Discovery of a Novel Ferredoxin from Azotobacter vinelandii Containing Two [4Fe-4S] Clusters with Widely Differing and Very Negative Reduction Potentials*

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EXPERIMENTAL PROCEDURES

Cell Growth and Protein Purification—The A. vinelandii strain used in this study is designated DJ138/pBS122. The parent strain DJ138 was described previously (15). The plasmid pBS122, which was constructed as described elsewhere (16, 17), is a derivative of pKT230 with insertion of a site-directed mutant fdxA gene encoding a C16S variant of FdII. For protein purification, cells were grown under N2-fixing conditions in a 200-liter New Brunswick fermentor (17). For the experiment to determine the effect of ammonium levels on the intracellular FdIII levels, a separate batch of cells was grown in the presence of excess ammonium acetate.

Cells were harvested, cell-free extracts were prepared, and the first DEAE-cellulose column was run as for the purification of nitrogenase (18) and FdI (16, 19) except that the heat step was omitted. FdIII eluted at 70% of the linear 0.1–0.5 M NaCl gradient as a well resolved brown peak exactly where the FdI peak was expected (16). This fraction was then diluted with 2 volumes of 0.1 M potassium phosphate buffer (pH 7.4) and loaded onto a 2.5 × 12-cm DEAE-cellulose column that was then washed slowly with 4 liters of 0.12 M KCl in the same buffer. The greenish brown fraction containing FdIII was then eluted with 0.3 M KCl, and ammonium sulfate was added to 75% saturation. After centrigugation at 8,000 × g for 20 min, the pellet was resuspended in 0.025 M Tris-HCl, pH 7.4, and 0.1 M NaCl.

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m Tris-HCl (pH 7.4), and the resulting solution was loaded onto a 2.5 × 100-cm Sephadex G-75 superfine gel filtration column. FdIII eluted as a well resolved greenish brown band.

**Protein Characterization—**NH$_2$-terminal protein sequencing was carried out after the protein was reduced and alkylated by β-mercaptoethanol and 4-vinylpyridine at the Biotechnology Resource Facility at the University of California, Irvine. For spectroscopic studies all samples were prepared anaerobically under argon in a Vacuum Atmospheres glove box using fully degassed buffers. The protein was initially purified in the presence of dithionite. To prepare oxidized samples the dithionite was first removed by gel filtration, and then the samples were exposed to oxygen for at least 2 h. Circular dichroism (CD) and electron paramagnetic resonance (EPR) samples of oxidized FdIII were then prepared by concentrating the protein and exchanging it into 0.1 M potassium phosphate (pH 7.4) using a Centricon-3 microconcentrator. The reduction of FdIII was carried out by mixing well degassed FdIII, 5′-deazadicarbocyanin and EDTA (final concentrations 100 μM, 200 μM, and 20 mM, respectively) in 0.1 M potassium phosphate (pH 7.4) and then illuminating for 1 min using white light from a slide projector. UV-visible absorption spectra were obtained with a Hewlett-Packard 8452 diode array UV-visible spectrophotometer, CD spectra were recorded using a JASCO J720 spectropolarimeter, and EPR spectra were obtained using a Bruker 300 Ex spectrophotometer. To determine iron content, samples were digested, and the analysis was carried out as described elsewhere (18) using FeCl$_3$·6H$_2$O to generate a standard curve with Fd (9) andFixFd (8) as controls. Matrix-assisted laser desorption ionization—time of flight mass spectrometry was conducted at the Protein/Peptide Micro Analytical Facility, California Institute of Technology.

