# A Three-Domain Iron−Sulfur Flavoprotein Obtained through Gene Fusion of Ferredoxin and Ferredoxin-NADP$^+$ Reductase from Spinach Leaves†

Alessandro Aliverti and Giuliana Zanetti*

*Dipartimento di Fisiologia e Biochimica Generali, and Centro Interuniversitario per lo Studio delle Macromolecole Informazionali, Università di Milano, Via Celoria 26, I-20133 Milano, Italy*

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ABSTRACT: Ferredoxin and ferredoxin-NADP$^+$ reductase are the two last partners of the photosynthetic electron-transfer chain, responsible for the final reduction of NADP$^+$ to NADPH. Herein, we report the engineering and characterization of a novel protein molecule in which the electron-carrier protein (ferredoxin I) and the reductase (a flavoprotein) were covalently linked in a single polypeptide chain by gene fusion. The gene was obtained by joining the cDNAs encoding the respective proteins and subsequently by deleting the intervening sequence between them by site-directed mutagenesis. No extra amino acid residues were introduced between the C-terminus of ferredoxin I and the N-terminus of the flavoenzyme. The chimera was purified to homogeneity and characterized. The $M_r$ of the chimera apoprotein was 45 800 as determined by mass spectrometry, in agreement with the expected value of 45 846. Both flavin and iron−sulfur cluster were in 1:1 ratio with respect to the apoprotein. The chimera was found active as a diaphorase and, more interestingly, highly efficient as a cytochrome *c* reductase, without need for free ferredoxin addition in the assay medium. Several lines of evidence indicate that the ferredoxin and the reductase in the chimera assume a configuration quite similar to that in the dissociable physiological complex. Thus, the fusion protein could be a useful tool for studying the mechanism of protein−protein recognition and electron transfer in the ferredoxin−ferredoxin-NADP$^+$ reductase system.

Electron transfer between redox proteins is fundamental to many outstanding biological processes as respiration, photosynthesis, and several redox reactions of primary and secondary metabolism pathways. For several years, our interests have focused on the redox-protein partners of the last step in the eukaryotic photosynthetic electron transport chain, namely ferredoxin I (Fd)[1] and ferredoxin-NADP$^+$ reductase (FNR) from spinach leaves (*1*). Together these proteins function in transferring electrons from photosystem I to NADP$^+$. FNR is the structural prototype of a large family of flavoproteins that function as transducers between nicotinamide nucleotides (two-electron carriers) and one-electron carriers (*2*, *3*). The one-electron reducible group resides in a separate protein or is part of the same molecule as a linked domain or subunit. The Fd−FNR couple is a system of the former type, *i.e.* a dissociable two-protein system. Fd contains a [2Fe-2S] cluster, whereas FNR has a tightly bound FAD (*1*). Complex formation between the two proteins has been under investigation since several years by many laboratories. By chemical cross-linking with a soluble carbodiimide, we succeeded in stabilizing a functional protein complex and could identify some residues of the two proteins involved in the cross-links (*4*). The cross-linked complex was not considered suitable for crystallization due to apparent heterogeneity (*5*). More recently, the two proteins from spinach leaves have been cloned and overexpressed in *Escherichia coli* (*6*, *7*). The three-dimensional structures of the spinach leaf FNR and the recombinant FNR have been determined at high resolution (*8*), whereas the spinach leaf Fd has not yet been studied crystallographically. Nevertheless, the X-ray structures of several cyanobacterial ferredoxins (*9*) and of one from a higher plant (*10*) are available in the PDB data bank and could be used as a model. In an attempt to obtain the three-dimensional structure of the Fd−FNR complex and to elucidate the mechanism of interprotein electron transfer, we thought to design a chimeric protein comprising the two functional units of Fd and FNR in the same polypeptide chain. The chimeric protein should thus acquire a domain organization similar to that of known members of the family as phthalate oxygenase reductase and xylene monooxygenase (*2*, *3*) where a Fd-like domain is linked to the basic two-domain motif of FNR. In the work here described, we have engineered a gene construct encoding the chimeric three-domain protein by linking at the 3′ end of the cDNA coding for the mature isoform I of spinach leaf Fd, the 5′ end of the cDNA encoding the mature form of the spinach leaf FNR. The overexpressed chimeric protein was endowed with NADPH-cytochrome *c* reductase activity, a property unique of the Fd−FNR complex. A detailed study of the structural and kinetic properties of the chimeric protein is here reported.

## EXPERIMENTAL PROCEDURES

*Materials*. Horse heart cytochrome *c*, INT, and NADP(H) were obtained from Sigma. Recombinant FNR and Fd were purified from *E. coli* as previously described (*11*, *7*). Restriction endonucleases and T4 DNA ligase were purchased from either Gibco BRL or Boehringer Mannheim.

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* Address correspondence to this author. Tel.: +39 2 7064 4505. Fax: +39 2 236 2451. E-mail: gzanetti@imiucca.csi.unimi.it.

