |
| All atoms                                 | 18.7                                |
| Protein atoms                             | 18.3                                |
| Main chain atoms                          | 16.7                                |
| FAD cofactor                              | 36.4                                |

* Rmerge = $\frac{\sum |F_{obs}| - |F_{calc}|}{\sum |F_{obs}|}$

**FAD Binding and Conformation**—The β-barrel of the FAD-binding domain exhibits a small gap between stands β4 and β5, providing space for the isoalloxazine and ribityl moieties of the FAD cofactor. The ribose and adenine moieties extend along the long FAD-binding loop between β3 and α1, which is surrounded on either side by two small β-strands (residues 167–177), and are well exposed to solvent. pFNR binds its prosthetic group FAD through direct and often water-mediated hydrogen bonds, van der Waals contacts, and stacking interactions between the bases and aromatic amino acids (Fig. 3). Many of these interactions involve main-chain atoms located in repetitive secondary structure elements.

The binding mode of the isoalloxazine ring is similar in all members of the superfamily. The isoalloxazine ring is tightly bound to the protein between two tyrosine residues, Tyr143 on the pro-si face and Tyr362 on the pro-re face (Fig. 4a). Main-chain atoms of residues Cys162 and Lys164 and the side chain of Ser144 form direct hydrogen bonds to the isoalloxazine ring, whereas Ser144 and Tyr362 interact through water-mediated hydrogen bonds. While a large part of the isoalloxazine ring is deeply nestled into the opening of the β-barrel, the edge of the isoalloxazine ring (with its methyl groups C7a and C8a) is exposed to solvent. This is presumably the site where the iron-sulfur cluster passes its electrons to the FNR-bound FAD (see below). In the structure of PDR, which exhibits a ferredoxin-like iron-sulfur domain covalently attached to a FNR-like pair of domains, the iron-sulfur cluster is indeed located very close to the C7a and C8a methyl groups of the isoalloxazine ring (8).

The ribityl moiety and the phosphate group are also tightly bound to the protein mostly through direct hydrogen bonds from protein side chains and main chains. The hydrogen bonding pattern resembles that described for spinach FNR (4). Main-chain atoms of Leu142 and the side chain of Tyr143 participate in direct hydrogen bonds to the hydroxyl groups of the ribityl moiety, while main-chain atoms of Val179 and Ser181 and side-chain atoms of Ser181 and Arg141 form hydrogen bonds to the phosphate groups. Furthermore, there exist four hydrogen-bonded water molecules.

While the binding of the isoalloxazine ring, the ribityl moiety, and the phosphate groups are well preserved in the reductase superfamily, ribose and adenine binding show variations. The binding mode among members of the superfamily is different. The FAD-binding loop (residues 167–177) between β5 and α1 is usually responsible for adenine binding. In contrast to spinach FNR (4), where the adenosine moiety is only loosely bound by a van der Waals contact with the side chain of Tyr120, the adenine part in pFNR is well fixed. When comparing the structures of pFNR, spinach FNR (4), and *Anabaena* FNR (5), the unique feature of the pFNR FAD-binding site is the bent conformation of the FAD-binding loop (residues 167–177 for pFNR) toward the adenine portion of FAD (Figs. 2c and 4b). This conformation brings the side chain of Tyr168 in optimal orientation for stacking interactions to the adenine ring. Besides that, Asn170 is in a position in which it can form a direct hydrogen bond to AN1 of the adenosine moiety, and Leu166 forms van der Waals contacts to AC5 and AN7 (Fig. 4, a and b).

The conformation of the ribityl portion of the FAD molecule bound to pFNR is different from that reported for spinach FNR (4), since its hydroxyl groups at position 3 and 4 point in different directions. However, the electron density is unambiguous in this region. The tighter interaction between the protein and the adenine moiety in pFNR may possibly force the switched conformation of the ribityl part in paprika FAD.