**Electrochemistry—**Purified water (~18 megohms-cm; Millipore) was used in all electrochemical experiments. The buffers Mes, Hepes, and Taps, and co-adsorbs neomycin sulfate and polymyxin B sulfate, were purchased from Sigma. Other reagents were purchased from Aldrich or the University of California, Irvine. For spectroscopic studies all samples were prepared anaerobically under argon in a Vacuum Atmospheres glove box using fully degassed buffers. The protein was initially purified in the presence of dithionite. To prepare oxidized samples the dithionite was first removed by gel filtration, and then the samples were exposed to oxygen for at least 2 h. Circular dichroism (CD) and electron paramagnetic resonance (EPR) samples of oxidized Fd III were then prepared by concentrating the protein and exchanging it into 0.1 M potassium phosphate (pH 7.4) using a Centricon-3 microconcentrator. The reduction of FdIII was carried out by mixing well degassed FdIII, 5′-deazadicarbocyanin and EDTA (final concentrations 100 μM, 200 μM, and 20 mM, respectively) in 0.1 M potassium phosphate (pH 7.4) and then illuminating for 1 min using white light from a slide projector. UV-visible absorption spectra were obtained with a Hewlett-Packard 8452 diode array UV-visible spectrophotometer, CD spectra were recorded using a JASCO J720 spectropolarimeter, and EPR spectra were obtained using a Bruker 300 Ex spectrophotometer. To determine iron content, samples were digested, and the analysis was carried out as described elsewhere (18) using FeCl$_3$·6H$_2$O to generate a standard curve with Fd (9) and FixFd (8) as controls. Matrix-assisted laser desorption ionization—time of flight mass spectrometry was conducted at the Protein/Peptide Micro Analytical Facility, California Institute of Technology.

**RESULTS AND DISCUSSION**

**The Discovery of FdIII and Its Relationship to FdI—**When crude extracts from nitrogen-fixing cells of wild-type *A. vinelandii* are separated on DEAE-cellulose with a 0.1–0.5 M NaCl gradient, three major brown peaks are observed (Fig. 1). The first two peaks correspond to the MoFe and Fe proteins of nitrogenase, respectively, and the third corresponds to FdIII (15). The size of the third peak is proportional to and therefore an indicator of the FdIII level present. In recent years we have constructed and purified many site-directed mutant variants of FdI (e.g. 9, 10, 17) some of which accumulate to much lower levels than the wild-type protein. We have observed a strong correlation between the size of the FdIII peak on the first column (Fig. 1) and the amount of material present in the cell-free extracts which cross-reacts with polyclonal antibodies raised against denatured gel-purified native FdI (2). We were therefore very surprised to find that cells expressing one particular variant, C16S FdI, which had only very low levels of FdI that cross-reacted to the antibody (Fig. 2), had a normal, wild-type FdI size peak on the first DEAE-cellulose column (Fig. 1).

Further purification of the brown “FdI” fraction from the C16S preparation (as described under “Experimental Procedures”) resulted in a greenish brown protein solution that exhibited a single band on Coomassie-stained SDS-polyacrylamide gels (Fig. 3). This protein did not cross-react with antibodies raised against FdI, and for reasons described below the new protein was designated FdIII. Once purified FdIII was available, polyclonal antibodies were raised, and we reexamined cell-free extracts from wild-type, FdI−, and C16S strains of *A. vinelandii* using the FdIII antibody. As illustrated in Fig. 2, the results show that the levels of FdIII are much greater in cells expressing the C16S variant of FdI than they are in either wild-type cells or in cells that make no FdI. The levels of FdIII in the C16S variant are also observed to be much higher than in all other FdI mutants tested to date based on Western analysis (data not shown). Thus FdIII specifically accumulates in response to expression of the C16S FdI variant. The reason for this is not currently understood. However, it should be noted that the [3Fe-4S]$^{2+}$ cluster is implicated in a regulatory function carried out by FdI in *A. vinelandii* and that C16 is a ligand to this cluster (9, 15, 17, 22–24). The purification yields

![Fig. 1. Elution profiles of DEAE-cellulose columns from three *A. vinelandii* strains. *wt* is wild-type. FdI− contains the *fdA* gene, which is interrupted with a kanamycin resistance gene. C16S, DJ38/pBS122, harbors a plasmid expressing a FdI mutant variant C16S.](image)
masses of FdI and FixFd apoproteins.