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[1] Abbreviations: FNR, ferredoxin-NADP$^+$ oxidoreductase; Fd, ferredoxin I; Fd−FNR, dissociable complex between ferredoxin I and ferredoxin-NADP$^+$ oxidoreductase; Fd/FNR, fusion protein between ferredoxin I and ferredoxin-NADP$^+$ oxidoreductase; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

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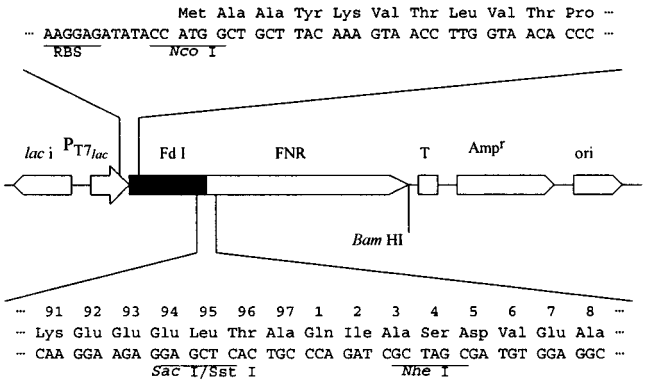


FIGURE 1: Functional map of pFd/FNR. Relevant regions are boxed. DNA sequences and corresponding amino acid sequences at the translation start position and at the junction between Fd and FNR are shown. $P_{T7lac}$, fusion between T7 RNA polymerase promoter and *lac* operator; RBS, ribosome binding site; T, T7 transcription terminator.

pET-11d was obtained from AMS Biotechnology. All other chemicals were of analytical grade.

*Construction of pFd/FNR*. The strategy outline for the construction of the expression plasmid of the Fd/FNR chimera is reported in Figure 1. A DNA fragment encoding the mature Fd sequence was excised from pUCFdI (*7*) by *Sac*I and *Sph*I double digestion, whereas a fragment coding for the mature FNR was obtained by restriction with *Sph*I and *Bam*HI of a pUC18 derivative harboring the reductase gene construct (*6*). The two fragments were ligated and inserted between the *Sac*I and *Bam*HI sites of pBluescript. The resulting recombinant plasmid was digested with *Kpn*I to yield a DNA fragment containing the two joined sequences, which was then recloned in the *Kpn*I site of M13mp18. To put the coding regions of Fd and FNR in frame as well as to remove the 280 nt intervening sequence between them, the M13 construct was subjected to a site-directed deletion using the oligonucleotide 5′GACCCA-CAAGGAAGAGGAGCTCACTGCCCAGATCG-CTAGCGATGTGGAGGCACCTCCACCT3′. This 61mer was designed so that its 5′ part is complementary to the last 28 nucleotides of the Fd gene and its 3′ region is complementary to the first 33 nucleotides of the FNR gene. The oligonucleotide contained a total of 5 silent base mismatches (underlined bases) with respect to the wild-type nucleotide sequences of Fd and FNR, in order to introduce two unique restriction sites (*Sac*I and *Nhe*I, see Figure 1) in the splicing region, which could be useful for future engineering of the protein−protein junction. The synthetic oligonucleotide was purified by PAGE followed by chromatography on a Sep-Pak C18 cartridge (Waters) prior to use in the mutagenesis procedure. The mutagenic reaction was carried out using the phosphorothioate-based method (*12*) with the Sculptor in Vitro Mutagenesis System by Amersham International. Some M13 clones were screened, by restriction analysis and nucleotide sequencing, for the presence of the desired deletion and mutations as well as for the absence of second site mutations. One of the M13 clones with the expected sequence was used for subsequent cloning steps. The Fd/FNR construction was excised from the M13 clone by *Nco*I and *Bam*HI and inserted between the same sites of pET-11d to yield pFd/FNR.

*Overexpression of the Fd/FNR Chimera*. *E. coli* strain BL21(DE3) was transformed with pFd/FNR. Cell growth and expression were performed essentially as described for Fd (*7*). Expression was induced by addition of 0.1 mM isopropyl $\beta$-D-thiogalactopyranoside, and growth was continued for 2 h at 30 and 26 °C or for 16 h at 12 °C. Analyses of total and soluble *E. coli* cell extracts were performed as already reported (*7*). In the preparation of soluble extract, ultrasonic disruption was used.

*Purification of the Fd/FNR Chimera*. All preparation steps were performed at 4 °C except for FPLC, which was carried out at room temperature. About 80 g of bacterial cells (fresh weight) was resuspended in 1.5 vol of 50 mM potassium phosphate, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mM $\beta$-mercaptoethanol. After cell sonication and centrifugation for 60 min at 43000*g* to remove cell debris, the solution was brought to 50% saturation of ammonium sulfate (2.05 M). After centrifugation, the supernatant was applied to a Sepharose 4B column equilibrated with 2.05 M ammonium sulfate in 50 mM potassium phosphate, pH 8.0. The chimera was eluted by a linear gradient from 1.64 to 0.41 M ammonium sulfate in the same buffer. After precipitation at 75% saturation of ammonium sulfate (3.08 M) and dialysis against 50 mM Tris-HCl, pH 7.4, the chimera preparation was loaded on a Q-Sepharose high-performance column (Pharmacia Biotech) equilibrated in the same buffer. The protein was eluted with a linear gradient from 50 mM to 1 M Tris-HCl, pH 8.0. The eluted sample was brought to 40% saturation of ammonium sulfate (1.64 M) and loaded on a phenyl-Sepharose high-performance column (Pharmacia Biotech) equilibrated in 1.64 M ammonium sulfate in 50 mM potassium phosphate, pH 8.0. The chimera was eluted with a linear gradient from 1.64 to 0 M ammonium sulfate in the same buffer. The protein was concentrated by ultrafiltration, desalted by gel filtration, and stored under nitrogen.