**Putative Ferredoxin Binding Site**—The redox partner of FNR, Fd, is a small acidic protein with an apparent molecular
Overall three-dimensional structure of pFNR. *a*, ribbon diagram of pFNR, showing the secondary structure elements. The N-terminal FAD-binding domain is blue, and the C-terminal NADP^+/-binding domain is red. β-Strands are represented by arrows; α-helices are
mass of 11 kDa, which contains a single [2Fe-2S] cluster coordinated by four cysteine residues as prosthetic group. Fd is involved in a number of different metabolic pathways (44) and in the photosynthetic electron transport chain, where Fd transfers its electron to FNR. The stability of the electron transfer complex between FNR and Fd depends on ionic strength, suggesting the involvement of intermolecular electrostatic interactions (13). Chemical modification experiments (45–48), chemical cross-linking experiments (49), and mutagenesis studies (50–52) on FNR and Fd of different species revealed a number of acidic residues in Fd and of basic residues in FNR that probably participate in complex formation. These experiments suggested that Fd binds within a large shallow cleft between the two domains of FNR. The FAD molecule is located in the center of this cavity, with its dimethylbenzene ring directed toward the putative Fd binding site.

The structure of pFNR exhibits this large concave cleft around the site of electron transfer. This cleft is surrounded by four major patches of positively charged residues grouped around the FAD cofactor. The first positively charged patch includes residues Lys136, His138, Lys139, and Arg141. A second patch of positively charged residues is located in the neighborhood of Lys201. The third patch is very large, including residues Lys348, Lys349, Lys352, and Lys353, while the fourth patch is located around the positively charged residue Lys323. Sequence comparison with spinach and Anabaena FNR show that most of these positively charged residues have been implicated in ferredoxin binding in the mentioned biochemical modification studies (45–52).

Modeling of Ferredoxin Binding Modes—Attempts to crystallize FNR in complex with Fd have not been successful in our hands. Therefore, the putative binding of the redox partner [2Fe-2S]-ferredoxin I from S. oleracea (32) to pFNR has been modeled assuming that larger conformational changes and domain movements do not occur. The structure of PDR (8), which exhibits a ferredoxin-like iron-sulfur domain covalently at-
attached to an FNR-like pair of domains, showed good correlation regarding the orientation of all secondary structure elements when superimposed with the structure of pFNR.

Models for the complex of spinach FNR with *Spirulina platensis* (3) and *Aphanothece sacrum* Fds (53) and a model (14) in which the spinach Fd sequence was fit to the structure of the Fd from *A. sacrum* have been obtained previously. Nevertheless, these models were obtained by manually placing the iron-sulfur cluster of Fd to the proposed binding cleft of FNR near the exposed portion of the flavin to account for the results of the biochemical studies (45–52). Here we present an FNR-Fd docking model generated by computational molecular modeling. We used the structure of [2Fe-2S]-ferredoxin I from *S. oleracea* (32) to dock onto pFNR. The relative starting orientation of the two proteins used for molecular modeling was not set in advance. The complex was calculated using a geometric surface recognition algorithm and an electrostatic correlation function. The resulting preliminary pFNR-Fd complex was then energy-minimized, allowing for side-chain conformational changes and rigid body movements of the interacting proteins. The final complex had minimized in total energy from $-220$ to $-1047$ kcal/mol including the internal and intermolecular interaction energy.

A ribbon drawing of the modeled pFNR-Fd complex is shown in Fig. 5. Fd fills the large shallow cleft between the two domains of FNR as predicted for ferredoxin binding and is located close to the FAD and NADP$^+$ binding sites. The center of the iron-sulfur cluster adjusts on the geometric plane, which is defined by the isoalloxazine ring of FAD, about 7.6 Å apart from the dimethylbenzene ring of the isoalloxazine moiety, the part of the cofactor through which the electrons are exchanged. In comparison with the structure of PDR (8), where the iron-sulfur cluster is 7.4 Å apart from the dimethylbenzene ring of the prosthetic group FMN, this distance seems to be suitable for effective electron transfer. Tyr$^{37*}$ of Fd, which is located close to the iron-sulfur cluster, faces directly into the active site of FNR. Its phenol ring is oriented nearly parallel to the plane defined by the isoalloxazine ring of FAD and faces toward the C-terminal Tyr$^{362}$ of pFNR (Fig. 6a). In this position, Tyr$^{37*}$ is

