Site-specific Mutagenesis of *Rhodobacter capsulatus* Ferredoxin I, FdxN, That Functions in Nitrogen Fixation

ROLE OF EXTRA RESIDUES*

Kazuhiko Saeki‡, Ken-ichiro Tokuda‡, Keiichi Fukuyama‡, Hiroshi Matsubara‡, Kazuhiko Nadanami‡, Mitiko Goi, and Shigeru Itoh**

From the ‡Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560, and the **Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa, Nagoya 464-01, and the **National Institute for Basic Biology, Myodaijicho, Okazaki 444, Japan

One of the two [4Fe-4S]-type clusters of the *Rhodobacter capsulatus* ferredoxin I, FdxN, was modified through site-specific mutagenesis of the distinctive features of the second cluster-binding motif, Cys-38-X2-Cys-41-X8-Cys-596-X4-Cys-59. First, various mutagenized products were tested to learn whether they could rescue the decreased capacity of an *fdxN*-null strain MSA1 to fix nitrogen: the phenotype of MSA1 was reassessed to Nif" (slow growth by nitrogen fixation) from our previous description of Nif" (Saeki, K., Suet-sugu, Y., Tokuda, K., Miyatake, Y., Young, D. A., Marrs, B. L. and Matsubara, H. (1991) J. Biol. Chem. 266, 12889–12895). Substitution of Cys-39 to Ser yielded an almost fully active product, while that of Cys-41 did not. Gradual deletions and deletion-substitution of the 8 residues between Cys-41 and Cys-50 also yielded active products. Second, three of the modified FdxN proteins were subjected to purification. Only the GA protein, whose 8 residues between positions 42 and 49 were replaced by the Gly-Ala sequence, was purified. The GA protein and the authentic FdxN showed similar optical properties. The two clusters in the former had $E_m$ values of $-490$ and $-430$ mV, while those in the latter had an identical value of $-490$ mV, when determined by EPR analysis. It was concluded that: 1) Cys-58 is not a ligand to [4Fe-4S] clusters but is important for structural integrity, 2) the residues between positions 42 and 49 may form a “loop-out” from a structure analogous to the *Peptococcus aerogenes* ferredoxin, and 3) the loop-out region does not have functional significance in nitrogen fixation but may be responsible for maintaining the highly negative redox potential of one of the two clusters.

Feredoxins are generally small iron-sulfur proteins that function in diverse metabolic pathways. They can be divided into several classes depending on the nature and number of the Fe-S clusters, i.e. the [2Fe-2S], [4Fe-4S], [2Fe-4S], and [4Fe-4S][3Fe-4S] types (1, 2). Most 2[4Fe-4S]-type ferredoxins consist of less than 60 amino acids and have internal sequence similarities between the amino-terminal and carboxyl (COOH)-terminal halves. They possess two cluster-binding motifs of a sequence consensus, Cys-X$_2$-Cys-X$_2$-Cys-X$_2$-Cys. Each [4Fe-4S] cluster is chelated by the first three Cys residues of one motif and the last Cys residue of the other motif. These properties reflect on their tertiary structures that display an approximate internal 2-fold rotation axis to relate the two clusters and most of the polypeptide chain (3–5).

Among the 2[4Fe-4S]-type ferredoxins, there is a subclass in that the second cluster-binding motif conforms to a distinct sequence consensus, Cys-X$_2$-Cys-X$_2$-Cys-X$_2$-Cys-X$_2$-Cys, as depicted in Fig. 1 (1). This consensus can be characterized by two features: 1) possession of “extra” residues, where the number of residues between second and third Cys is larger than the common number two; and 2) possession of an “additional” fifth Cys, which can be a potential ligand to the Fe-S cluster. Members of this subclass have been purified from photosynthetic bacteria that are diazotrophs (6–11). In addition, several members have also been identified as products of potential ferredoxin genes, which have often been designated as *fxdN*, in the *nif* (or *anf*) gene region of photosynthetic (12) and non-photosynthetic diazotrophs (13–17). Here, we tentatively designate this subclass as photosynthetic bacterial and *nif*-associated ferredoxins. The distribution among such bacterial species and the results of gene disruption experiments performed with *Bradyrhizobium japonicum* (13), *Rhizobium meliloti* (15), and *Rhodobacter capsulatus* (18, 19) may lead to the inference that these members have a function(s) in nitrogen fixation. Nevertheless, a gene to encode a ferredoxin of this subclass is also identified in a non-diazotrophic bacterium *Haemophilus influenzae*, whose whole nucleotide sequence of the genome has recently been determined (20).