*Electrophoresis*. PAGE in the presence of sodium dodecyl sulfate was carried out on 12% polyacrylamide gels, essentially as described in (*7*). Nondenaturing PAGE was run on 12−22.5% polyacrylamide gradient gel slabs omitting sodium dodecyl sulfate. Isoelectrofocusing was carried out on polyacrylamide gels in the pH range 2.5−6.5.

*Gel Filtration*. Gel filtration was performed by FPLC on a Superdex 75HR 10/30 column (Pharmacia Biotech) using 50 mM sodium phosphate, pH 7.2, containing 150 mM NaCl.

*Mass Spectrometry*. Matrix-assisted laser-desorption/ionization mass spectrometry was carried out in a Vestec Focus mass spectrophotometer using ferulic acid as a matrix. The apoprotein of the Fd/FNR chimera was prepared by trichloroacetic acid precipitation.

*Spectral Analyses*. Absorption spectra were recorded with a Hewlett-Packard 8453 diode-array spectrophotometer. Fluorescence measurements were performed with a Jasco FP-777 spectrofluorometer at 15 °C. The extinction coefficient of the chimera was determined by releasing FAD from the protein under conditions in which the spectral properties of the iron−sulfur cluster were unaffected. Solutions (ca. 20 $\mu$M) of Fd/FNR chimera, FNR, and Fd were prepared in 10 mM Tris-HCl, pH 7.4, and their spectra were recorded. Sodium dodecyl sulfate was added to 0.2% final concentration. Spectral changes due to FAD release from FNR or Fd/FNR were completed in a few minutes at 25 °C, whereas no spectral changes were observed in the case of Fd, even after prolonged incubation. The spectrum, in the visible region, of the chimera after treatment with sodium dodecyl sulfate was analyzed using the multicomponent analysis

feature of the Hewlett-Packard UV−Visible ChemStation software. FAD and iron−sulfur cluster contents were determined using the $\epsilon_{450}$ of 11.3 $mM^{-1}$ $cm^{-1}$ for free FAD and the $\epsilon_{420}$ of 9.68 $mM^{-1}$ $cm^{-1}$ of Fd.

*Catalytic Studies*. Standard diaphorase assays were performed using as electron acceptor either 1 mM $K_3Fe(CN)_6$ in 0.1 M Tris-HCl, pH 8.2, or 0.1 mM INT in 0.2 M Tris-HCl, pH 9.0, containing of 70 mM NaCl and 0.1% Triton X-100. Cytochrome *c* reductase activity was assayed with 50 $\mu$M cytochrome *c* as the final electron acceptor in 0.1 M Tris-HCl, pH 8.2. Unless otherwise stated, 8 $\mu$M Fd was present. In all standard assays, 0.1 mM NADPH was present and kept constant by a glucose 6-phosphate/glucose 6-phosphate dehydrogenase regenerating system. Steady-state kinetics were carried out by varying the concentrations of both electron donor and acceptor; the resulting data were fitted to a ping-pong mechanism using the Hewlett-Packard enzyme kinetic software. For studying the ionic strength effect on enzyme activity, the cytochrome *c* reductase activity was assayed in 20 mM Tris-HCl, pH 8.2, in the presence of NaCl concentrations varying over the 0−1 M range.

## RESULTS

*Production of the Chimeric Protein by Gene Fusion and Heterologous Expression.* The gene fusion between spinach Fd and FNR was obtained by joining two cDNA fragments encompassing the regions coding for the mature form of the two proteins, and then deleting by oligonucleotide-directed mutagenesis the intervening sequence between the C-terminal codon of Fd and the N-terminal codon of FNR, as described in the Experimental Procedures (see Figure 1). Several clones with the expected nucleotide sequence were obtained from the mutagenesis procedure. The Fd/FNR cDNA fusion excised from one of these clones was inserted in the pET11d vector. The resulting pFd/FNR plasmid was used for expression in *E. coli*. If required, different peptides can be designed and easily introduced as linkers in the chimera, due to the presence of two restriction sites strategically engineered at both sides of the junction point between the Fd and FNR cDNAs (see Figure 1).