![Diagram of the modeled pFNR-Fd complex](image)

Fig. 4. FAD-binding site of pFNR. a, stereo view of the FAD-binding site of pFNR. b, stereo view of the superposition of the FAD binding site structures of pFNR (green) and spinach FNR (blue). For clarity reasons, only the atomic model of the FNR-bound FAD and the labels for pFNR are shown. Both images were produced with SETOR (43).

Amino acids denoted with an asterisk are residues of Fd from *S. oleracea* within the modeled FNR-Fd complex.
able to form a weak hydrogen bond (OH-OE1, 3.8 Å) to Glu\textsubscript{360} and is in close van der Waals contact to the dimethylbenzene ring of FAD. Tyr\textsubscript{37*} represents the only residue of Fd that directly interacts with active site residues of pFNR. In the protein-protein interface of the modeled complex, however, numerous interactions, mainly of an electrostatic nature, can be identified. Most of the interprotein interactions are formed between residues of the NADP\textsuperscript{+}-binding domain of pFNR and of Fd, whereas residues of the FAD-binding domain are less involved in interprotein interactions. Herein, we focus on some selected interprotein interactions. Lys\textsubscript{139} of the FAD-binding domain of pFNR interacts with Glu\textsubscript{360}, which is located at the C terminus of Fd, while five important interactions are present between the NADP\textsuperscript{+}-binding domain of pFNR and Fd as follows: Lys\textsubscript{201} interacts with Asp\textsubscript{26*} and Ser\textsubscript{48*}, Lys\textsubscript{348} with Glu\textsubscript{30*}, Lys\textsubscript{352} with Asp\textsubscript{26*}, and Lys\textsubscript{353} with Asp\textsubscript{60*}. The pFNR residues involved in these interprotein interactions correspond to the four major patches of positively charged residues on the surface of pFNR described above. The Fd residues can be grouped into three major regions of negative charge on the surface of Fd. Two distinct negative patches are located on both sides of the iron-sulfur cluster, and a third patch is present near the C terminus of Fd.

The structure of the pFNR-Fd complex is absolutely consistent with the results of the biochemical studies on electron transfer between FNR and Fd (45–52), while these were not able to give evidence for detailed interprotein interactions, and seems to meet all requirements for efficient electron transfer. Superposition of the structure of the pFNR-Fd complex and the structure of PDR (8) reveals no major structural differences between the two domains of pFNR and the two FNR-like domains of PDR, as expected. The orientations of the complexed Fd and the Fd-like domain of PDR are not identical, while the positions of both iron-sulfur clusters are nearly the same. The iron-sulfur cluster of PDR is moved a little bit out of the plane that is defined by the isoalloxazine ring of the prosthetic group. In comparison with the Fd-like domain of PDR, the complexed Fd seems to be rotated nearly 90° around a vertical axis running through its iron-sulfur cluster. However, a PDR-like rotation of the complexed Fd would no longer be consistent with the observed cross-linking results (45–52).

The modeling studies strengthen the postulated mode of FNR-Fd complex formation (14). Both protein surfaces possess several patches of oppositely charged residues that help in properly orientating the partner molecules. Interaction of the above mentioned basic residues on pFNR and acidic residues on Fd may then help in improving the orientation, as do the many short range forces, such as intermolecular van der Waals contacts, hydrophobic packing interactions, and hydrogen bonding. All of these interactions may contribute to fine structural rearrangements of both redox partners to optimize the interprotein electron transfer.

