Purification and Biophysical Characterization of a New [2Fe-2S] Ferredoxin from Azotobacter vinelandii, a Putative [Fe-S] Cluster Assembly/Repair Protein*

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During the purification of site-directed mutant variants of Azotobacter vinelandii ferredoxin I (FdI), a pink protein, which was not observed in native FdI preparations, appeared to associate specifically with variants that had mutations in ligands to FdI [Fe-S] clusters. That protein, which we designate FdIV, has now been purified. NH₂-terminal sequence analysis revealed that the protein is the product of a previously described gene, herein designated fdxD, that is in the A. vinelandii iseSU operon that encodes proteins involved in iron-sulfur cluster assembly or repair. An apoprotein molecular mass of 12,434.03 ± 0.21 Da was determined by mass spectrometry consistent with the known gene sequence. The monomeric protein was shown to contain a single [2Fe-2S]²⁺/+² cluster by UV/visible, CD, and EPR spectroscopies with a reduction potential of −344 mV versus the standard hydrogen electrode. When overexpressed in Escherichia coli, recombinant FdIV holoprotein was successfully assembled. However, the polypeptide of the recombinant protein was modified in some way such that the apoprotein molecular mass increased by 52 Da. Antibodies raised against FdIV and EPR spectroscopy were used to examine the relative levels of FdIV and FdI in various A. vinelandii strains leading to the conclusion that FdIV levels appear to be specifically increased under conditions where another protein, NADPH:ferredoxin reductase is also up-regulated. In that case, the fpr gene is known to be activated in response to oxidative stress. This suggests that the fdxD gene and other genes in the iron-sulfur cluster assembly or repair operon might be similarly up-regulated in response to oxidative stress.

In 1962 the isolation of the first iron-sulfur ([Fe-S]) protein, Clostridium pasteurianum ferredoxin, was reported (1). Within a few years it was clear that a new class of proteins had been discovered containing clusters composed of Fe and S²⁻ atoms ligated to the protein by cysteine ligands (2). Since that time there has been an exponential growth in the discovery of new [Fe-S] proteins that has exploded recently due to gene sequencing. Today, over 100 different types of [Fe-S] proteins have been isolated from all life forms, from the most primitive archaea and bacteria to the most advanced eucaryotes (3–9). These structurally diverse proteins are involved in critical electron transfer reactions, chemical catalysis of hydration/dehydration reactions (10–11), regulation of gene expression (12–18), oxygen and iron sensing (12–16, 19), or the generation and stabilization of radical intermediates (20–23).

The multiplicity of [Fe-S] cluster structures is remarkable (3–9, 24–25) with the simplest clusters containing 1, 2, 3, or 4 iron atoms. Much of what we know about these protein-bound clusters comes from studies of a class of [Fe-S] proteins collectively known as ferredoxins. Ferredoxins are loosely defined as small, soluble, generally acidic [Fe-S] proteins that function to transfer electrons from one protein to another (2–9). In the past, it was believed that a single ferredoxin was a relatively nonspecific electron transfer agent that could participate in a number of different cellular processes (26), and it is still common to see the term “bacterial ferredoxin” or “plant ferredoxin” used in this generic sense. However, it is now clear that a single organism can contain numerous different types of ferredoxins, each of which is expected to have a specific function in the cell.

Table 1 shows that the organism of interest in this study, Azotobacter vinelandii, appears to synthesize at least 12 different small, ferredoxin-like proteins. Only a few of these proteins have been purified and characterized to date (28–31, 34–35, 43–44) with others identified based on sequence motif and/or homology with proteins that have been purified from other organisms. One protein of particular relevance to this study was initially identified by Zheng et al. (32) who sequenced a gene cluster from A. vinelandii that encoded proteins that are likely to be involved in the assembly of [Fe-S] clusters. Within that gene cluster was a gene they designated fdx (Table I). That gene was expected to encode a [2Fe-2S] ferredoxin because it was homologous both in sequence and gene location to a [2Fe-2S] ferredoxin that had been purified previously from Escherichia coli (45).

For several years now we have been using a different ferredoxin, the extremely well characterized 7Fe FdI† from A. vinelandii (Table I), as a model to address certain basic questions in [Fe-S] protein biochemistry (46–50). The general approach has been to construct, purify, and characterize numerous site-directed mutant variants of FdI. In the course of these studies we have observed that some, but not all variants of FdI, appear to

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† The abbreviations used are: Fd, ferredoxin; E₉, redox potential of the system relative to that of a normal hydrogen electrode; E₉₉, midpoint redox potential; FPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
copurify with a contaminating pink protein that is not seen during the purification of native FdI. Here we report the purification and characterization of that pink protein and its identification as the product of the fdx gene that is cotranscribed with genes encoding proteins that appear to be involved in the assembly of [Fe–S] clusters.

We have chosen to designate the pink protein as FdIV because FdI (28, 33–36), a [2Fe–2S]-containing protein that is designated [Fe–S] II but is sometimes referred to as FdII (28, 33–36), a [2Fe-2S]–containing protein that is also produced by wild-type strains. The overproduction strains are approximately equivalent to, or less than, the levels of native FdI produced by wild-type strains. For this reason the first third of the peak was collected and concentrated using a Centriplus-10 concentrator (Amicon) prior to further purification on a 1-ml Mono-Q/FPLC column (Amersham Pharmacia Biotech) with a flow rate of 1 ml/min and a linear gradient of 0.3–0.8 M NaCl in 0.05 M Tris-HCl, pH 8.0, over 20 ml. Two fractions were well resolved as follows: the red-colored FdIV fraction that eluted between 0.46 and 0.48 M NaCl and a brown-colored FdII fraction that eluted between 0.54 and 0.58 M NaCl. The pink-colored FdIV fraction became red-colored when it was air-oxidized. Each fraction was collected, buffer-exchanged with 0.05 M Tris-HCl, pH 7.4, concentrated to 0.5 ml, and kept in the −20 °C freezer until use. The yield for FdIV is ~0.5 mg per kg C42D cell.