Upon induction, BL21(DE3) *E. coli* cells harboring pFd/FNR expressed a polypeptide showing an apparent $M_r$ of 52 000 in denaturing PAGE (Figure 2), which was immunoreactive toward anti-FNR antibodies in a western blot (not shown). When bacterial cultures were grown at 37 °C, most of the Fd/FNR chimera accumulated in insoluble form. Several growth and induction conditions were tested to optimize the production of the recombinant protein in soluble form. As shown in Table 1, FNR-related activities in soluble cell extracts were found to substantially increase by lowering the growth temperature during induction. A correlation was clearly found between the diaphorase activity of the cell extract and its cytochrome *c* reductase activity assayed without Fd addition in the reaction mixture. The high level of the latter activity was taken as an indication that the recombinant protein, in addition to maintaining the INT reductase activity of its FNR component, had acquired the ability to catalyze cytochrome *c* reduction at high rate, without need of free Fd as a mediator. Cytochrome *c* reductase activity was thus used as a typical assay for the Fd/FNR chimera, due to its greater specificity with respect to diaphorase assays. A temperature of 10−12 °C was chosen for subsequent expression of the Fd/FNR gene fusion in *E. coli* and production of the chimeric protein. Under these conditions, the recombinant product accounted for about 1.3% of soluble *E. coli* proteins.

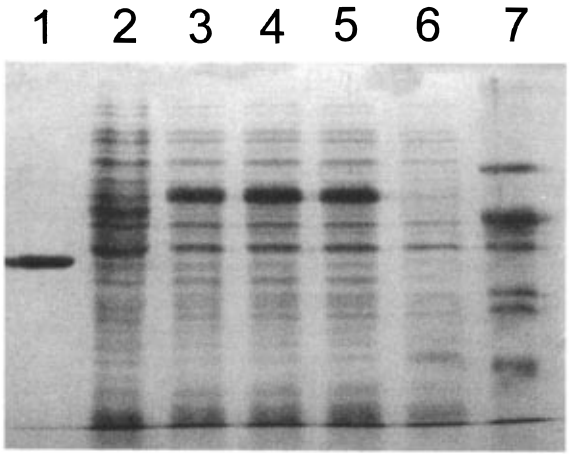


FIGURE 2: Sodium dodecyl sulfate−PAGE of total *E. coli* cell extracts: analysis of the Fd/FNR fusion protein synthesis at 37 °C. Lane 1, purified FNR; lane 2, nontransformed BL21(DE3) host; lanes 3−5, cells transformed with pFd/FNR (three different clones); lane 6, cells transformed with pETFdI expressing Fd (see ref 7); lane 7, molecular weight markers (mass values in kDa are as follows: 66, 45, 36, 29, 24, 20.1).

Table 1: Effect of the Growth Temperature on the Production of the Fd/FNR Fusion Protein

| | specific activity[a] (units/mg) | |
|---|---|---|
| growth temperature (°C) | INT | cyt *c* |
| 30 | 0.59 | 0.34 |
| 26 | 0.65 | 0.46 |
| 12 | 1.78 | 1.19 |

[a] Activities were measured in the soluble cell extracts.

Table 2: Purification of the Fd/FNR Fusion Protein

| step | protein (mg) | activity (units) | specific activity (units/mg) | yield (%) |
|---|---|---|---|---|
| crude extract | 7480 | 7800 | 1.04 | 100 |
| Sepharose 6B | 492 | 4290 | 8.72 | 55 |
| Q-Sepharose | 78.5 | 4250 | 54.1 | 54 |
| phenyl-Sepharose | 40.7 | 3210 | 78.9 | 41 |

*Purification of the Fd/FNR Chimera.* As expected, the recombinant protein showed a behavior intermediate between that of Fd and that of FNR in the various chromatographies used. The most efficient purification procedure for the chimera was similar to that used to purify the recombinant Fd, with some modifications required by the substantial increase in hydrophobicity of the chimera due to the FNR component. As shown in Table 2, the major increase in purification was produced by the first two steps, namely, ammonium sulfate fractionation coupled to salt-promoted adsorption chromatography on Sepharose 6B and anion-exchange chromatography on Q-Sepharose. At this stage, a 50-fold purification resulted in substantial removal of protein contaminants from the chimera as shown in Figure 3 (lane 4). Final purification was achieved by hydrophobic interaction chromatography on phenyl-Sepharose, which yielded a homogenous preparation of the chimera as judged by denaturing PAGE (Figure 3, lane 5). Removal of contaminant nucleic acids, which were a major concern in

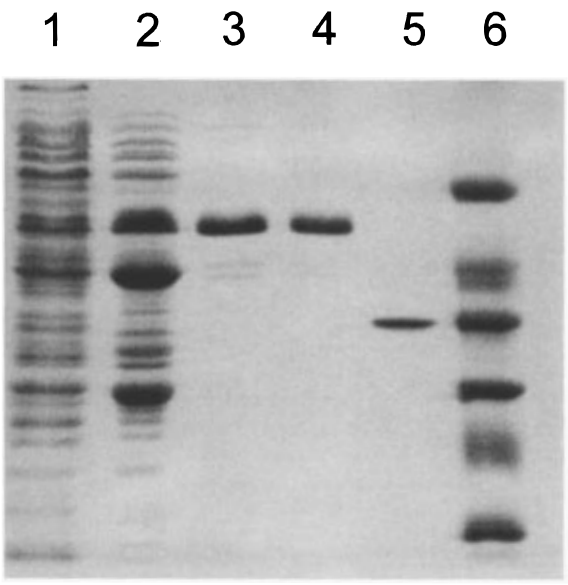


FIGURE 3: Purification of the Fd/FNR chimera as analyzed by sodium dodecyl sulfate−PAGE. Lane 1, crude extract; lane 2, after Sepharose 4B and dialysis; lane 3, after Q-Sepharose; lane 4, after phenyl-Sepharose and desalting; lane 5, purified FNR; lane 6, molecular weight markers (mass values in kDa are as follows: 66, 45, 36, 29, 24, 20.1, 14.2).

