Structure of C42D Azotobacter vinelandii FdI

A Cys-X-X-Asp-X-X-Cys MOTIF LIGATES AN AIR-STABLE [4Fe-4S]2+/+ CLUSTER

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All naturally occurring ferredoxins that have Cys-X-X-Asp-X-X-Cys motifs contain [4Fe-4S]2+/+ clusters that can be easily and reversibly converted to [3Fe-4S]2+/0 clusters. In contrast, ferredoxins with unmodified Cys-X-Cys-X-Cys motifs assemble [4Fe-4S]2+/+ clusters that cannot be easily interconverted with [3Fe-4S]2+/0 clusters. In this study we changed the central cysteine of the Cys39-X-X-Cys42-X-X-Cys45 of Azotobacter vinelandii FdI, which coordinates its [4Fe-4S]2+/+ cluster, into an aspartate. UV-visible, EPR, and CD spectroscopies, metal analysis, and x-ray crystallography show that, like native FdI, aerobically purified C42D FdI is a seven-iron protein retaining its [4Fe-4S]2+/+ cluster with monodentate aspartate ligation to one iron. Unlike known clusters of this type the reduced [4Fe-4S]+ cluster of C42D FdI exhibits only an S = 1/2 EPR with no higher spin signals detected. The cluster shows only a minor change in reduction potential relative to the native protein. All attempts to convert the cluster to a 3Fe cluster using conventional methods of oxygen or ferricyanide oxidation or thiol exchange were not successful. The cluster conversion was ultimately accomplished using a new electrochemical method. Hydrophobic and electrostatic interaction and the lack of Gly residues adjacent to the Asp ligand explain the remarkable stability of this cluster.

A fundamental question in [Fe-S] protein biochemistry concerns how cysteine ligand and neighboring residue organization determines [Fe-S] cluster type, and whether or not one type can be converted to another (for reviews, see Refs. 1–9). These issues are important, in part, because there are physiological situations where 3Fe to 4Fe or 4Fe to 2Fe cluster interconversion reactions modulate the activity of an enzyme or a regulatory protein (1, 8–13). In addition, understanding these reactions is of importance in attempts to create clusters with new reactivities (14), to the de novo design of [Fe-S]-containing proteins (14, 15), and to the study of [Fe-S] cluster assembly (16).

This study focuses on the simplest of these reactions, the interconversion of [3Fe-4S]2+/0 and [4Fe-4S]2+/+ clusters. As shown in Fig. 1, these two cluster types differ only by the presence or absence of a single Fe atom at one corner of the cube. The well characterized seven-iron ferredoxin from Azotobacter vinelandii, which contains one [3Fe-4S]2+/0 and one [4Fe-4S]2+/+ cluster, is proving to be an excellent model system for elucidating the properties of these redox centers. As shown in Fig. 2, the amino acid sequence Cys-X-X-Cys-X-X-Cys, which provides three of the four cysteine ligands to a [4Fe-4S]2+/+ cluster, is a very common motif. Interestingly, with one exception, all naturally occurring ferredoxins or ferredoxin variants that contain 4Fe clusters, which can be easily interconverted with 3Fe clusters have instead a Cys-X-X-Asp-X-X-Cys motif. The Asp serves as the ligand that is lost during the cluster interconversion process (23, 25–29). The exception is DgFdII, where the central Cys is covalently modified in the [4Fe-4S] cluster-containing state and where the interconversion reaction is complicated by a change in subunit composition (10, 30). Aconitase, which also easily interconverts, also has a non-Cys (water or OH−) ligand (31, 32). In contrast, ferredoxins or variants that contain [4Fe-4S]2+/+ clusters with four Cys ligands, attempts to convert the 4Fe center to a 3Fe center more often produce only poor yields or result in complete degradation accompanied by denaturation (33–38). This is certainly the case for the protein of interest in this study, AvFdI, where the oxidative destruction of its [4Fe-4S]2+/+ cluster has been studied in some detail (38–40). In this study, we changed the central cysteine of the Cys39-X-X-Cys42-X-X-Cys45 motif, which coordinates the [4Fe-4S]2+/+ cluster, into an aspartate. This paper describes the redox and spectroscopic properties of the new [4Fe-4S]2+/+ cluster, and our various attempts to convert it into [3Fe-4S]2+/0. It also is the first report of the x-ray structure of an aspartate-ligated [4Fe-4S]2+/+ cluster.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of fdx—The oligonucleotide used for the mutagenesis has the sequence 5′-TGATCGCGACGCAGCTTGC-3′, which differs from the wild-type sequence by the substitution of TGC (encoding Cys) for GAC (encoding Asp). The oligonucleotide-directed mutagenesis procedure was similar to that previously described (41, 42) except that a pBluescript SK− phagemid was used as the mutagenesis vector instead of M13mp18. The mutation was confirmed at the DNA level by DNA sequencing. A 1.9-kilobase pair ClaI-XhoI fragment of A. vinelandii carrying wild-type fdxA gene was inserted into pBluescript SK−. The fragment containing the mutation was subcloned into pKT230 as described elsewhere (43), and electroporation was used to
strains 7, Ref. 24), and Ta conversion motifs (FdI), which eluted between 0.54 and 0.58 M NaCl. Each fraction was 4Fe clusters that do not interconvert (FdIV), which eluted between 0.54 and 0.58 M NaCl. Each fraction was monitored by their absorbance at 405 nm and by cross-reactivity with antibodies raised against purified FdI, FdIII, or FdIV. The purified protein and red-colored FdIV eluted as a single broad peak as observed in 0.05 M Tris-HCl, pH 7.5; FdI was incubated at room temperature with K3Fe(CN)6 at a molar ratio of 1:3 for 2 h prior to freezing (39, 40). For NMR, samples were exchanged into a 0.05 M Tris-buffered D2O solution, pH 7.8, by passage through a Sephadex G-25 column (1.5 × 15 cm). The FdI fraction was then concentrated to 0.6–1.0 mM by microfiltration with a Centrifree-10 (Amicon, Beverly, MA). 1H NMR spectra were recorded at room temperature using 90° pulses of 8 μs with 0.5-s delay times, using a Bruker GN-500 Fourier transform NMR spectrometer at the NMR facility, University of California, Irvine, CA. About 6000 scans were accumulated for each spectrum over a 2.5-h period. UV-visible absorption spectra were each recorded in a 0.5-mL quartz cuvette on a Hewlett Packard 8452A diode array spectrophotometer. CD spectra were obtained using a Jasco J-500C spectropolarimeter, with the sample contained in a small volume cylindrical cell with fused quartz window.