**Overproduction of FdIV in E. coli**—Overproduction of FdIV in E. coli was accomplished by constructing a fdxI gene in vitro and cloning this cartridge into the pT7–7 plasmid (53) such that fdxI gene expression was controlled by the T7 phage transcriptional and translational regulatory elements. The fdxI gene cartridge was constructed by using an isolated 1.0-kilobase pair DNA fragment that was generated by EcoRI restriction enzyme digestion of an isolated fragment of A. vinelandii strain DJ16 (∼98% homologous to A. vinelandii, GenBank™ accession number M20568). The amino acid coding region for FdIV was cloned into the pT7–7 plasmid pDB946. pDB946 was subsequently used as a template for PCR amplification of the fdxI coding sequence. PCR amplification of fdxI was performed essentially as recommended by the supplier of AmpliTaq (Perkin-Elmer). Cycling temperatures were 95 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. The PCR primers used were 5′-CATGGATATTGACGATCGCTTCTCC-3′ and 5′-CTGGGATCCCTCTAGGCTGTGCTGTTTCTCC-3′. The amino acid coding region for FdIV is underlined in the first primer sequence shown below. Following fdxI amplification, the gene cartridge was digested with the restriction enzymes Ndel and BamHI and ligated to Ndel– and BamHI-digested pT7–7 DNA. Proper orientation of fdxI within the resulting hybrid plasmid (pDB1024) such that the T7 gene promoter directs fdxI transcription was confirmed by restriction enzyme digestion of isolated plasmid DNA. For isolation of FdIV overproduced in E. coli, plasmid pDB1024 was transformed into the host strain BL21(DE3), and the transformed cells were maintained on LB media supplemented with 100 µg/ml ampicillin. Cells were grown in 500-ml batches in LB media at 30 °C and were induced for FdIV production at approximately 160 Klett units (red filter) by the addition of lactose to 1% (w/v) final concentration. Following addition of lactose, cells were cultured for an additional 2 h, harvested by centrifugation, and frozen at −20 °C until use.

The purification of recombinant FdIV was carried out aerobically. E. coli cells containing the overproduced FdIV were ruptured by sonication in 0.025 M Tris-HCl, pH 7.4. Extracts were then ultracentrifuged in a Beckman Ty3 rotor at 35,000 rpm for 20 min at 4 °C. Cell-free lysate was then loaded onto a 1.5 × 14-cm Q-Sepharose (Amersham Pharmacia Biotech) column equilibrated in 0.025 M Tris-HCl, pH 7.4, and 0.1 M NaCl. After loading, a gradient of 5 column volumes from 0.1 to 0.6 M...
NaCl was run. The red protein eluted at approximately 0.5 M NaCl, and the peak fractions were loaded onto a 5 × 44 cm Sepharose S-300HR (Amersham Pharmacia Biotech) equilibrated and run in 0.025 M Tris-HCl, pH 7.4, and 0.1 M NaCl. Column eluents were monitored at A

using an Amersham Pharmacia Biotech UV-1 optical detector and controlled using a Shimadzu FC 1000 universal pump.

Protein Sequencing and Determination of Molar Extinction Coefficient—The NH₂-terminal protein sequencing was carried out at the Biotechnology Resource Facility at the University of California, Irvine, and also at the W. M. Keck Facility at Yale University. The protein samples used for protein sequencing at the University of California, Irvine, were obtained by excising the protein bands from a Coomassie Blue-stained SDS-12% PAGE and eluting the protein into 10 mM ammonium bicarbonate. The protein sample was then modified using 2-mercaptoethanol and 4-vinylpyridine so that the modified cysteines could be detected. The sample used for sequencing at Yale University was prepared by excising the protein band from a Coomassie Blue-stained polyvinylidene fluoride membrane (Millipore) onto which an FdIV sample resolved in an SDS-12% PAGE was electroblotted. Twenty seven residues were solved at the University of California, Irvine (PQIVFPLHEVHCPCQGRVVEAATQGSL), note that the underlined residues were identified with ambiguity). Twenty residues were identified at Yale University, PQIVFPLHEVHCPCQGRVVEA.

The molecular weight of FdIV was determined at the W. M. Keck Facility at Yale University using quadrupole-time-of-flight mass spectrometry using an electrospray interface. The electrospray ionization technique was preferentially chosen with the hope of detecting signals corresponding to both holoprotein with the [Fe-S] cluster bound and the apoprotein. The mass spectrometry using MALDI ionization technique was performed at the Biotechnology Resource Facility at the University of California, Irvine. Quantitative triplicate amino acid analyses of Mono-Q/FPLC purified FdIV was carried out at the W. M. Keck Facility at Yale University. The protein content for determination of the molar extinction coefficient was estimated from the nanomoles of Ala, Leu, and Phe using the composition and M₁₂,375 (for apoprotein) calculated from the gene sequence and was corrected for holoprotein. Here the cluster composition was determined.