the case of recombinant Fd (*7*), was accomplished in the first step of the procedure. After chromatography on Sepharose 6B and ammonium sulfate precipitation, a value of 1.35 for the $A_{280}/A_{260}$ ratio was calculated from the spectrum of the chimera-containing fraction, which is the same shown by the purified fusion protein. By this purification protocol, about 0.5 mg of Fd/FNR chimera per gram (fresh weight) of *E. coli* cells was obtained in homogenous form.

*Molecular Properties.* In denaturing PAGE, the Fd/FNR chimera showed a $M_r$ of about 52 000 (Figure 3), a value significantly higher than that resulting from the sum of the individual masses of Fd and FNR ($M_r$ 45 846). However, it is well-known that Fd, as well as other ferredoxins, yields a higher $M_r$ value by sodium dodecyl sulfate−PAGE (about 20 kDa), due to their high content of negative charges (*13*). Thus, it is not surprising that the ferredoxin-containing chimera also yields a higher $M_r$ value by the same technique. A single, symmetrical peak was obtained by analytical gel filtration of the purified protein on a Superdex 75 column, yielding an approximate value of 54 000 Da (data not shown). A more precise value for the $M_r$ of the chimera apoprotein was determined using matrix-assisted laser-desorption/ionization mass spectrometry. As shown in Figure 4, the purified protein preparation gave a single sharp peak with a $M_r$ of 45 800 in close agreement with the expected value of 45 846 Da. This value is consistent with the *in vivo* removal of the N-terminal methionine from the neosynthesized polypeptide chain, as was previously observed for the spinach Fd expressed in *E. coli* (*7*).

The chimera was also analyzed by nondenaturing PAGE. A sharp band was obtained which showed a mobility intermediate between those of Fd and FNR. In isoelectrofocusing, the chimera focused as a single band at pH 4.4, a pI value identical to that of the physiological complex between Fd and FNR.

*Spectral Properties.* The absorption spectrum of the purified protein is shown in Figure 5. The absorbance in

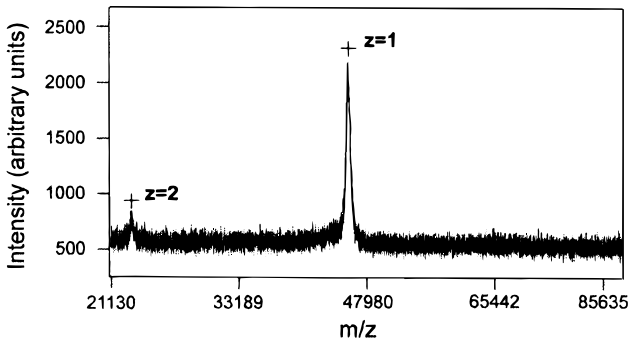


FIGURE 4: Matrix-assisted laser desorption/ionization mass spectrum of the Fd/FNR chimera. The two peaks correspond to the singly (*m/z* 45 800) and doubly charged (*m/z* 22 900) molecular ions of the Fd/FNR chimera.

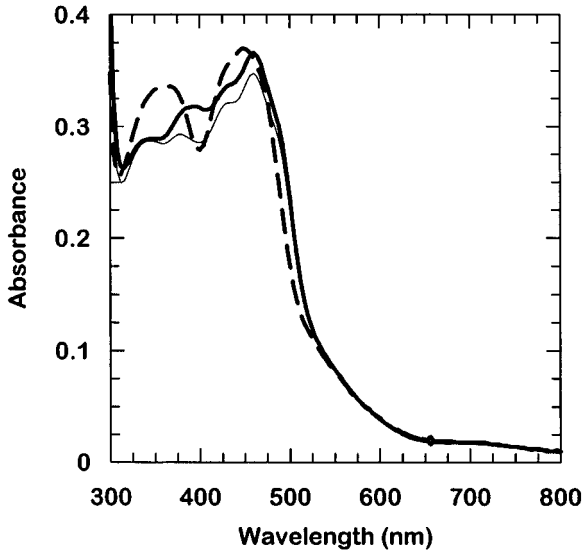


FIGURE 5: Spectrum in the near-ultraviolet and visible regions of the Fd/FNR chimera before and after release of the bound FAD. Solid line, native Fd/FNR chimera; dashed line, the same sample after 5 min of incubation with 0.2% sodium dodecyl sulfate at 25 °C; thin line, computed spectrum of equal amounts of Fd and FNR. Experimental details are reported in the Experimental Procedures.