It is of interest if the above two characteristics of the unique second cluster-binding motif have physiological and/or physicochemical significance, but there have been only a few studies on this subject. At the physiological level, the site-specific mutagenesis of *R. meliloti* FdxN has shown that the substitution of the additional Cys to Ser abolishes the capacity to support nitrogen fixation *in planta*, while the deletion of the extra residues has little effect (21). At the structural level, the nuclear magnetic resonance (NMR) analyses of the *Chromatium vinosum* ferredoxin, which has a unique COOH-terminal extension of 22 amino acids, have revealed that this extension sequence forms an α-helix and interacts with a turn formed by the extra residues between the Cys residues. It was also shown that the additional Cys residue is not a ligand to the [4Fe-4S] clusters (22). These studies designed under different disciplines remain to be interrelated.

*This work was supported in part by a grant from the Nissan Foundation for Science Promotion (to K. S.) and by Grants-in-aid for Scientific Research 07640862 (to K. S.), 04225218 (to K. F.), and 07228266, 12889–12895, and 07640862 (to K. S.), 04225218 (to K. F.).*
The photosynthetic bacterium *R. capsulatus* contains as many as six ferredoxins of different classes (23–28); among them, the ferredoxin I encoded by the *fdxN* gene belongs to the photosynthetic bacterial and *nif*-associated subclass (29, 30). Despite the possession of a variety of ferredoxins and a flavodoxin (31), the disruption of *fdxN* severely decreases the capacity to fix nitrogen (18, 19), and this defective phenotype can be complemented by the reintroduction of the gene (18, 32). In this paper, we have site-specifically mutagenized the *R. capsulatus* ferredoxin I gene to investigate the functional significance of the additional Cys residue and the extra residues. We further purified the engineered gene product that lacks the extra residues and compared its physicochemical properties with those of the engineered gene product that lacks the extra residues and Cys residue and the extra residues.

**Experimental Procedures**

**Bacterial Strains and Culture Conditions—*Escherichia coli* strains DH5α (33) for plasmid construction, TG1 (33) for preparation of single strand DNA (ssDNA),1 CJ236 (34) for preparation of uracil-containing ssDNA, and Top10 (35) for conjugal transfer of pBR322 derivative to *R. capsulatus* were grown at 37°C in either 2×YT or LB medium (33). The *R. capsulatus* strains used were wild-type SB1003 (36) and *fdxN*-null MSA1 (18). Photosynthetic growth on either malate-(NH₄)₂SO₄, or peptone yeast extract medium PYS, was performed using an Applied Biosystems DNA sequencer (model 373A) after the thermal cycling reaction with 15 ng template DNA and was used to replace the *Amp r* gene. The resulting vector pTSV1 has the *puf* promoter followed by the *pufQ* gene, and the EcoRI/SacI*KpnI* Smal/BamHI multi-cloning site originating from pUC118 (41). The plasmid pMFM3, which was the template plasmid in site-specific mutagenesis, was constructed by inserting a BamHI linker, pCG-GATCCG, into the *pFAd3* (18), which was digested with *Xhol* and *HindIII* and then blunted with Klenow polymerase.

Site-specific mutagenesis of *fdxN* was performed according to Kunkel et al. (34) using unradiated ssDNA produced by *E. coli* CJ236 harboring the plasmid pMFM3 (pMFM3/CJ236). The mutated oligonucleotide primers used are listed below.

| Primer Name | Sequence | Function |
|-------------|----------|----------|
| MT3:       | 5'-ACC GAA TGC GGC GGC TGC GTC AAG G-3' | Forward primer for site-directed mutagenesis |
| MT4:       | 5'-GTC AAC GCC TCG ATG ACC GAC A-3' | Reverse primer for site-directed mutagenesis |
| MT5:       | 5'-ACC GAC AAC TCG ATC AAC CCG G-3' | Mutant primer for site-directed mutagenesis |
| K3:        | 5'-GAA TGC GAA GGC CAA TGC GTC AAC-3' | Mutant primer for site-directed mutagenesis |
| K4:        | 5'-GAA TGC GAA GGC CCA TGC GTC AAC-3' | Mutant primer for site-directed mutagenesis |
| K4p:       | 5'-GAA TGC GAA GGC CCC CAA TGC GAC-3' | Mutant primer for site-directed mutagenesis |
| K5:        | 5'-GAA TGC GAA GGC CTA CCG CAA TG-3' | Mutant primer for site-directed mutagenesis |
| K7:        | 5'-TGC GAA GGC GAT CTC CCG CAA TG-3' | Mutant primer for site-directed mutagenesis |

**Sequences 1–9**

(Altered nucleotides and deleted positions are underlined and double-underlined, respectively.)