To identify FdIII, the first 56 NH2-terminal amino acid sequence of the protein standards. The numbers correspond to the molecular masses of the protein standards. The numbers on the right indicate the molecular masses of FdI and FixFd apoproteins.

of FdIII from cells expressing C16S FdI are typically 15 mg/1 kg, wet weight, of cells, compared with the approximately 8 mg of FdI usually obtained from 1 kg, wet weight, of wild-type cells (19).

FdIII Is Closely Related to C. vinosum Ferredoxin—To identify FdIII, the first 56 NH2-terminal amino acid sequence of the purified protein was obtained after modification of cysteine residues by 4-vinylpyridine (Fig. 4). Before this study 11 small [Fe-S] proteins had been identified in A. vinelandii, many by gene sequencing (3). Surprisingly, the sequence shown in Fig. 4 did not correspond to any of the known ferredoxin-like proteins from this organism. Data base searches, however, revealed that the first 56 amino acids of FdIII exhibit 77% identity and 88% similarity with the sequence shown in Fig. 4 contain the interrupted fdxN gene. C16S contains the plasmid expressing a Fd mutant variant C16S. Equal volumes of the Fd fractions from the DEAE-cellulose column were loaded in each lane. Upper panel, detected by the anti-FdIII antibodies; lower panel, detected by the anti-FdI antibodies.

The ligand assignment shown in Fig. 4 is derived from the x-ray structure of C. vinosum ferredoxin (11). All eight ligand cysteine residues lie within the available FdIII sequence and coincide in position with the [Fe-S] cluster ligand cysteines of C. vinosum ferredoxin, thereby eliminating the possibility that FdIII is a clostridial-type ferredoxin. Most of the ferredoxins shown in Fig. 4 contain nine Cys residues, one of which is not a cluster ligand. This ninth Cys is also conserved in FdIII and is in a position identical to that of the ninth Cys of C. vinosum ferredoxin.

The FdIII sequencing data presented here appear to bring to four the number of ferredoxins from one organism, A. vinelandii, which have a chromatium-type sequence (3). The other three were identified originally by gene sequencing, and all appear to be related somehow to nitrogen fixation based on their relationships to other genes. For example, fixN is cotranscribed with fixF (25), vnf/Fd is cotranscribed with vnf/H (26, 27), and fixFd is cotranscribed with fixABCX (28). Only FixFd has been purified to date (3). Because of the relationship of these other proteins to nitrogen fixation we tested whether or not FdIII was nif-regulated by growing the cells in the presence and absence of ammonia. There was no difference in the amounts of FdIII present under the two conditions as measured either by Western analysis or by the purification of the protein. Thus, like FdI (29), FdIII is not nif-regulated.

FdIII Is a Monomer with a Molecular Mass of 9,920 Da—Although SDS-polyacrylamide gel electrophoresis is often used to determine subunit molecular masses, we have found that it is not useful when studying small acidic [Fe-S] proteins. This is illustrated in Fig. 3, which compares the migration of the denatured FdIII with the migration of molecular mass standards and two A. vinelandii ferredoxins of known molecular mass, FdI and FixFd. As shown in Fig. 3, FdI and FixFd migrate as if their molecular masses were 18,800 and 9,000 Da, respectively, whereas their actual polypeptide molecular masses are known to be 12,071 (29) and 7,758 (28). Therefore, other methods were employed to determine the molecular mass.

First, matrix-assisted laser desorption ionization–time of flight mass spectrometry shows that the FdIII apoprotein has a mass of 9,220 ± 2 Da. Based on a composition of 2[4Fe-4S]2+ clusters (see below) this method gives a molecular mass of 9,924 Da for the holoprotein. To obtain a second estimate and to compare the native protein with the denatured protein, an FPLC-Superdex 75 gel filtration method was used, with FdI and FixFd as standards in addition to the commercially available standards. Again the migration of FdI and FixFd deviated significantly from the standard curve derived from the other four non–iron-sulfur proteins. Using only FdI and FixFd as standards, the native molecular mass of FdIII by this method was calculated to be 10,600 ± 680 Da. Thus, like the other proteins in Fig. 4 and most ferredoxin-like proteins, FdIII is a monomer.