**Electron Transfer Pathway**—Our modeling studies reveal a potential orientation of the redox partners in the electron transfer complex and also suggest a pathway for electron transfer between the iron-sulfur cluster of Fd and the isoalloxazine ring of FNR-bound FAD. The rate of electron transfer and the pathway of electrons within a protein complex is controlled by distance, location, and environment of the redox components (54). It is generally accepted that the main pathway of long distance electron transfer in proteins involves a chain of covalently bonded atoms. Aromatic residues placed in appropriate positions to the two redox centers may enhance electron transfer through proteins by a more effective coupling through their extended π*-orbitals, since the energy gap between the tunneling electron and the π-system is significantly smaller than between the tunneling electron and σ-orbitals (54). We suggest that the position of Tyr\textsubscript{37*} in the modeled electron transfer complexes may enhance the interaction between the iron-sulfur cluster and the isoalloxazine ring, since the two ring systems are in van der Waals contact, providing an electronic overlap and enabling electron tunneling through the aromatic rings. Our FNR-Fd complex therefore suggests the following electron transfer pathway. Starting at the iron-sulfur cluster, Fe\textsuperscript{3+} transfers one electron through the iron-ligating sulfur atom of Cys\textsubscript{39*} along the peptide chain to the aromatic π-system of Tyr\textsubscript{37*}. Tyr\textsubscript{37*} is in close van der Waals contact (2.4–3.1 Å) to the aromatic isoalloxazine ring, and by their electronic overlap tunneling to FAD is enabled. In other systems that have been selected by evolution for efficient electron transfer, aromatic residues have been found in similar positions, probably enhancing electron transfer. Examples are the tryptophan-mediated reduction of quinone in the photosynthetic reaction center (55, 56) and in the MADD/amicyanin system, where a tryptophan residue is placed at the interface of the two proteins (57).

**NADP\textsuperscript{+} Binding and Catalytic Mechanism**—The structures of two mutants of pea FNR (Y308S and Y308W, in which the C-terminal tyrosine residue is mutated) in complex with NADP\textsuperscript{+} and NADPH revealed a productive NADP\textsuperscript{+}-binding mode for members of the FNR family, in which the nicotinamide ring is stacked against the flavin isoalloxazine ring at an angle of −30° and occupies the same place as the side chain of the C-terminal tyrosine in native FNR (6). Due to the necessary displacement of this C-terminal tyrosine side chain, none of the previous structural studies on native FNR yielded productive NADP\textsuperscript{+} binding. NADP\textsuperscript{+}-binding to wild type FNR occurs...
through the 2′-phospho-5′-AMP part of NADP⁺, whereas the nicotinamide part of NADP⁺ faces into the solvent (4, 5). Three mechanisms are possible for productive NADP⁺ binding. In the first binding mechanism, Fd binding to FNR provides a favorable environment for tyrosine displacement, followed by productive NADP⁺ binding (ordered sequential mechanism–Fd binding increases the affinity of FNR for NADP⁺). In the second mechanism, the tilted geometry of the isalloxazine ring in reduced FAD forces tyrosine displacement and productive NADP⁺ binding (ping-pong mechanism). The third possibility is that both complex formation between FNR and Fd and the geometry of the reduced FAD force productive NADP⁺ binding (ordered sequential mechanism–Fd binding and the redox state of FAD both increase the affinity of FNR for NADP⁺). Here we present a model for productive NADP⁺-binding upon complex formation between pFNR and Fd following an ordered sequential reaction mechanism. Minor structural rearrangements upon pFNR-Fd complex formation may favor displacement of

**Fig. 6. Active site of the modeled electron transfer complexes after energy minimization.** The atomic model of pFNR is shown in green, and that of the mutant E92K of [2Fe-2S]-ferredoxin I of S. oleracea (32) is shown in blue. FAD and the [2Fe-2S]-cluster are represented by balls and sticks colored by element type. a, stereo view of the electron transfer site of the modeled pFNR-Fd complex. b and c, these stereo plots show the active site of the pFNR-Fd-NADP⁺ complex with plausible orientations for the displaced C-terminal tyrosine 362 of pFNR after energy minimization in complexes 2 and 4, respectively. These images were produced with SETOR (43).
the C-terminal tyrosine, and the complex interface provides space in which the displaced tyrosine side chain is energetically stabilized. Tyr$^{37a}$, which faces into the catalytic pocket of the complex, can also interact with the bound NADP$^+$. In the modelled pFNRFd complex (Fig. 6a), four positions are stericly accessible for the side chain of Tyr$^{362}$. It could face into the gap between the adenine moiety of FAD and Fd exposed to solvent (position 1). On the other hand, Tyr$^{362}$ could rotate toward the main hydrophobic side chain of Tyr$^{362}$, forming a hydrogen bond to one of the hydroxyl groups of NADP (OH-OD1, 3.8 Å), while the carboxyl group forms a weak hydrogen bond to one of the hydroxyl groups of NADP (O-OH9, 3.5 Å).