Protein Characterization—The antibody for FdIV was produced in rabbits in Bethyl Laboratories, Inc. (Montgomery, TX), using routine immunization protocols. The final antiserum was obtained 1 week after the sixth antigen was injected into the rabbit. To determine iron content, samples were digested, and the analysis was carried out as described previously using FeCl₃, 6H₂O to generate a standard curve with FdI and FdIII as controls (54). Redox titration of FdIV was performed spectrophotometrically at room temperature using 5-deazariboflavin as a chemical mediator (E°' = −320 mV versus standard hydrogen electrode) as described (55). The sample was anaerobically prepared in a glove box (oxygen level < 1 ppm) and was sealed with a rubber stopper and an aluminum cap. A 1-cm light path cuvette, in a 1-ml volume, contained 5 μM 5'-deazariboflavin, 15 mM potassium oxalate, 45 mM benzyl viologen, and 40 μM FdIV in 0.05 M Tris-HCl, pH 8.0. The mixture was exposed to the light of a slide projector (5–300 s), and absorbance changes were monitored after 30 s equilibration in darkness. A water-containing vessel was installed between the sample and light source to avoid overheating the sample during the illumination.

The percentage of [2Fe-2S] cluster reduction was determined from the absorbance change at 440 nm, which is an isosbestic point between the oxidized and reduced forms of benzyl violagen. The total reduction of [2Fe-2S] was estimated from the absorbance change after 2 mM Na₂S₂O₄ was added to sample. The extent of benzyl violagen reduction was calculated from the absorbance at 600 nm after correction for the absorbance of Fd at this wavelength.

Absorption spectra were obtained using a Jasco J-500C spectrophotometer. CD measurements were obtained using small volume cylindrical cells with fused quartz windows.

Attempted Insertional Inactivation of fxs Gene in A. vinelandii— Kanamycin resistance cassettes were inserted into the fxs gene in order to disrupt its transcription. Plasmid pDB946 was digested with EcoRV, and kanamycin resistance cassettes derived from pUC-KAPA were inserted. Kanamycin resistance cassettes were inserted so that they are transcribed in the same (pDB1015) or opposite orientation (pDB1016) as the fxs gene.

The Level of Both FdIV and FdI in FdI Variants—EPR and Western blot methods were performed to quantify the amount of FdIV and FdI present in the cell. Because of the low abundance of protein in whole cells, we prepared concentrated protein samples in the following way. The same amount (350 g) of cell paste from site-directed variants (C42D, C20S, and ΔT14/ΔD15), LM100 (FdI deletion), and AvO (containing wild-type FdI) was thawed, broken, and centrifuged at 8,000 rpm for 2 h. The supernatant was loaded onto a 2.5 × 15-cm anion exchange (DE52, Whatman) column equilibrated with 0.05 M Tris-HCL, pH 8.0. The column was washed with 2 volumes of 0.025 M Tris-HCL, pH 7.4, followed by 300 ml linear gradient from 0.1 to 0.5 M NaCl. FdIV and FdI eluted together as a single peak as monitored by A

The peak was collected and concentrated to ~5 ml in an Amicon cell fitted with a YM-3 membrane.

RESULTS AND DISCUSSION

How FdIV Came to Our Attention—Over the past several years we have purified, or attempted to purify, a large number of site-directed mutant variants of the 7Fe FdI from A. vinelandii (47–50, 52). Many of these proteins presented unique purification challenges, but in all cases our initial attempts began with a procedure that was developed for native FdI involving a first column separation on DEAE-cellulose with a linear NaCl gradient. In all cases the columns were monitored by measuring the absorbance at 405 nm, and in all cases an FdI peak was observed in the expected position, relative to internal marker proteins, the MoFe and Fe proteins of nitrogense (51, 54). The size of the absorbance peak varied depending upon how much of a particular FdI variant accumulated in vivo, but the position of the peak was invariant. It was upon going to the second step, using another DEAE-cellulose column, or the third step, using a size exclusion column (Sephadex G-50), that significant differences in the color of the FdI fractions were observed. For some, but certainly not all variants, the protein fractions appeared pink, instead of the normal brown color for native FdI. Often in these cases it was never possible to purify successfully the FdI variant, or the FdI variant was ultimately purified only with great difficulty and in very low yield. Table II summarizes the qualitative observations obtained by us over the past several years.

While purifying the FdI [4Fe-4S] cluster ligand variant, C42D, we observed that even the first column FdI fraction appeared to be pink and that a large amount of pink material eluted from subsequent columns in the same fractions as the C42D variant which we were identifying by cross-reaction to FdI antibodies. To determine whether the pink color arose from an unusual form of FdI or from a contaminating protein, a variety of purification methods was employed in attempts to separate the pink color from C42D FdI. As described under “Experimental Procedures,” FdIV was ultimately separated from partially purified C42D FdI which we were identifying by cross-reaction to FdI antibodies. To determine whether the pink color arose from an unusual form of FdI or from a contaminating protein, a variety of purification methods was employed in attempts to separate the pink color from C42D FdI. As described under “Experimental Procedures,” FdIV was ultimately separated from partially purified C42D FdI which we were identifying by cross-reaction to FdI antibodies. To determine whether the pink color arose from an unusual form of FdI or from a contaminating protein, a variety of purification methods was employed in attempts to separate the pink color from C42D FdI. As described under “Experimental Procedures,” FdIV was ultimately separated from partially purified C42D FdI which we were identifying by cross-reaction to FdI antibodies. To determine whether the pink color arose from an unusual form of FdI or from a contaminating protein, a variety of purification methods was employed in attempts to separate the pink color from C42D FdI. As described under “Experimental Procedures,” FdIV was ultimately separated from partially purified C42D FdI which we were identifying by cross-reaction to FdI antibodies.
TABLE II
Intracellular FdI and FdIV levels of AvFDI mutation variants