Fig. 4 compares the molecular mass of FdIII with values reported for homologous chromatium-type ferredoxins. Clearly this class can be subdivided further into two groups, proteins with molecular masses of 6,000–7,000 Da and proteins with molecular masses of 9,000–10,000. The increase in molecular mass results from a COOH-terminal extension. Even when only the NH2-terminal sequences are considered, however, the four proteins having greatest sequence similarity to FdIII, including C. vinosum ferredoxin, also fall into the same molecular mass class (Fig. 4).

FdIII Contains Two [4Fe-4S]2+ Clusters—The UV-visible absorption spectra of FdIII are shown in Fig. 5. The spectrum of air-oxidized FdIII contains a broad peak at 390 nm and a shoulder at 315 nm. The shape of this spectrum is indistinguishable from that obtained for air-oxidized C. vinosum ferredoxin (30), FixFd (3), and other proteins that contain two [4Fe-4S]2+ clusters and is quite different from that obtained for the 7Fe FdI (10). The spectrum shown in Fig. 5 has an
A. vinelandii Ferredoxin FdIII

A<sub>390</sub>/A<sub>280</sub> ratio of 0.74, which is the same as reported for C. vinosum ferredoxin (48); FdIII, translated from the Franciscella tularensis ferredoxin gene (49); Fd, translated from the H. influenzae ferredoxin homolog gene (50); EcORF86, translated from the Escherichia coli ORF-086 (G. Plunkett III, GenBank™ locus ECU36841); CFDI, protein sequence of Chlorobium limicola FdIII (51); CFDI, protein sequence of C. limicola Fd (52); RfDxN, Rhodopseudomonas rubrum Fd (53); AvFix, translated from the A. vinelandii fixFd gene (28); RpFdI, protein sequence of Rhodopseudomonas palustris FdI (54); RfDxN, translated from the R. capsulatus fdxN gene (55); RfDxN, translated from the Rhodobacter sphaeroides fdxN gene (56); RfDxN, translated from the R. meliloti fdxN gene (57); AvFdxN, translated from the A. vinelandii fdxN (25); AvVnfFd, translated from the A. vinelandii vnfFd gene (27). The cluster is based on the crystal structure of C. vinosum ferredoxin (11). The calculated molecular mass of polypeptides (the NH<sub>2</sub>-terminal Met is not included) is from the amino acid sequence or translated nucleotide sequence of the gene.

### Table 1

| Cluster | 1 1 1 1 2 2 2 2 1 1 M<sup>+</sup> | 1 1 1 1 2 2 2 2 1 1 M<sup>+</sup> |
|---------|---------------------------------|---------------------------------|
| AvFDIII | -SLL/1T-DC1NCGYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | -SLL/1T-DC1NCGYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |
| CvFd | MALLITD-EC1NGYYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | MALLITD-EC1NGYYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |
| FtFd | MALLITD-EC1NGYYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | MALLITD-EC1NGYYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |
| HiFd | MALLITD-EC1NGYYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | MALLITD-EC1NGYYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |
| EcORF86 | -ALL1RT-DC1NCGYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | -ALL1RT-DC1NCGYCEP+EC1AGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |
| ClFDI | -AGRTTE-ETC1GAACCEP+CGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | -AGRTTE-ETC1GAACCEP+CGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |
| ClFDI | -ALX1RT-ETC1GAACCEP+CGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... | -ALX1RT-ETC1GAACCEP+CGA1-SQGERIUYVDSNLCTEGVHYDEPCQCVQFVDP-Cl... |