The obtained plasmids were subjected to sequence determination using an Applied Biosystems DNA sequencer (model 373A) after the thermal cycling reaction with Taq DNA polymerase. Those confirmed for the expected mutagenesis events were named pMTM3–pMTM5 and pMTM3–pMTM7, respectively, according to the primers used. Plasmid pMTM4 was used to generate unradiated ssDNA, and a second mutagenesis was performed with the MT5 primer to obtain plasmid pMTM45.
was then subcloned in pTSV1 to make plasmid pFPN. The other 10 expressed pPM3–pPM5, pKT3–pKT7, and pPM45 to the pTSV1 vector.

Construction of the plasmid, pYSA111, to express the fdxN* gene under the control of the puf promoter will be described elsewhere.3 Nitrogenase Activity—Nitrogenase activity with R. capsulatus cells under anaerobic conditions was measured by the acetylene reduction method as described previously (38). R. capsulatus cells were grown in RCV-E medium for 24 h and washed twice with anaerobic RCV-NF medium before use.

Purification of Engineered Ferredoxin—R. capsulatus MSA1 cells harboring either plasmid pFPN, pPM3, pPM4, or pPM5 were photosynthetically grown for overnight in a PYS medium supplemented with 40 μM iron sulfate and 1 μg/ml spectinomycin, harvested by centrifugation, and stored at −70 °C until use. All the procedures, except the cell disruption and centrifugation steps, were performed in a Coy anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) using anaerobic buffers prepared by bubbling with N2 gas and degassing. Cells, approximately 80 g (wet weight), were thawed and suspended with 100 ml of 0.1 M Tris-Cl buffer (pH 8.0) containing 5 mM dithiothreitol. The suspension was passed through a French pressure cell at approximately 20,000 lb/in2 and was anaerobically transferred to a screw-cap tube under a flow of N2 gas, then centrifuged at 180,000 × g for 90 min. The supernatant was degassed and applied to a DEAE-Sepharose fast-flow column (2.5 × 10 cm). The column was first washed with 100 ml of 0.1 M Tris-Cl buffer (pH 8.0) containing 5 mM sodium dithionite (DT buffer) and then with 100 ml of DT buffer containing 0.1 M NaCl (0.1SDT buffer). The brown fraction containing ferredoxin was eluted by the 0.5SDT buffer. The eluate, usually 5–6 ml, was chromatographed on a Sephadex G-75 column (2.5 × 50 cm) with 0.1SDT buffer. The ferredoxin fraction was diluted with an equal volume of DT buffer and applied to a second DEAE-Sepharose column (1.5 × 10 cm). The column was developed by a linear gradient of NaCl to 0.5 M in DT buffer. At this step, authentic or engineered FdxN was separated from other proteins that included ferredoxin II. The FdxN fraction was concentrated using a small DEAE-Sepharose column and chromatographed on tandemly connected two Toyopearl HW-50F columns (2.5 × 50 cm × 2) with 0.1 M Tris-Cl (pH 8.0) buffer.

When the exchange of the buffer system was needed, the sample was adsorbed on a small DEAE-Sepharose column, eluted with a new buffer containing 0.5 M NaCl and passed through a Sephadex G-25 column to remove NaCl.

Protein Chemical Analysis—Amino acid composition of the purified sample and of its derivatives was determined by the method of E. T. Adman, unpublished results. Site-specific Mutagenesis of Cys Residues of fdxN—We have made engineered genes to substitute Cys 54 and/or Cys 59 to Ser (Fig. 3). The three fdxN* genes, fdxNC54S, fdxNC59S, and fdxNC54S/C59S, were subcloned, respectively, into the vector.

RESULTS

This study aims to analyze the physical and chemical properties of engineered FdxNs (FdxN* proteins). Before such analysis, the capacity of R. capsulatus fdxN-null disrupted mutant MSA1 (18) to utilize molecular nitrogen was re-investigated first at 5 W/m2-intensity of the illumination for growth that was at least 3-fold stronger than that used in our previous study (18). The strain displayed no growth on the solid RCV-NF medium, which lacked a nitrogen source, in 3 days as reported previously (18), while the wild-type SB1003 had fully grown during the same period. However, the mutant displayed a trace of, but evident, growth after 7 days, unlike our previous results with lower light intensity. The nitrogenase activity of MSA1 cells grown anaerobically on RCV-E medium was about 5% of that of the wild-type cells (Fig. 2). Thus, the disruption of fdxN* was less critical on the capacity to fix nitrogen under stronger light. We, therefore, re-visited the phenotype of MSA1 from NifA to NifB (slow growth by nitrogen fixation) and concluded that FdxN is not absolutely required for nitrogen fixation by R. capsulatus.