| Mutant strains       | Mutation position | FdI levela | Pink protein observed during purification |
|----------------------|-------------------|------------|------------------------------------------|
| Wild-type            |                   | +++        | No                                       |
| C16S                 | [3Fe-4S] ligand   | +          | Yes                                      |
| C49S                 | [3Fe-4S] ligand   | +          | Yes                                      |
| ΔT14/ΔD15            | [3Fe-4S] ligand   | +++        | No                                       |
| T14C                 | [3Fe-4S] ligand   | +++        | No                                       |
| C11S                 | Free Cys          | None       | Yes                                      |
| C20A                 | [4Fe-4S] ligand   | +          | No                                       |
| C20S                 | [4Fe-4S] ligand   | +          | Yes                                      |
| C20A/C24A            | [4Fe-4S] ligand   | +/−        | Yes                                      |
| C24A                 | Free Cys          | +++        | No                                       |
| C29S                 | [4Fe-4S] ligand   | +          | No                                       |
| C42D                 | [4Fe-4S] ligand   | +          | Yes                                      |
| D23N                 | Surface charge    | +++        | No                                       |
| H35D                 | Surface charge    | +++        | No                                       |
| E38S                 | Surface charge    | +++        | No                                       |
| E46A                 | Surface charge    | +++        | No                                       |
| P25Y                 | Near [4Fe-4S]     | +++        | No                                       |
| P25H                 | Near [4Fe-4S]     | +          | No                                       |
| D15N                 | Near [3Fe-4S]     | +++        | No                                       |
| D15K/K54D            | Near [3Fe-4S]     | +++        | No                                       |
| K54Q                 | Near [3Fe-4S]     | ++         | No                                       |

a The FdI levels are determined based upon Western analysis. +/−, barely detectable in the assay; +, contains less than 1 mg of FdI per kg of cell paste; ++, contains 1–4 mg of FdI per kg of cell paste; +++, contains 4–8 mg of FdI per kg of cell paste.

FIG. 1. SDS-12% polyacrylamide gel electrophoresis separation of the purified FdIV. Lane a, Mono-Q/FPLC-purified FdIV; lane b, native FdI; lane c, FdIII; fast lane, molecular marker. The numbers shown on the right indicate the molecular mass (kDa) represented by the marker.

Reported for other small and highly charged proteins (the isoelectric point of FdI calculated from amino acid sequence is 4.03) (58). The similar purification properties and electrophoretic behaviors of FdIV and FdI suggested that they were likely to have similar charges and molecular weights. As expected, FdIV is a monomer that it runs on Superdex 75/FPLC with almost the same retention time as FdI, which is monomer. It is also a highly acidic protein. The isoelectric point calculated from the amino acid sequence as shown in Fig. 2 is 4.29. Once FdIV was purified, antibodies were raised against it, and the antiserum does not cross-react with FdI (data not shown).

Identification of FdIV—For identification, the gel-purified protein was subjected to NH₂-terminal protein sequencing with cysteine modification. Twenty residues were unambiguously identified, PQYVLPHEVHCPEGRVVEA. Sequence homology searches in the data base Protein Data Base + SwissProt + PIR + GenBankTM translations were performed. As shown in Fig. 2, the results revealed the presence of a sequence with a perfect match in A. vinelandii (32), and sequences with high identity in Pseudomonas aeruginosa (59), E. coli (45, 60), Hemophilus influenzae (61), and Buchnera aphidicola (62). Searches in the Unfinished Microbial Genome data base have found similar sequences in Vibrio cholerae, Shewanella putrefaciens, Salmonella typhi, Yersinia pestis, Pasteurella multocida, Bordetella pertussis, Neisseria meningitidis, Neisseria gonorrhoeae, and Actinobacillus actinomycetemcomitans. Sequence comparisons indicate that FdIV is the gene product of the fdx gene in the A. vinelandii iscSUA operon and consists of 111 residues with 12,435 Da (deduction from the amino acid sequence) with the first 2 Mets missing in the purified protein (Fig. 2). The molecular mass determined by electrospray mass spectrometry was 12,434.03 ± 0.21 Da, which can be attributed to apoprotein (average mass 12,435 Da) (data not shown). We further confirmed this by observing the same molecular mass for the fdx gene product with MALDI ionization techniques (data not shown). The MALDI technique requires use of an organic chemical matrix (saturated sinapinic acid was used in this study) dissolved in 30% acetonitrile and 0.1% trifluoroacetic acid (63). That procedure is expected to destroy the iron-sulfur cluster. Therefore the molecular mass obtained from the MALDI techniques should correspond to the apoform, which it does. Unlike what has been reported for other [Fe-S] proteins (64–66), in this electrospray mass spectrometry study we could not detect the second peak corresponding to holoprotein at 178 Da above that of the apoprotein, 12,435 Da.