the visible region is that typical of an iron−sulfur flavoprotein. A simple spectrophotometric method was developed for quantitative determination of FAD contained in the chimera, taking advantage of Fd stability toward sodium dodecyl sulfate treatment. Whereas FNR-bound FAD was readily and quantitatively released in the presence of 0.2% sodium dodecyl sulfate at room temperature, prolonged incubation of Fd under these conditions did not result in spectral changes. When the Fd/FNR chimera was incubated in the same conditions, a spectrum was recorded (Figure 5), which could be exactly fitted by a linear combination of the spectra of free FAD and Fd. Computer-based fitting of the experimental spectral data allowed us to calculate the concentrations both of the free FAD and of the ferredoxin iron−sulfur cluster; they were found to be present in a 1:1 ratio. Thus, a calculation of the extinction coefficient of the chimera yielded a value of 20 300 $M^{-1}$ $cm^{-1}$ at 460 nm, which was significantly larger than that expected. Indeed, comparison of the chimera visible spectrum with that computed for an equimolar mixture of the two proteins revealed the discrepancy (Figure 5). Since it is well-known that Fd binding to FNR increases the absorbance of the reductase-bound flavin, these observations suggest that a

Table 3: Catalytic Activity (in Units/FAD) with Various Electron Acceptors of FNR and the Fd/FNR Fusion Protein

| enzyme | INT | $K_3Fe(CN)_6$ | cyt *c* |
|---|---|---|---|
| FNR | 2800 | 20 000 | 3800[a] |
| Fd/FNR | 3830 | 17 392 | 3325 |

[a] The cytochrome *c* reductase activity of FNR was assayed in the presence of 8 $\mu$M Fd.

protein−protein interaction similar to that in the physiological complex was occurring in the chimera. Furthermore, the protein fluorescence of the Fd/FNR chimera was found to be much lower than that of FNR (30% less on a molar basis). Fd has been shown to quench the protein fluorescence of the reductase upon binding (*14*). Thus, the lower protein-fluorescence emission found for the chimera again supports the structural similarity between the chimera and the dissociable complex.

*Catalytic Properties.* The reductase moiety of the Fd/FNR chimera maintained most of its functionality as a diaphorase, although with a somewhat altered electron-acceptor specificity. When assayed in nonsaturating standard conditions (Table 3), apparently, the ferricyanide reductase activity of the chimera was smaller than that of FNR and *vice versa* for the INT reductase activity. By steady-state kinetic studies, it was found that the apparent $K_m$ values for the two electron acceptors of the chimera were somewhat different from those of FNR, *i.e.* the $K_m$ for ferricyanide was increased whereas that for INT was decreased. A fairly high ferredoxin concentration is required in the assay mixture for the catalysis of NADPH-cytochrome *c* reduction by FNR, which is by itself inactive in such a reaction. As noted earlier, the chimera was found to be able to catalyze this reaction without addition of free ferredoxin. The chimera showed about 90% of the activity of FNR in the presence of 8 $\mu$M Fd (Table 3). Addition of the same amount of Fd to the chimera assay increased only slightly the cytochrome *c* reduction rate. Steady-state kinetic studies allowed us to determine kinetic parameters both for the ferricyanide and the cytochrome *c* reductase activities of the chimera (Table 4). It can be seen that the chimera is fully active as cytochrome *c* reductase in saturating conditions, with a catalytic efficiency that closely approaches that of the dissociable system.

*Catalyst Concentration Dependence of Cytochrome c Reductase Activity.* The high turnover number of the chimera as a NADPH-cytochrome *c* reductase suggested that it transfers electrons from NADPH to cytochrome *c* via intramolecular transfer from FAD to the [2Fe-2S] cluster. However, an alternative pathway could also be envisaged. The chimera could catalyze the reaction via intermolecular electron transfer, *i.e.* through the involvement of two chimeric molecules per catalytic cycle. The NADPH-reduced flavin of a chimera molecule could transfer an electron to the [2Fe-2S] of another chimera molecule, which would then be reoxidized by cytochrome *c*. To ascertain the actual catalytic mechanism of cytochrome *c* reduction by the chimera, the dependence of the reaction rate on catalyst concentration was studied. As shown in Figure 6, the Fd/FNR chimera and an equimolar ratio of Fd and FNR behaved quite differently, the former system being up to 50-fold more active than the latter at low enzyme concentration. More interestingly, the dissociable system yielded a concave curve with the turnover number increasing with increasing catalyst concentration, whereas for the chimera a straight line was obtained in the same plot. These data clearly ruled out any intermolecular, concentration-dependent electron-transfer step in the case of the reaction catalyzed by the chimera.

*Ionic Strength Dependence of Cytochrome c Reductase Activity.* The ionic strength dependence of the cytochrome *c* reductase activity of the Fd/FNR chimera was found similar to that of the dissociable Fd−FNR complex (Figure 7). Indeed, the activity of both systems was influenced by ionic strength in a complex biphasic fashion. Nevertheless, the ionic strength optimum for the cytochrome *c* reductase activity of the chimera was found to be higher than that of the dissociable complex (100−150 vs 50−100 mM, respectively). Furthermore, the activity of the chimera remained substantially high at ionic strengths greater than 150 mM, showing still 35% of the maximal activity at $I = 500$ mM.