Restoration of Nitrogenase Activity of fdxN-Null Mutant by puf Promoter-driven Expression of Authentic fdxN—We employed a system that enabled us to test the functional competency of an fdxN* and would also facilitate the purification of its product. Namely, we used a vector pTSV1 with R. capsulatus puf promoter to express an engineered ferredoxin gene in the strain MSA1. The promoter is strongly induced under anaerobic conditions regardless of the nitrogen sources, so that any fdxN* gene subcloned should be highly transcribed even when other nif-related genes are repressed. For the validation of this system, the authentic fdxN* gene was subcloned in the vector to construct plasmid pFPN and conjugatively transferred into MSA1. The conjunctivus strain pFPN/MSA1 showed full NifB phenotype in terms of both growth rate and nitrogenase activity as pCP-BH2/MSA1 did (Fig. 2). About 20 mg of FdxN could be purified from 80 g (wet weight) cells of pFPN/MSA1 grown in PYS medium under illuminated anaerobic conditions. The yield was comparable with that from the wild-type cells grown under the nif-repressed conditions.

Effects of Overexpression of fdxA on NifB Phenotype of fdxN-Null Mutant—Although fdxN is disrupted in MSA1, the strain still contains ferredoxin II that is a [4Fe–4S]3+ and 4Fe-4S-type ferredoxin encoded by the fdxA gene and can function as electron donor to nitrogenase in vitro (24). The cellular concentration of FdxA is relatively low (29) and constant regardless of the nitrogen source (40). Therefore, the effect of the overexpression of fdxA in MSA1 was investigated. We transferred the plasmid pYSA111, which was a pTSV1 derivative. Immunoblot analysis using anti-FdxA antibodies revealed more than a 5-fold increase in FdxA concentration in cells of pYSA111/MSA1 grown under anaerobic conditions (data not shown). The elevated expression of fdxA from pYSA111 enabled us to purify 10 times more FdxA protein from SB1003 harboring the plasmid than from the wild-type. However, the conjunctivus strain pYSA111/MSA1 showed only a slight increase in nitrogenase activity, and its growth rate on nitrogen-free medium was comparable with those of MSA1 and pTSV1/MSA1 (Fig. 2). These results indicated that the level of increase in the [4Fe–4S]3+ to [4Fe–4S]-type FdxA was still insufficient to compensate for the loss of the [2Fe–3S]-type FdxN, in vitro.

Site-specific Mutagenesis of FdxA and FdxN—We have made engineered genes to substitute Cys54 and/or Cys59 to Ser (Fig. 3). The three fdxN* genes, fdxNC54S, fdxNC59S, and fdxNC54S/C59S, were subcloned, respectively, into the vector.

2 Y. Suetsumi, K. Saeki, and H. Matsubara, manuscript in preparation.
3 E. T. Adman, unpublished results.
4 The dielectric constant was set to 2.5, and the effective distance between two atoms. The coordinates of the [4Fe–4S] clusters and sulfur atoms of ligand cysteines were fixed during the calculation.
pTSV1 to construct plasmids, pPM4, pPM5 and pPM45, and conjugatively transferred to R. capsulatus MSA1. The resulting transconjugants were surveyed for their capacity to fix nitrogen (Fig. 3). The strain pPM5/MSA1, which possessed fdxNC59S, grew on RCV-NF plates as fast as SB1003 and pFNP/MSA1. This strain exhibited a nitrogenase activity approximately 90% of those of the strains with the authentic fdxN. In contrast, pPM4/MSA1 and pPM45/MSA1, which have fdxNC54S/C59S, respectively, showed the Nifs phenotype indistinguishable from MSA1 and pTSV1/MSA1, in terms of growth rates and nitrogenase activities. These results demonstrated that the substitution of Cys54 eliminates the functional capacity, while that of additional Cys59 does not.

Site-specific Mutagenesis of Extra Residues of fdxN—To survey the physiological significance of the extra residues, we made seven mutant genes, fdxN42–49/7r–fdxN42–49/3r and fdxN* genes were subcloned in pTSV1 to make plasmids pPK7–pPK3 and pPM3 and introduced into the strain MSA1. All the resulting transconjugant strains grew on N2 at rates comparable with SB1003 and pFNP/MSA1. Most of them displayed nitrogenase activities comparable with those of the wild-type and pFNP/MSA1, though three strains, pPK4p/MSA1, pPK4a/MSA1, and pPK3/MSA1, which should have FdxN* proteins with the deletion of 4–5 residues, displayed slightly decreased activities (Fig. 3). The decrease in activity apparently correlated to the extent of the deletion, but it must be noted that the strain pPM3/MSA1 which has FdxNGA protein lacking whole extra residues exhibited activity equal to those of pFNP/MSA1 and SB1003. These results clearly showed that the extra residues of FdxN are not...
indispensable to function in nitrogen fixation.