FdIV Contains a [2Fe-2S]2+/+ Cluster—Of the proteins shown in Fig. 2 only the protein from E. coli has been purified and characterized and shown to contain a single [2Fe-2S]2+/+ cluster (45). In that case the four cysteine ligands to the cluster were suggested to be those shown in Fig. 2 based on comparison with human (67) and Pseudomonas putida (68) ferredoxins whose [2Fe-2S]2+/+ clusters are known to be ligated by a Cys-X₅-Cys-X₉-Cys-X₃₀-Cys motif. This pattern is highly conserved among all 14 sequences shown in Fig. 2. In addition, the sequences around the four cysteine residues in the motif are also highly similar. If these four cysteines are indeed the ligands for [2Fe-2S]2+/+ clusters, this pattern represents a subclass of ferredoxin-type [2Fe-2S]2+/+ cluster binding motifs, which is shared by “vertebrate or bacterial”-type ferredoxins (45, 69–70). This is in contrast to the Cys-X₅-Cys-X₉-Cys-X₃₀ or X₅₀-Cys motif found in “plant”-type Fds, which participate in photosynthetic electron transfer processes in plants and in blue-green algae (71–72). The pink color of Fd IV, as well as its sequence, is consistent with the protein containing a [2Fe-2S]2+/+ cluster as a prosthetic group. The nature of this center was therefore investigated and identified by spectroscopic analyses and biochemical methods.

Iron content analysis for FdIV gives 2.0 ± 0.1 iron atoms per molecule when the ε₄₅₀ = 7,071 M⁻¹ cm⁻¹, determined from triplicate amino acid analysis for protein quantitation. In the same assay, the 7Fe containing FdI and 8Fe containing FdIII gave 7.0 ± 0.1 and 7.7 ± 0.1 iron atoms per molecules, respectively. These results indicate that FdIV contains either a [2Fe-2S] cluster or two rubredoxin-like 1Fe centers. The possibility of having two rubredoxin-like iron centers was ruled out because FdIV contains only 6 cysteine residues per molecule, which cannot accommodate formation of two rubredoxin-like 1Fe centers. This is further supported by the observation that we could not detect any EPR signal with g values of 4.3 and 9.5, which arise from the 3/2 and 1/2 kramers’ doublets, respectively, of an S = 5/2 multiplet of rubredoxin in the dithionite-reduced state (data not shown) (73–74). The UV/visible spectrum of oxidized FdIV, shown in Fig. 3, is distinct from those obtained for 7Fe FdI and 8Fe ferredoxins (such as FdIII) (43) and is indistinguishable from that of bacterial type [2Fe-2S] ferredoxins, exhibiting peaks at 414 and 458 nm and a broad maximum with a shoulder near 322 and 337 nm (45). The extinction coefficient of 7,071 M⁻¹ cm⁻¹ at 458
nm is a little lower than those obtained for other [2Fe-2S] Fds that are reported to be between 8,000 and 10,000 M⁻¹ cm⁻¹ (75–76). Upon reduction of FdIV with 2 mM dithionite, the absorbance in the visible region is greatly reduced, and char-

able CD compared with other biological [Fe-S] clusters, and CD

spectra are more sensitive than the corresponding absorption

spectra to the protein folding in the vicinity of the cluster

(78–79). For the oxidized protein the negative peaks at 550 and

350 nm are also used to differentiate between plant-type [2Fe-

and bacterial-type 2Fe ferredoxins. Again the environment around the [2Fe-2S] 2

cluster in FdIV, as observed by CD, is quite similar to verte-

brate or bacterial-type ferredoxins. This [2Fe-

ferredoxin gene (45, 60); Ht, translated from the H. influenzae unfinished fragment of complete genome; Sp, translated from the S. putrefaciens unfinished fragment of complete genome; St, translated from the S. typhi unfinished fragment of complete genome; Yp, translated from the Y. pestis unfinished fragment of complete genome; Pm, translated from the P. multocida PM70 unfinished fragment of complete genome; Bp, translated from the B. pertussis unfinished fragment of complete genome; Nw, translated from the N. meningitidis serogroup A unfinished fragment of complete genome; Ns, translated from the N. gonorrhoeae unfinished fragment of complete genome; Aa, translated from the A. actinomycetemcomitans unfinished fragment of complete genome. The predicted cysteine ligands to the [2Fe-2S] cluster are indicated by both bold letters and arrows.

Fig. 2. Comparison of partial FdIV sequence to the translated sequences of several [2Fe-2S] ferredoxin genes. Av*, amino acid sequence of FdIV obtained in this study; Av, translated from the A. vinelandii fds gene (32); Pa, translated from the P. aeruginosa fds gene (59); Ec, translated from the E. coli ferredoxin gene (45, 60); Hi, translated from the H. influenzae Rd fds-1 gene (61); Ba, translated from the B. aphidicola ferredoxin gene (62); Vc, translated from the V. cholerae unfinished fragment of complete genome; Sp, translated from the S. putrefaciens unfinished fragment of complete genome; St, translated from the S. typhi unfinished fragment of complete genome; Yp, translated from the Y. pestis unfinished fragment of complete genome; Pm, translated from the P. multocida PM70 unfinished fragment of complete genome; Bp, translated from the B. pertussis unfinished fragment of complete genome; Nw, translated from the N. meningitidis serogroup A unfinished fragment of complete genome; Ns, translated from the N. gonorrhoeae unfinished fragment of complete genome; Aa, translated from the A. actinomycetemcomitans unfinished fragment of complete genome. The predicted cysteine ligands to the [2Fe-2S] cluster are indicated by both bold letters and arrows.

Fig. 3. UV/Visible absorption spectrum of FdIV. Solid line, air-oxidized; dotted line, reduced in the 2 mM sodium dithionite for 30 min. The main peak and the separate bands in the range from 250 to 350 nm are also used to differentiate between plant-type [2Fe-2S]²⁺ ferredoxins and vertebrate or bacterial-type [2Fe-2S]²⁺ ferredoxins. Again the environment around the [2Fe-2S]²⁺ cluster in FdIV, as observed by CD, is quite similar to vertebrate or bacterial-type ferredoxins.