## DISCUSSION

In several enzymes of the FNR family that use for catalysis both a flavin and an iron−sulfur cluster, the iron−sulfur domain acts as an independent modular unit. This unit can be either a separate protein or a domain linked in different combinations to flavin and pyridine nucleotide binding domains. Furthermore, there is a growing list of enzymes comprising different subunits or proteins that are synthesized as independent units in one organism and fused together in another. A well-known case is that of the bacterial P450 reductase, which comprises in one polypeptide chain both the reductase and the cytochrome P450 (*15*). Indeed, several groups have produced synthetic chimeric proteins between these proteins from the same organism or even from different organisms (*16*, *17*). A more recently reported case is that of thioredoxin reductase, which in *Mycobacterium leprae* is present as a fusion between the flavoprotein component and the protein substrate thioredoxin (*18*).

The primary goal of obtaining a functional chimeric protein by expression of a cDNA construct encoding the mature forms of ferredoxin and the reductase, has been successfully met. This success was probably due to the choice of putting first the Fd gene and joining in 3′ to it the FNR gene under the control of an inducible promoter in the expression plasmid. This gene arrangement was borne out by two considerations. First, the N-terminal 18 residues of the spinach FNR are not visible in the X-ray structure (*8*, *19*), suggesting that this part of the polypeptide chain is disordered and unrestrained. Thus, it could have worked as a natural spacer arm to allow for a proper interaction of the two joined proteins. Second, the C-terminal residue of FNR is Tyr314, the side chain of which is positioned right at the active site opposite to the isoalloxazine ring of FAD (*19*). It has been proposed by Karplus (*19*, *8*) that the Tyr314 ring should move away from its position to allow the interaction of the nicotinamide ring of NADP(H) with the flavin so that hydride transfer could occur. Indeed, pea FNR mutants, in which this Tyr has been changed to Ser or Gly or deleted, showed a highly decreased activity (*20*). Furthermore, the best expression plasmid for FNR was that coding for a fusion protein with the maltose binding protein (*11*). The chosen orientation (Fd first, then the reductase) is opposite to that found in phthalate oxygenase reductase (*2*), the best known representative of the FNR family in which the reductase and ferredoxin constitute two domains of a single polypeptide

Table 4: Comparison of the Kinetic Parameters for the Ferricyanide and Cytochrome *c* Reductase Reactions of the Fd/FNR Chimera with Those of FNR

| | $K_3Fe(CN)_6$ | | | cyt *c* | | |
|---|---|---|---|---|---|---|
| enzyme | $k_{cat}$ ($e^-$eq $s^{-1}$) | $K_m^{NADPH}$ ($\mu$M) | $k_{cat}/K_m$ ($e^-$eq $s^{-1}$ $M^{-1}$) | $k_{cat}$ ($e^-$eq $s^{-1}$) | $K_m^{NADPH}$ ($\mu$M) | $k_{cat}/K_m$ ($e^-$eq $s^{-1}$ $M^{-1}$) |
| FNR[a] | 500 | 35 | $1.43 \times 10^7$ | 76 | 9.3 | $8.2 \times 10^6$ |
| Fd/FNR | 420 | 32 | $1.33 \times 10^7$ | 80 | 13.2 | $6.1 \times 10^6$ |

[a] Values taken from refs 1 and 11 for the ferricyanide and cytochrome *c* reductase reactions, respectively. "$e^-$eq" refers to "electron equivalent".

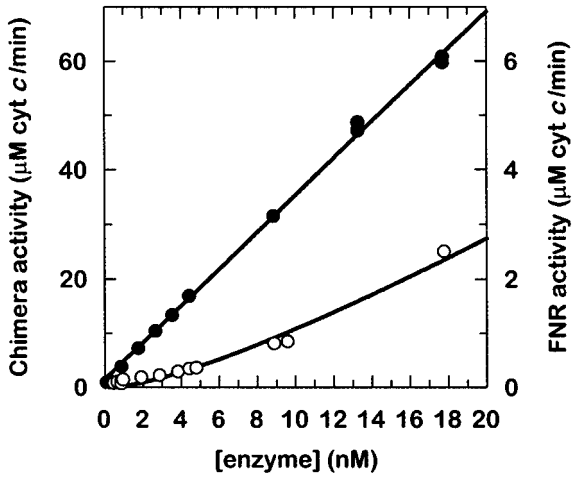


FIGURE 6: Comparison between the NADPH-cytochrome *c* reductase activities of the Fd/FNR chimera and the dissociable Fd−FNR system. The fusion protein (●) and an equimolar mixture of Fd and FNR proteins (○) were assayed at various catalyst concentrations in the conditions described in the Experimental Procedures.