Finally, Tyr$^{362}$ could face into the gap between the ribityl moiety of FAD and the bound Fd (position 4). For modeling ternary complexes between Fd, FAD, and NADP$^+$ according to an ordered sequential reaction, we positioned NADP$^+$ according to the binding mode reported by Deng and coworkers (6) and Fd binds to FNR according to the binding mode reported for FNR, whereas the nicotinamide part still faces into the solvent as described above. Its carboxyl group faces toward the ribityl part of NADP$^+$ (described above). Its carboxyl group faces toward the ribityl part of NADP$^+$, forming a hydrogen bond to one of the hydroxyl groups (O-OH9, 3.1 Å). Furthermore, the productive conformation of NADP$^+$ is stabilized by the side chain of Tyr$^{37a}$, which forms two strong interactions to the nicotinamide group (OH-O, 2.6 Å; OH-NH$_2$, 3.5 Å) of NADP$^+$. Similar stabilization conditions are present in complex 4 (Fig. 6c), and the side chain of Tyr$^{362}$ faces into the gap between the ribityl moiety of FAD and Fd, forming a hydrogen bond to Asp$^{336}$ (OH-OD1, 3.8 Å), while the carboxyl group forms a weak hydrogen bond to one of the hydroxyl groups of NADP$^+$ (O-OH9, 3.5 Å).

These ternary complexes suggest a reasonable ordered sequential mechanism for productive NADP$^+$ binding involving complex formation between FNR and its leading substrate Fd. Primarily, NADP$^+$ binds through its 2'-AMP portion to the uncomplexed FNR, whereas the nicotinamide part still faces into the solvent as reported for Arabana FNR (5). In the leading step of the ordered sequential reaction, Fd binds to FNR according to the binding mode described above, producing a favorable environment for Tyr$^{362}$ displacement, which increases the affinity of the enzyme for NADP$^+$. In a second step, the nicotinamide portion of NADP$^+$ finally binds to FNR. The energetic cost of displacing the tyrosine can presumably easier be compensated by creation of an energetically favorable environment for the tyrosine side chain, by the energetic gain due to nicotinamide binding and due to its interaction with Tyr$^{37a}$. Therefore, we favor this electron transfer mechanism, including the formation of a ternary complex.

Acknowledgments—We thank Gärtneri Kamlah (München, Germany) for providing C. annuum Yolo Wonder fruit leaves. Silvia Gaertner and Irmgard Sures are gratefully acknowledged for giving advice on RNA isolation and cDNA synthesis.