EPR analysis of purified FdIV in the oxidized form shows no signal (Fig. 5A). Upon reduction by dithionite, the spectrum exhibits a nearly axial signal with apparent g values of 2.03 and 1.95. This signal may be attributed to a [2Fe-2S]₂⁺ cluster. This [2Fe-2S]₂⁺ cluster showed an optimum of 30k, and almost half of the

experimental data indicate that FdIV is a nearly axial [2Fe-2S]²⁺ cluster.
signal intensity was still detectable at temperatures as high as 70k (data not shown). The power saturation dependence is further consistent with a [2Fe-2S]2+ cluster (data not shown). Taken together, the power and temperature dependence rule out the possibility that the EPR signal arises from a [4Fe-4S]2+ cluster.

The oxidation-reduction potential of the FdIV [2Fe-2S]2+/+ cluster was measured by anaerobic titration using benzyl viologen as a potential indicator and 5-'deazariboflavin/potassium oxalate as a photoreductant. The absorbance change was measured at 440 nm to eliminate the contribution of benzyl viologen to the signal as described under “Experimental Procedures.” By using the Nerst equation (Equation 1) and plotting the $E_m$ versus the log([FdIVox]/[FdIVred]), the midpoint redox potential was calculated to be approximately $-344 \pm 10$ mV versus standard hydrogen electrode at pH 7.8 (best fitted with a slope of 52 mV in case of $n = 1$) (Fig. 6).

$$E_m (mV) = E_n + 59/h \log([FdIV_{ox}]/[FdIV_{red}]) \quad \text{(Eq. 1)}$$

The potential is similar to the related $E. coli$ [2Fe-2S]2+/+ Fd, which was known to be approximately $-380$ mV (77) and is within the range of other [2Fe-2S]2+/+ ferredoxins.

Comparison of FdIV Purified from A. vinelandii and Recombinant FdIV from E. coli—In order to obtain larger quantities of FdIV for crystalization and affinity chromatography experiments, the $fdxD$ gene was used to construct a hybrid plasmid where the expression of the $fdxD$ was placed under the control of the $T_7$ transcriptional and translational control elements. As described under “Experimental Procedures,” FdIV was successfully expressed in $E. coli$ in soluble form using this system, and the protein was easily purified with yields of $0.8$ mg per g of cells. The air-oxidized, isolated gene product appeared to be red-colored, indicating that the [2Fe-2S]2+ cluster was correctly assembled into the polypeptide in its native forms (Fig. 6A). Disruption of the $fdxD$ gene was desired not only as a probe of the function of the protein but also as a means of eliminating a tenacious contaminant encountered during the purification of some FdI variants (Table II). Wild-type $A. vinelandii$ was transformed with plasmids that would result, upon recombination, in strains that would have the $fdxD$ gene disrupted by the insertion of a kanamycin resistance cartridge (see “Experimental Procedures”). However, after initial selection for the kanamycin resistance phenotype, the selected colonies were observed to revert back to kanamycin sensitivity. This behavior indicates that the $fdxD$ gene is required for proper cellular function of $A. vinelandii$.

Relative Amounts of FdI and FdIV in Strains Expressing FdI Variants—The purification and characterization of FdIV described above provides two new tools to examine the relative...
amounts of FdIV and FdI in various *A. vinelandii* strains. First, antibodies raised separately against the two proteins can be used because each antibody only cross-reacts with the protein it was raised against. Based on the qualitative information shown in Table II, we selected three strains expressing FdI variants to compare with the wild-type strain *AcOP*. The strains expressing C20S FdI, C42D FdI, and ΔT14ΔD15 were chosen because the levels of FdIV accumulated by these strains appeared to cover wide ranges of FdI accumulation, roughly similar expression (ΔT14ΔD15), a little lower FdIV expression (C20S FdI), and significantly lower FdI expression (C42D FdI) when compared with the levels of FdI accumulated by the wild-type strain, an observation that is confirmed by the comparison shown in Fig. 8A. Therefore, it might be possible to probe the relationship between FdI and FdIV by looking at FdIV expression level in these strains. In addition, all three of these proteins were stable enough to be purified (50, 52). Initially as a control, we also compared the FdIV levels in LM100, an *A. vinelandii* strain that does not synthesize FdI.

Fig. 8B compares the levels of FdIV in the FdI fractions collected from the first column for the *A. vinelandii* strains we tested. The first surprise in this comparison is that the levels of FdIV seem to be the greatest in LM100, the strain that does not synthesize any FdI. Another surprise is that significant FdIV was observed using antibodies for wild-type and ΔT14ΔD15 even though none of the protein was observed during the purification of FdI from those strains. In contrast, for C20S, which has FdI (Fig. 8A) and FdIV levels comparable to wild-type (Fig. 8B), FdIV was observed during the purification of FdI. At present we are unable to explain why FdIV presents itself as a serious problem during the purification of some FdI variants and not for others, but clearly there is more to the problem than simply the relative levels of the two proteins. The one observation shown in Fig. 8B that was predicted in advance is that C42D does have more FdIV than the other strains.

One disadvantage of the Western blot method of comparison is that it only provides semiquantitative information about the total protein present and cannot distinguish apoprotein and/or cluster-converted protein from holoprotein. Quantitative information about the relative amounts of FdI and FdIV holoproteins in the various strains can be obtained by EPR. This is possible because the [3Fe-4S] cluster of FdI exhibits a characteristic $g = 2.01$ EPR signal in the oxidized state (Fig. 9A), conditions where FdIV is diamagnetic. Upon reduction with dithionite the [3Fe-4S]$^{10}$ cluster becomes EPR silent in the perpendicular mode, whereas the [2Fe-2S]$^{11}$ cluster from FdIV exhibits a characteristic $g = 1.95$ EPR signal (Fig. 9B).