**Purification of Products of Engineered fdxN Genes—**Three engineered ferredoxins, FdxNC54S, FdxNC59S, and FdxNGA, were subjected to anaerobic purification from the cells of appropriate strains. We could usually purify approximately 25 mg of FdxNC54S from 80 g (wet weight) cells of pPM3/MSA1; the yield was about 125% of that for authentic FdxN from the cells of pFNP/MSA1. On the other hand, we could not purify FdxNC54S and FdxNC59S protein by the same procedures, respectively, from cells of pPM4/MSA1 and pPM5/MSA1. Because the strain pPM5/MSA1 showed diazotrophic growth comparable with SB1003 (Fig. 3), the results indicate that the FdxNC59S is functional in vivo, but is unstable in vivo or becomes unstable during the purification.

The amino acid composition of the purified FdxNGA protein differed, as expected, from the authentic FdxN protein (data not shown). The molecular mass of the fully carboxymethylated FdxNGA as determined by electrospray ionization method was 6476.0 Da and matched within 2 Da to the theoretically predicted mass of 6477.3 Da, while the experimental and theoretical values for the authentic Cm-FdxN were 7272.3 and 7270.1, respectively. The FdxNGA protein was used for spectroscopic analysis.

**Optical Spectroscopic Properties of the FdxNGA Protein—**The ultraviolet (UV)-visible light absorption and CD spectra of FdxNGA protein were almost similar to those of the authentic FdxN protein (Fig. 4). The CD spectra of the two proteins were almost the same in near-UV and visible regions; the apparent difference in near-UV region might be due to the strong difference in far-UV region (Fig. 5). Both proteins exhibited peaks at 295, 320, 428, and 567 nm, suggesting little difference in the structures of the [4Fe-4S]-clusters. In contrast, far-UV CD spectra of FdxNGA and the authentic FdxN significantly differed, suggesting a change in the secondary structure by the removal of extra residues (Fig. 5).

**EPR Analysis—**Similar to clostridial 2[4Fe-4S]-type ferredoxins, oxidized forms of FdxN and FdxNGA were EPR-silent (data not shown; see Fig. 7). Their fully reduced forms with excess sodium dithionite exhibited EPR spectra significantly distinct from each other (Fig. 6). Fully reduced authentic FdxN showed a complex EPR spectrum with signals at g values 2.10, 2.02, 1.95, and 1.83, which suggested spin coupling between the two reduced [4Fe-4S] clusters (46). In contrast, fully reduced FdxNGA showed another form of complex EPR spectrum with signals at g values 2.13, 2.01, 1.97, 1.89, and 1.82. The spectrum was similar to that of reduced Clostridium pasteurianum ferredoxin (47) and appears also to be produced by the spin coupling between two reduced [4Fe-4S] clusters (46).

We performed redox titrations at pH 10.0 at 283 K (typical EPR spectra measured at 10 K are shown in Fig. 7); titrations conducted at pH 8.0 gave essentially the same results (data not shown). The authentic FdxN exhibited a very weak rhombic signal, which should correspond to one reduced [4Fe-4S] cluster with spin = 1/2 at the $E_h$ range between -360 mV and -460 mV. Some fraction of FdxN should be singly reduced, i.e. at the state that one of the two clusters was reduced. This signal was overtaken by the complex signals of the interacting two reduced [4Fe-4S] clusters at the $E_h$ values more negative than -460 mV. The peak at $g = 1.89$ detected above -469 mV in Fig. 7 seemed to represent the fraction of the protein of singly reduced form, although the signal intensity became a mixture of fractions of singly and doubly reduced forms below this potential. The signal intensity at $g = 1.92$, on the other hand, seemed to represent primarily the fraction of doubly reduced form. Therefore, the signal intensities at $g = 1.89$ and 1.92 were plotted against the redox potential after the normalization of the amplitudes to those at the fully reduced conditions (Fig. 8, upper). The two plots were almost similar but showed a difference above -500 mV. The apparent $E_m$ read as an $E_h$ value for 50% normalized amplitude was about -510 mV and matched the value electrochemically determined by Naud et al. (48). The solid and broken lines in the same figure represent the theoretical titration curves of the fractions of singly and doubly reduced FdxN, respectively. They were calculated by

![Fig. 4. Light absorption spectra of FdxN and FdxNGA.](image)

![Fig. 5. CD spectra of oxidized FdxN and FdxNGA.](image)
assuming that the two [4Fe-4S] clusters have an identical redox midpoint potential ($E_m$) of $-490$ mV and are independent, i.e. behave as the case of twice as many molecules each bearing a single [4Fe-4S] cluster (47). The theoretical curve for the doubly reduced fraction fits the data derived from the peak intensities at $g = 1.92$. The curve for the singly reduced fraction does not correlate to the two plots, but it resembles the deviation between them. The relatively weak signal of the singly reduced form, however, made further analysis to be rather difficult under the present experimental conditions.