REFERENCES
1. Carrillo, N., and Vallejos, R. H. (1987) Topics Photosynth. 8, 527–560
2. Arakaki, A. K., Cecarelli, E. A., and Carrillo, N. (1997) PASB J. 11, 133–140
3. Karplus, P. A., Daniele, M. J., and Herriott, J. R. (1991) Science 251, 60–66
4. Bruns, C. M., and Karplus, P. A. (1995) J. Mol. Biol. 247, 125–145
5. Serre, L., Vellieux, M. D., Medina, M., Gómez-Moreno, C., Fontecilla-Camps, J. C., and Frey, M. (1999) J. Mol. Biol. 283, 20–39
6. Deng, Z., Aliverti, A., Zanetti, G., Arakaki, A. K., Ottado, J., Orellano, E. G., Calceterra, N. B., Ceccarelli, E. A., Carrillo, N., and Karplus, P. A. (1999) Natur. Struct. Biol. 6, 847–853
7. Correll, C. C., Batic, C. J., Ballou, D. P., and Ludwig, M. L. (1992) Science 258, 1604–1610
8. Lu, G., Campbell, W. H., Schneider, G., and Lindquist, Y. (1994) Structure 2, 809–821
9. Nishida, H., Inaka, K., Yamanaka, M., Kaida, S., Kobayashi, K., and Mikki, K. (1990) Biochemistry 34, 1583–1597
10. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. B., and Kim, J. J. P. (1997) Pro. Natl. Acad. Sci. U. S. A. 94, 8411–8416
11. Ingelman, M., Bianchi, V., and Eklund, H. (1979) J. Mol. Biol. 133–140
12. Ingelman, M., Bianchi, V., and Eklund, H. (1979) J. Mol. Biol. 111, 109–121
13. Karplus, P. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
14. Katchalski-Katzir, E., Shirar, I., Eisenstein, M., Friess, A. A., Affalo, C., and Valkov, I. A. (1995) Pro. Natl. Acad. Sci. U. S. A. 92, 2195–2199
15. Gabb, H. A., Jackson, R. M., and Sternberg, M. J. E. (1997) J. Mol. Biol. 272, 106–120
16. Binda, C., Coda, A., Aliverti, A., Zanetti, G., and Mattevi, A. (1998) Acta Crystallogr. Sec. D 54, 1355–1358
17. Jackson, R. M., Gabb, H. A., and Sternberg, M. J. E. (1998) J. Mol. Biol. 276, 265–285
18. Jones, T. A., and Kjelgaard, M. (1991) O: The Manual, University of Uppsala, Uppsala, Sweden
19. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Grosse-Kunstleve, R. W., Jiang, S.-J., Kuzebewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1990) Acta Crystallogr. Sec. D 54, 905–921
20. Barton, G. J. (1993) Protein Eng. 6, 37–40
21. Luzzati, V. (1952) Acta Crystallogr. 5, 283–291
22. Read, R. J. (1998) Acta Crystallogr. Sec. A 54, 140–149
23. Karplus, P. A., and Bruns, C. M. (1994) J. Bioenerg. Biomembr. 26, 802–810
24. Aliverti, A., Corrado, M. E., and Zanetti, G. (1994) Protein Sci. 3, 86–90
25. brilliant, N., and Sasisekharan, V. (1962) Adv. Protein Chem. 23, 263–437
26. Zanetti, G., Aliverti, A., and Curti, B. (1984) J. Mol. Biol. 29, 423–437
27. Zanetti, G., Aliverti, A., and Curti, B. (1984) J. Mol. Biol. 29, 6153–6157
28. richter, G., Morelli, D., Ronchi, S., Negri, A., Aliverti, A., and Curti, B. (1988) Biochemistry 27, 3753–3759
29. Hurley, J. K., Hazzard, J. T., Martinez-Júlvez, M., Medina, M., Gómez-Moreno, C., and Tullin, G. (1999) Protein Sci. 8, 1614–1622
30. Aliverti, A., Corrado, M. E., and Zanetti, G. (1993) FEBS Lett. 334, 247–250
31. Schmitz, S., Martinez-Júlvez, M., Gómez-Moreno, C., and Bohme, H. (1998) Biochim. Biophys. Acta 1407, 85–93
32. Karplus, P. A., and Bruns, C. M. (1994) J. Bioenerg. Biomembr. 26, 89–99
33. Marcus, R. A., and Sutin, N. (1985) Biochim. Biophys. Acta 811, 265–322
34. Deisenhofer, J., Petsko, A. H., Huber, R., and Michel, H. (1984) J. Mol. Biol. 180, 385–398
35. Plaut, M., Michel-Beyerle, M. E., Buxon, M., and Jortner, J. (1989) FEBS Lett. 249, 70–74
36. Chen, L., Durley, R. C. E., Mathews, F. S., and Davidson, V. L. (1994) Science 264, 86–90