![Fig. 7. SDS-12% polyacrylamide gel electrophoresis separation of the recombinant FdIV (a) and purified FdIV (b). The samples were run on the same gel but not next to each other. The numbers shown on the right indicate the molecular mass (kDa) represented by the marker.](Image 127x651 to 220x729)

Fig. 9A compares the EPR spectra exhibited by the first column FdI fractions for the strains of interest. Clearly the amount of [3Fe-4S]$^{10}$ containing FdI is highest in wild-type followed by C20S with much lower levels observed for both C42D and ΔT14ΔD15. In the latter case, however, it should be noted that the low level of [3Fe-4S]$^{10}$ cluster is not expected to correlate with the levels of cross-reactive material present (Fig. 8A) because that mutation resulted in converting the FdI [3Fe-4S]$^{10}$ cluster to a [4Fe-4S]$^{11}$ cluster which does not exhibit an EPR signal in the oxidized state (50).2 As expected, no FdI was detected in LM100. The good correlation between FdI levels observed with antibodies (Fig. 8A) and FdI levels observed by EPR (Fig. 9B) for C42D and C20S FdI indicate that the fractions contain mainly holoprotein. This is also consistent with the final yields of holoprotein for these two mutants once these fractions were purified to homogeneity.

The levels of FdIV observed by cross-reactivity with antibodies and by EPR are also similar. Fig. 9B shows that the highest levels of FdIV are observed in LM100 but significant levels are also observed in the wild type. Also consistent with the information in Fig. 8B C42D has higher levels of FdIV than does the wild type, and the levels of FdIV in C20S and ΔT14ΔD15 are lower.

Physiological Considerations—Here we have reported the purification and characterization of a new [2Fe-2S]$^{2+/+}$ ferredoxin from *A. vinelandii*, FdIV, and the identification of the

![Fig. 8. Relative amounts of FdI (A) and FdIV (B), detected by Western blot, in strains expressing FdI variants. The same amount of protein (7 μg for FdI and 30 μg for FdIV) was loaded in each lane. Top antibodies were raised against FdI, and bottom antibodies were raised against FdIV. WT, wild type *AcOP*.](Image 319x402 to 543x591)

![Fig. 9. Relative amounts of FdI (A) and FdIV (B), detected by EPR, in strains expressing FdI variants. Spectrum a, AcOP; spectrum b, LM100; spectrum c, C24D; spectrum d, C20S; and spectrum e, ΔT14ΔD15 FdI variants. The same amounts of protein were used, 0.36 and 0.18 mg, respectively, in A and B. EPR conditions for FdI are as follows: microwave frequency, 9.49 GHz; modulation amplitude, 0.64 mT; microwave power, 1 milliwatt; and temperature, 10 K; receiver gain, $5 \times 10^3$. For measurement of FdIV [2Fe-2S]$^{10}$, the sample was first reduced in 2 mM sodium dithionite, and EPR was carried out under the same condition as in FdI except change in temperature and receiver gain into 35 K and $2 \times 10^3$, respectively. A minor signal observed near $g_0$ of [2Fe-2S]$^{10}$ in all FdI mutants is unidentified but, considering that the same signal is also observed in FdI-less LM100 strain, it does not represent [3Fe-4S]$^{10}$ EPR signal of FdI.](Image 368x648 to 495x729)

2The redox potential of the [4Fe-4S] cluster of FdI is too low to be reduced by dithionite so it is also not observed in Fig. 9B.

![Image](Image 127x651 to 220x729)
protein as the product of a previously sequenced gene which we now designate fdxD. This gene is located immediately downstream of the iscSUA-hcsBA gene cluster and appears to be cotranscribed with this cluster (32). The iscSUA gene products are likely to catalyze critical reactions in the assembly and/or repair of [Fe-S] clusters. The hcsBA gene products may serve as the molecular chaperone proteins that assist in the maturation of these [Fe-S] proteins (32). In this context it therefore seems likely that FdIV is somehow involved in the processes of [Fe-S] cluster formation and/or repair possibly by carrying out an essential electron transfer step. Our inability to construct a knock-out mutant further suggests that FdIV is essential for the survival of the cell.

FdIV originally came to our attention because it appeared to associate specifically with certain Fd variants that had altered [Fe-S] ligand composition, an observation that would be consistent with its role in [Fe-S] cluster assembly or repair. On the other hand we have now established that the protein has a very similar charge and molecular weight to FdI such that its apparent “association” with the FdI ligand variants could be attributed to the coincidental copurification of the two proteins. Indeed unlike the situation with FdI and its redox partner NADPH:ferredoxin reductase (57), all attempts to obtain cross-linked complexes of FdI and FdIV have been unsuccessful.