The titration conducted with FdxN$^{GA}$ gave somewhat different results. The protein showed a rhombic signal ($g_{x,y,z} = 1.85, 189$ and $2.08$) at the $E_h$ range between $-360$ mV and $-460$ mV. The signal can be assigned to one reduced [4Fe-4S] cluster with spin $= 1/2$. A considerable fraction of FdxN$^{GA}$ should be at the singly reduced form in this $E_h$ range. As the potential was lowered below $-460$ mV, the signal was replaced by the complex signals that have features of spin coupling between two reduced [4Fe-4S] clusters and represent the fraction of FdxN$^{GA}$ of the doubly reduced form. The peaks at $g = 2.08$ and 1.90 seemed to represent the fractions of singly and doubly reduced forms, respectively. Their intensities were plotted against $E_h$ (Fig. 8, lower); the heights of $g = 2.08$ peak were plotted after the subtraction of the minor contribution of the doubly reduced form. Simulations of the titration curves were achieved by assuming the two [4Fe-4S] clusters with different $E_m$ values of $-490$ and $-430$ mV, respectively. Each theoretical curve fits the corresponding plot. This indicates that the $E_m$ of one Fe-S cluster shifted by approximately $+60$ mV with a minor effect on the other cluster that retains an $E_m$ value of $-490$ mV.

**DISCUSSION**

The protein engineering in this study indicated the functional and physicochemical significance of the unique cluster-binding motif of the ferredoxin I of *R. capsulatus*. We used the fdxN-null mutant MSA1, which has a deletion-insertion of the kanamycin-resistant gene in fdxN, with an expression vector pTSV1 that has the puf promoter. The phenotype of the mutant MSA1 that was revised from Nif$^+$ to Nifs (18) agrees with that of the fdxN-null mutant with the insertion of gentamycin-resistant gene reported by Schmehl et al. (19). The absence of FdxN was less critical under the high light intensity. The role of FdxN must be, therefore, more important under non-optimal conditions for nitrogen fixation. The [2Fe-2S]-type ferredoxin encoded by the fdxC gene that situates upstream of fdxN may also be required for optimization of diazotrophic growth, because we observed that the fdxN-null mutant MSB1 grew very slowly under increased illumination with the nitrogenase activity of nearly 10% of that of the wild-type strain (49).

The remaining capacity to fix N$_2$ of the fdxN-null mutants of *R. capsulatus* might depend on either direct electron transfer between the still unidentified reductase and oxidase of FdxN, possibly the Fe protein of nitrogenase (32, 50), or other ferredoxin(s) (24–28). We had previously pointed out the possibility that the cellular concentration of FdxA is simply too low to support the capacity (18). This [4Fe-4S]/[3Fe-4S]-type ferredoxin is constitutively expressed (40) and can donate electrons

---

**Fig. 6.** EPR spectra of fully reduced FdxN and Fdx$^{GA}$. Concentration of each sample was 210 $\mu$m ferredoxin in 100 mM Tris-Cl buffer (pH 8.0) supplemented with excess sodium dithionite. Conditions of measurements: temperature, 8 K; microwave power, 10 mW; microwave frequency, 9.63 GHz; modulation amplitude, 1 millitesla; receiver gain, $1 \times 10^4$; scan rate, 200 millitesla/s; time constant, 0.16 s.

**Fig. 7.** Redox titration of FdxN (left) and Fdx$^{GA}$ (right) at pH 10.0. Concentration of each sample was 50 $\mu$m ferredoxin in 25 mM Tris, 75 mM cyclohexylaminopropane sulfonate, 100 mM NaCl, 40 $\mu$m methyl and benzyli viologens (pH 10.0). 1.0 M sodium dithionite was used as reductant. Samples were incubated at the indicated $E_h$ for at least 8 min before being frozen in liquid nitrogen. Conditions of measurements were essentially the same as in Fig. 6, except that the temperature was 10 K.
to nitrogenase in vitro with the apparent \( K_m \) value higher than that for FdxN (24, 51). However, the 10-fold overexpression of FdxA showed practically no effect on the Niif phenotype in MSA1. Therefore, the functional difference between the [4Fe-4S]-type FdxN and the [3Fe-4S][3Fe-4S]-type FdxA must be based not only on the differential control of gene expressions but also on their structural difference.