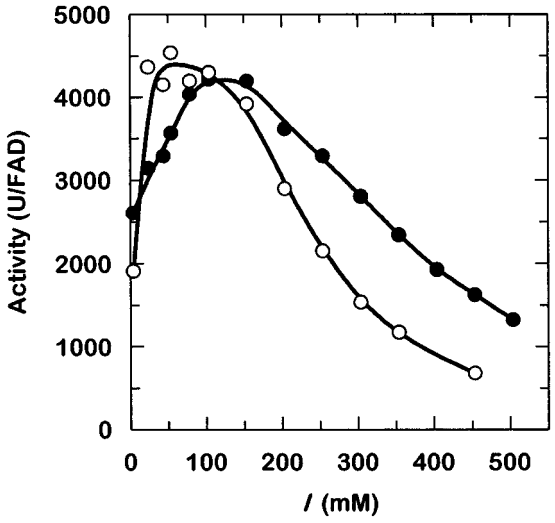


FIGURE 7: Ionic-strength dependence of the NADPH-cytochrome *c* reductase activities of the Fd/FNR chimera and of the dissociable Fd−FNR complex: (●) Fd/FNR chimera; (○) FNR assayed in the presence of 4 $\mu$M Fd.

chain. Nevertheless, other members of the family as xylene monooxygenase reductase (*21*) have the same orientation as the artificial chimera. Unfortunately, no three-dimensional structure is available for this latter type. The N-terminal part of ferredoxin is far away from the iron−sulfur center and thus, it could have been the best choice for manipulation. The C-terminal region instead contains a residue, Glu92, which is conserved in all the chloroplast-type ferredoxin and it has been shown to be important for the protein function by site-directed mutagenesis (*22*, *23*). Yet, the constraints dictated by the FNR structure were considered more important for the function of the chimeric protein and only the first orientation was attempted.

The expression of the chimeric gene was found to be more critical than either one of the single genes. Growth of *E. coli* harboring the pFd/FNR plasmid at 37 °C produced a high amount of insoluble chimeric protein, which was worthless because of the nearly impossible task of reconstituting a functional holoenzyme with its FAD and iron−sulfur cluster from the apoprotein. By decreasing the growth temperature, a procedure already set up for FNR production (*11*), it was possible to obtain the fusion protein, in soluble form, in high yield. It was envisaged that by slowing down the synthesis process, additional time is given for correct folding and prosthetic groups insertion of the three-domain protein, thus avoiding protein precipitation by hydrophobic interchain interactions. It is well-known that the apoprotein form of FNR is quite unstable and tends to form aggregates (*24*). The purification procedure has been set up on the basis of the known properties of the two proteins joined in the chimera. Its purification was more demanding than that of the single proteins. However, a very good yield (about 40%) allowed us to reproducibly obtain 40 mg of purified protein from 80 g of *E. coli* cells.

Several analyses revealed that the isolated chimeric protein comprises the properties both of ferredoxin and the reductase. Moreover, it showed a catalytic activity unique of the dissociable and cross-linked complexes between the proteins, *i.e.* the catalysis of electron transfer from NADPH to cytochrome *c* (*1*, *5*). It was felt particularly important to demonstrate that the fusion protein had a stoichiometric content of FAD and iron−sulfur cluster. The molar extinction coefficient at 460 nm (20.3 $mM^{-1}$ $cm^{-1}$) determined for the chimera, which is higher than the sum of each protein values, suggests that the two chromophores in the chimeric protein are influencing each other as found in the dissociable and in the cross-linked complexes (*25*, *5*). Furthermore, the protein fluorescence of the fusion protein is 71% of that of FNR, confirming that the interaction between the Fd and reductase domains mimics that of the dissociable complex between the free proteins in which the quenching of the FNR protein fluorescence by Fd reached about 40% at 1:1 stoichiometry (*14*). A similar quenching was found in the cross-linked complex (*14*). The chimeric protein maintained all the enzymic activities of the free reductase and most importantly it gained the ability to directly transfer electrons to cytochrome *c*. The reductase acquires this activity only in the presence of ferredoxin [or flavodoxin (*25*,*26*)]. By comparing the activity of the chimeric protein with that of the dissociable complex (1:1 ratio of the two proteins) as a function of concentration, it was shown that electron transfer takes place intramolecularly between the two prosthetic groups of the chimeric protein at high rate, since a perfectly linear correlation was found in the plot. Furthermore, the

activity of the chimera was always found much higher than that of the dissociable complex, suggesting a higher efficiency of the chimera. The effects of ionic strength on the cytochrome *c* reductase activity of the chimera and of the dissociable complex were analyzed. In the case of the dissociable Fd−FNR, the complex ionic strength effects have been explained in terms of two distinct molecular mechanisms (*27*). At very low ionic strength there is a stabilization of a Fd−FNR "tight complex", in which electron transfer is inhibited. By increasing the ionic strength, the weakening by salts of the electrostatic interactions between ferredoxin and FNR (as well as with cytochrome *c*) produces a complex optimal for activity, which then becomes less active at higher ionic strength. The same hypothesis seems to hold for the chimera, although the covalent linking within the same polypeptide chain constrains the conformational freedom of the Fd and FNR moieties, thus resulting in a higher ionic strength optimum and a higher stability of the chimera toward disruption of the catalytic complex by salts.

In conclusion, the fusion protein has been shown to possess several properties of the dissociable complex such as the spectral perturbations, the quenched protein fluorescence and the catalysis at high rate of electron transfer between NADPH and cytochrome *c*. This would indicate a spatial configuration of the two prosthetic groups very similar to that present in the physiological dissociable complex. On the other hand as expected, the chimera was less influenced by high ionic strengths and was much more efficient, at low concentration, in the electron transfer from NADPH to cytochrome *c* in comparison with the dissociable complex.

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