Although these observations do not rule out direct contact between the two proteins, when combined with the observation that FdIV levels are dramatically increased in the FdI minus strain LM100, they do provide an alternative explanation, namely that FdIV levels are specifically increased in response to deletion of FdI (or some function of FdI disrupted in specific mutant strains). In this context it is important to note that in A. vinelandii another protein, ferredoxin reductase, has been shown to be overexpressed to the same levels via the same specific DNA sequence, in response to either deletion of FdI (33) or to addition of the superoxide propagator paraquat to specific DNA sequence, in response to another protein, ferredoxin reductase, has been shown to be overexpressed to the same levels via the same specific DNA sequence, in response to either deletion of FdI (33) or to addition of the superoxide propagator paraquat to specific DNA sequence, in response to either deletion of FdI (33) or to addition of the superoxide propagator paraquat to specific DNA sequence, in response to another protein, ferredoxin reductase, has been shown to be overexpressed to the same levels via the same specific DNA sequence, in response to another protein, ferredoxin reductase, has been shown to be overexpressed to the same levels via the same specific DNA sequence, in response 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overexpressed to the same levels via the same specific DNA sequence, in response to another protocol.

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REFERENCES
1. Mortenson, L. E., Valentine, R. C., and Carnahan, J. E. (1962) Biochem. Biophys. Res. Commun. 7, 448–452
2. Lovenberg, W. (1973) Iron-Sulfur Proteins, Vol. I and II, Academic Press, New York
3. Beinert, H., Helm, R. H., and Münck, E. (1997) Science 277, 653–659
4. Holm, R. H., Kennewohl, P., and Solomon, E. I. (1996) Chem. Rev. 96, 2239–2314
5. Johnson, M. K. (1994) in Encyclopedia of Inorganic Chemistry (King, R. B., ed.) Vol. 4, pp. 1896–1915, Wiley InterScience, New York
6. Cambre, M. R. (1992) Adv. Inorg. Chem. 38, 281–322
7. Howard, J. B., and Rees, D. C. (1991) Adv. Protein Chem. 42, 199–280
8. Van Aubel, H. (1996) FEBS Lett. 382, 218–219
9. Beinert, H., Beinert, K. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 1875–1880
Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G. G., FitzHugh, W., Fields, C. A., Gocayne, J. D., Scott, J. D., Shirley, R., Lin, L.-I., Glodex, A., Kelley, J. M., Wendman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S., Gnehm, C. L., McDonald, L. A., Smith, H. O., and Venter, J. C. (1995) Science 269, 496–512

62. Clark, M. A., Baumann, L., and Baumann, P. (1998) Curr. Microbiol. 36, 158–163

63. Siuzdak, G. (1996) Mass Spectrometry for Bio/Technology, pp. 56–76, Academic Press, San Diego

64. Pétillot, Y., Forest, E., Meyer, J., and Mouli, J-M. (1995) Anal. Biochem. 228, 56–63

65. Meyer, J., Gagnon, J., Gaillard, J., Lutz, M., Achim, C., Münck, E., Pétillot, Y., Colangelo, C. M., and Scott, R. A. (1997) Biochemistry 36, 13374–13380

66. Atta, M., Lafferty, M. E., Johnson, M. K., Gaillard, J., and Meyer, J. (1998) Biochemistry 37, 15974–15980

67. Mittal, S., Zhu, Y. Z., and Vickery, L. E. (1988) Arch. Biochem. Biophys. 264, 383–391

68. Peterson, J. A., Lorence, M. C., and Amarneh, B. (1990) J. Biol. Chem. 265, 6066–6073

69. Cupp, J. R., and Vickery, L. E. (1988) J. Biol. Chem. 263, 17418–17421

70. Uhmann, H., Beckert, V., Schwarz, D., and Bernhard, R. (1992) Biochem. Biophys. Res. Commun. 188, 1131–1138

71. Tsukihara, T., Fukuyama, K., Mizushima, M., Harioka, T., Kusunoki, M., Katsume, Y., Hase, T., and Matsubara, H. (1990) J. Mol. Biol. 216, 399–410

72. Jacobson, B. L., Chae, Y. K., Markley, J. L., Raymond, I., and Holden, H. M. (1993) Biochemistry 32, 6788–6793

73. Peisach, J., Blumberg, W. E., Lede, E. T., and Coon, M. J. (1971) J. Biol. Chem. 246, 5877–5881

74. Farinas, E., and Regan, L. (1998) Protein Sci. 7, 1399–1406

75. Stephenson, P. J., Thomson, A. J., Dunn, J. R., Keiderling, T. A., Rawlings, J., Rao, K. K., and Hall, D. O. (1978) Biochemistry 17, 4770–4778

76. Cheng, H., Xia, B., Reed, G. H., and Markley, J. L. (1994) Biochemistry 33, 3155–3164

77. Knoell, H.-E., and Knapp, J. (1974) Eur. J. Biochem. 50, 245–252

78. Crouse, B. R., Yano, T., Finnegan, M. G., Yagi, T., and Johnson, M. K. (1994) J. Biol. Chem. 269, 21030–21036

79. Golinelli, M.-P., Chatlet, C., Duin, E. C., Johnson, M. K., and Meyer, J. (1998) Biochemistry 37, 10429–10437

80. Isas, J. M., and Burgess, B. K. (1994) J. Biol. Chem. 269, 19404–19409

81. Coghill, V. M., and Vickery, L. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 835–839

82. Böhme, H., and Haselkorn, R. (1989) Plant Mol. Biol. 12, 667–672

83. Baur, J. R., Graves, M. C., Feinberg, B. A., and Ragsdale, S. W. (1990) Biofactors 2, 197–203

84. Davasse, V., and Mouli, J.-M. (1992) Biochem. Biophys. Res. Commun. 183, 341–349

85. Jouanneau, Y., Duport, C., Meyer, C., and Gaillard, J. (1992) Biochem. J. 286, 267–273

86. Yannone, S. M., and Burgess, B. K. (1998) J. Biol. Inorg. Chem. 3, 253–258