A. vinelandii Ferredoxin FdIII

FIG. 4. Sequence alignment of FdIII and bacterial [4Fe-4S]<sup>2+</sup> ferredoxins. CvfD, protein sequence of C. vinosum ferredoxin (48); FdIII, translated from the Franciscella tularensis ferredoxin gene (49); Fd, translated from the H. influenzae ferredoxin homolog gene (50); EcORF86, translated from the Escherichia coli ORF-086 (G. Plunkett III, GenBank™ locus ECU36841); CFDI, protein sequence of Chlorobium limicola FdIII (51); CFDI, protein sequence of C. limicola Fd (52); RfDxN, Rhodopseudomonas rubrum Fd (53); AvFix, translated from the A. vinelandii fixFd gene (28); RpFdI, protein sequence of Rhodopseudomonas palustris FdI (54); RfDxN, translated from the R. capsulatus fdxN gene (55); RfDxN, translated from the Rhizobium leguminosarum biovar trifolii fdxN gene (56); RmFdxN, translated from the R. meliloti fdxN gene (57); AvFdxN, translated from the A. vinelandii fdxN (25); AvVnfFd, translated from the A. vinelandii vnfFd gene (27). The cluster is based on the crystal structure of C. vinosum ferredoxin (11). The calculated molecular mass of polypeptides (the NH<sub>2</sub>-terminal Met is not included) is from the amino acid sequence or translated nucleotide sequence of the gene.

### Figure 5

UV-visible absorption spectra of FdIII in 0.025 M Tris-HCl, pH 7.4. Thick line, air-oxidized; thin line, incubated in the presence of 2 mM sodium dithionite for 45 min.

A<sub>390</sub>/A<sub>280</sub> ratio of 0.74, which is the same as reported for C. vinosum FdI and for other 8Fe ferredoxins. The iron content was confirmed by direct iron analysis using A. vinelandii 7Fe FdI and the 8Fe FdxN as controls. The results gave 7.7 ± 0.4 atoms of iron/molecule of FdIII, 7.7 ± 0.1 atoms of iron/molecule of FdxN, and 6.8 ± 0.4 atoms of iron/molecule of Fd.

The visible-near UV CD spectrum of oxidized FdIII is shown in Fig. 6. It exhibits two major positive features in the visible region, one at 420 nm and the other at 580 nm. The wavelength dependence and form of the CD spectrum are typical of [4Fe-4S]<sup>2+</sup> clusters and quite different from the spectra exhibited by [1Fe-0S], [2Fe-2S], and [4Fe-4S]<sup>2+</sup> clusters (30).

[4Fe-4S]<sup>2+</sup> clusters do not exhibit EPR signals at low temperature (31). As shown in Fig. 7a, oxidized FdIII is EPR-silent at liquid helium temperatures consistent with the presence of [4Fe-4S]<sup>2+</sup> clusters and showing that FdIII does not contain a [3Fe-4S]<sup>2+</sup> cluster, which would exhibit a very characteristic g = 2.01 EPR signal under the conditions used (32). This further eliminates the possibility that FdIII is a 7Fe protein and also shows that unlike some 8Fe ferredoxins that lose iron to form 3Fe clusters upon exposure to air (5), the 4Fe clusters of FdIII are extremely stable. Some protein-bound [4Fe-4S] clusters can also convert to [3Fe-4S]<sup>1+</sup> clusters upon addition of ferricyanide (33). However, incubation with a 10-fold excess of ferricyanide produced no new EPR signals attributable to [3Fe-4S]<sup>1+</sup> or indeed to [4Fe-4S]<sup>2+</sup> (34), the latter observation eliminating the albeit remote possibility (based on sequence comparisons and visible spectra (1, 30)) that FdIII is actually a HiPIP.

Taken together the above data lead to the conclusion that the new protein isolated here is a [4Fe-4S]<sup>2+</sup> ferredoxin of the chromatium-type. Ferredoxins that are first identified by gene sequencing are often named based on the location of the gene relative to other known genes (e.g. FdxN (25), FixFd (28)). Ferredoxins that are first identified by protein purification, as
is the case here, are generally numbered in the order in which they are identified. We have chosen to name this ferredoxin FdIII because FdI and a [2Fe-2S]-containing protein that is designated [Fe-S]II but is sometimes referred to as FdII have both been characterized extensively from *A. vinelandii* (1, 35–37).