The FdxN\(^{C59S}\) protein was almost fully functional in vivo, whereas neither FdxN\(^{C54S}\) nor FdxNC54S/C59S was functional. FdxNGA were assumed to have different expressions but also on their structural difference. Thus the two [4Fe-4S] clusters should be chelated by two sets of Cys residues: Cys9, Cys12, Cys15, and Cys54 for the first cluster (cluster I), and Cys38, Cys41, Cys50, and Cys19 for the second cluster (cluster II), as depicted in Fig. 1. Consequently, the extra 8 residues between Cys41 and Cys50 of the \( R. \) capsulatus ferredoxin should form a “loop-out” from a structure analogous to that of the Peptococcus aerogenes (i.e. \( Peptostreptococcus \) asaccharolyticus) ferredoxin (3, 52) (Fig. 9 and description below), as estimated for the case of the \( C. \) vinosum ferredoxin that has a COOH-terminal extension of 22 amino acids, which interacts with the extra region (22). Although the FdxN\(^{C59S}\) protein was functional in vivo, we failed to isolate this protein. This implies that the additional Cys\(^{59}\), which is not essential for function, is important for the structural integrity of the protein. Such an interpretation may explain the contradictory report that a similar substitution of the additional Cys to Ser in \( R. \) meliloti FdxN suppresses its capacity to support nitrogen fixation in planta (21). The modification might have destabilized the rhizobial FdxN protein even in vivo. It is known for the Azotobacter vinelandii [4Fe-4S][3Fe-4S]-type ferredoxin that site-specific substitution of Cys\(^{59}\), which is normally involved in chelating the [4Fe-4S] cluster, to either Ala or Ser causes a rearrangement of the protein structure and forces Cys\(^{59}\), which is normally a free cysteine, to be a substitute ligand to the [4Fe-4S] cluster (53, 54). This type of protein rearrangement does not seem to have occurred in the engineered FdxN\(^{C54S}\) protein of \( R. \) capsulatus.

The gradual deletion experiments have demonstrated that the loop-out or extra residues are not indispensable for \( R. \) capsulatus FdxN to support diazotrophic growth. Some MSA1 derivatives containing FdxN\(^{∗}\) proteins with fewer extra residues, such as FdxN\(^{42–49/4rP}\), FdxN\(^{42–49/4rA}\), and FdxN\(^{42–49/3r}\), displayed slightly decreased nitrogenase activity. Their extra residues might be too short to form stable proteins that fulfill the physiological function. This may explain why we have not found extra residues with less than 6 residues among the natural ferredoxins of the subclass. Our results confirm the report by Masepohl et al. (21), who showed that the deletion and substitution of the extra residues of \( R. \) meliloti FdxN has almost no effect on the capacity to support nitrogen fixation in planta. On the other hand, \( Rhodospirillum \) rubrum is reported to produce FdxN protein even under nif-repressed growth conditions (11). A gene to encode a ferredoxin with similar extra residues has also been identified in the non-diazotrophic bacterium \( H. \) influenzae (20). These findings, together with the results of the protein engineering studies with \( R. \) meliloti (21) and \( R. \) capsulatus (this study), suggest that the possession of loop-out and extra residues in the second clus-

---

**Fig. 8.** Dependence of the EPR signal intensities on the redox potential of the medium in the authentic FdxN (upper) and mutant FdxN\(^{42–49}\) (lower). The data were taken from titrations shown in Fig. 7 and those performed under similar conditions. Solid and broken lines represent theoretical fractions of the ferredoxins with one and two reduced clusters, respectively, calculated with assumptions described below. Upper, open circles and filled triangles represent signal intensities at \( g \approx 1.92 \) and 1.92, respectively. The two clusters of FdxN were assumed to be independent and have an identical \( E_m \) of \(-490 \text{ mV} \). Lower, open circles and filled triangles represent signal intensities at \( g \approx 2.08 \) and 1.90, respectively. The two clusters of FdxN\(^{42-49}\) were assumed to have different \( E_m \) of \(-490 \text{ mV} \) and \(-430 \text{ mV} \).