Unlike All Known 8Fe Ferredoxins, FdIII Has Two Very Different Reduction Potentials for the Two [4Fe-4S]2+/3+ Clusters—Of the proteins shown in Fig. 4, the following have been isolated and at least partially characterized with respect to reduction potentials: *Rhodobacter capsulatus* FdI (the *fxN* gene product) (38); *C. vinosum* ferredoxin (11–14, 39, 40); FixFd from *A. vinelandii* (3) and recombinant *Rhizobium meliloti* FdxN (41). As monitored by the appearance of a g = 1.94 EPR signal due to [4Fe-4S]2+ clusters, at least three of these proteins could be at least partially reduced in solution by dithionite or a combination of dithionite, methyl viologen, and zinc (14). As originally reported for *C. vinosum* ferredoxin (12), the addition of dithionite (at different pH values ranging from 6.0 to 9.0) or electrochemically reduced methyl viologen to FdIII did not lead to the reduction of its [4Fe-4S]2+ clusters as evidenced by the lack of change in either the UV-visible (Fig. 5), CD, or EPR spectra. Attempted reduction with dithionite/methyl viologen/zinc, or Ti(III) citrate unfortunately led to irreversible denaturation of the protein. Fig. 6 shows that the protein could be reversibly reduced with a 5‘deazariboflavin/EDTA/light system. However, the CD data show that unlike the situation with 8Fe ferredoxins that have two clusters with potentials around -400 versus SHE even this powerful reductant fails to reduce the protein fully (3).

Using the same 5‘deazariboflavin/EDTA/light system, EPR samples were prepared of FdIII (Fig. 7b). In general, [4Fe-4S]+ clusters exhibit EPR signals with the g values and the intensity depending on the spin state. Most [4Fe-4S]+ clusters exhibit S = ½ spin and anisotropic EPR with g < 1.94. If two S = 1/2 clusters are present, separated by short distances, the signals are broadened and show additional structure (3, 14, 31). The complex

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**Fig. 7. EPR spectra of FdIII.** The protein samples are 100 μM FdIII in 0.1 M potassium phosphate, pH 7.4. Trace a, air-oxidized, 10 K; trace b, after reduction with 200 μM 5‘-deazariboflavin, 20 mM EDTA, and white light 5.2 K. The microwave power was 2 milliwatts, the modulation amplitude was 5.1 G, and the microwave frequency was 9.43 GHz. mT, millitesla.

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**Fig. 8. Cyclic voltammograms of *A. vinelandii* FdIII at a pyrolytic graphite edge electrode.** Buffer/electrolyte consists of 0.1 M NaCl, 60 mM mixed buffer (15 mM each in Hepes, Mes, acetate, and Taps), 0.1 mM EGTA, pH 7.0, 0 °C. Panel a, solution voltammetry with scan rate 10 mV/s. [FdIII] = 60 μM. Neomycin (2 mM) was added to promote and stabilize the response. Reduction potentials are -466 ± 10 and -644 ± 10 mV versus SHE for couples A and B, respectively. Panel b, film voltammetry with scan rate 100 mV/s. Polymyxin (200 μg/ml) was used as a coadsorbate. Reduction potentials are -466 ± 5 mV and -681 ± 10 mV versus SHE for couples A’ and B’, respectively.
The data presented here establish for the first time that a ferredoxin can contain two [4Fe-4S] Clusters having two very different reduction potentials. In contrast to typical 8Fe ferredoxins in which the two clusters have indistinguishable reduction potentials, the [4Fe-4S] Clusters in this study are reported to have similar or even identical reduction potentials (44). This is true not only for the clostridial-type ferredoxins that have the same Cys motif (although different sequences) for both clusters, but also for the chromatium-type ferredoxins that have two very different motifs for the two clusters. For example, FixFd, which is in the 6,000–7,000-Da molecular mass chromatium-type class, very clearly shows only a single signal in cyclic voltammetry, i.e., two couples having indistinguishable reduction potentials. For C. vinsonum ferredoxin, which is in the larger molecular mass group, the two clusters are also reported to have the same reduction potential; this is surprising since the local structures around the two clusters are now known to be very different (11).

The involvement of the fix ABCX genes in the respiratory chain in the electron transport to nitrogenase, Azotobacter vinelandii. Ph.D. thesis, pp. 48–72, Agricultural University, Wageningen, The Netherlands

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