**Fig. 9.** Modeled three-dimensional structures of \( R. \) capsulatus FdxN and FdxN\(^{42-49}\). The structures were obtained from the averaged conformations in the last 20-ps trajectory of the molecular dynamics simulation. Upper left, backbone of FdxN is shown in tube model. The Glu\(^{49}\)-to-Gln\(^{49}\) loop is colored in purple and the left part in blue. Ball and stick models are two [4Fe-4S] clusters. In [4Fe-4S] clusters, Fe atom is in red and the sulfur atom in yellow. The letter \( N \) indicates the NH\(_2\) terminus. Upper right, the same as upper left, but backbone of FdxN\(^{42-49}\) with the Gly-Ala loop in purple. Lower left, space-filling model of FdxN corresponding to upper left. Lower right, space-filling model of FdxN\(^{42-49}\) corresponding to upper right.
Site-specific Mutagenesis of R. capsulatus Ferredoxin I

ter-binding motif does not necessarily indicate a functional link to nitrogen fixation.
The FdxNGA protein that had the Gly-Ala sequence in replacement of the extra residues was essentially indistinguishable from the authentic FdxN in its capacity to support nitrogen fixation in vivo, as well as in the optical absorption spectra and the visible CD spectrum. However, the two proteins showed distinct paramagnetic and redox properties. The authentic FdxN showed a weak signal of a singly reduced [4Fe-4S] cluster in a narrow range of $E_m$. The EPR spectrum in its fully reduced state suggests an interaction between two clusters that have essentially identical $E_m$ values at around $-490$ mV. The shape of the signal was somewhat different from those of typical 2[4Fe-4S]-type ferredoxins (46, 47). In contrast, FdxNGA showed a clear signal of a singly reduced [4Fe-4S] cluster in a relatively wide range of $E_m$ and a spectrum similar to that of the typical clemential 2[4Fe-4S]-type ferredoxin (46, 47) in its fully reduced state. The deletion of the extra loop seems to have shifted the $E_m$ value of one of the two clusters from $-490$ to $-430$ mV and modified the spin interaction mode of the two clusters. The different signal shapes of fully reduced FdxN and FdxNGA may represent the modified spin interaction.

We simulated the tertiary structure of the FdxN on the basis of the known structure of P. aerogenes ferredoxin $\beta$ as shown in Fig. 9. The putative structure of the authentic FdxN obtained by energy minimization and molecular dynamics simulation indicated that the extra loop shields cluster II from the outer medium. The deletion of the extra loop, thus, would expose cluster II to the medium with a slight effect on cluster I. Because the shielding of a [4Fe-4S] cluster from the medium is expected to destabilize the electrons on the cluster and to shift its $E_m$ to the more negative side according to theoretical investigations (55, 56), mutagenesis might induce the positive shift of the $E_m$ of cluster II. It can be concluded that the extra loop together with its potential interaction with the COOH-terminal residues is responsible for keeping the negative $E_m$ of cluster II.

This conclusion may be a clue in explaining the reason why ferredoxins with the extra residues are found in many diazotrophs and some non-diazotrophs, even though the feature is dispensable for the diazotrophic growth of R. capsulatus, could be an evolutional advantage. Namely, it can be speculated that the redox properties of ferredoxin be-sides nitrogenase, could be an evolutional advantage. Namely, it might be an advantage to have a redox system, which can be used for the survival of those bacteria.

Acknowledgment—We thank Dr. B. L. Marrs for the gift of a puf promoter fragment, Drs. T. Kakuno and T. Hase for assistance in large scale culturing of R. capsulatus strains, K. Kuriyama-Matsubara for mass spectrometry analysis, and Drs. Y. Hagiwara, Y. Gotoh, and S. Kurumitsu for assistance and discussion in CD spectrum analysis. We are also grateful to Dr. E. T. Adman for permission to use unpublished structural data for P. aerogenes ferredoxin.

REFERENCES
1. Matsubara, H., and Saeki, K. (1992) Adv. Inorg. Chem. 38, 223–280
2. Camrack, R. (1992) Adv. Inorg. Chem. 38, 281–322
3. Adman, E. T., Sieker, L. C., and Jensen, L. H. (1973) J. Biol. Chem. 248, 3987–3996
4. Fukuyama, K., Nagahara, Y., Tsukihara, T., Kusabe, Y., Hase, T., and S. Matsubara. (1980) J. Biol. Chem. 242, 8397–8401
5. Howard, J. B., and Rees, D. C. (1991) Adv. Protein Chem. 39, 199–280
6. Tanaka, M., Haniu, M., Yasunobu, K., Evans, M. C. W., and Rao, K. K. (1974) Biochemistry 13, 2953–2969
7. Tanaka, M., Haniu, M., Yasunobu, K. T., Evans, M. C. W., and Rao, K. K. (1974) Biochemistry 14, 1938–1943
8. Hase, T., Matsubara, H., and Evans, M. C. W. (1977) J. Biochem. (Tokyo) 81, 1745–1749