**Electrochemistry—Purified water of resistivity ~18 megohm-cm**

(Millipore, Bedford, MA) was used in all experiments. The buffers MES, HEPES, and TAPS and the co-adsorbates neomycin or polymyxin (sulfate, Sigma). An Autolab electroanalytical analyzer (Eco Chemie, Utrecht, The Netherlands) was used to record DC cyclic voltammograms. All experiments were carried out under anaerobic conditions in a Vacuum Atmospheres glove box with an inert atmosphere of N2 (<1 ppm). The three-electrode configuration featuring all-glass cells has been described previously (47); this feature a jacketed main cell compartment (typically holding 500 μL), which could be maintained at 0 °C to optimize protein film stability. The saturated calomel reference electrode was held in a Luggin side arm. Prior to each experiment, the pyrolytic graphite “edge” electrode (surface area typically 0.18 cm2) was polished with an aqueous alumina slurry (Buehler Micropolish; 1.0 μm) and sonicated extensively to remove traces of A12O3. Reduction potentials (Ered) from cyclic voltammetry were determined from the average of the anodic and the cathodic peak potentials, Ered = ½(Epa – Epc) and adjusted to the standard hydrogen electrode scale using E_SCE = E_SHE + 243 mV at 22 °C (where SCE is saturated calomel reference electrode and SHE is standard hydrogen electrode).

Bulk electrochemistry solutions contained between 0.05 and 0.1 mM protein in 60 mM mixed buffer (15 mM HEPES, 15 mM MES, 15 mM TAPS, and 15 mM acetate), with 0.1 mM NaCl as supporting electrolyte and 4 mM neomycin. Neomycin stabilizes the protein-electrode interactions. For film solution experiments, solution used to coat the electrode contained approximately 0.1 mM protein in 60 mM mixed buffer, 0.1 mM NaCl, and 200 μg/mL polymyxin at pH 7.0. The cell solution in each case was of similar composition but contained no protein and was adjusted to the required pH with either HCl or NaOH. The pH of the cell solution was measured before and immediately after the experiment. For detailed analysis, voltammograms were corrected for non-faradaic back.

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1 The abbreviations used are: EPR, electron paramagnetic resonance; FdI. ferredoxin; FPLC, fast protein liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; TAPS, N-tris(hydroxymethyl)methyl-3-aminoipropanesulfonic acid; W, watt(s); T, tesla.
ground current by subtracting a polynomial base line (48). Pulsing experiments were carried out as follows. Films were prepared and then scanned over the normal potential range until the film had stabilized (usually three scans). This voltammogram was then recorded. The protein film was then subjected to an oxidative potential pulse of 0.643 V for 1–4 s. A scan was then taken immediately afterward in the normal potential region, in order to observe the changes that had occurred.

**Crystallization**—Crystals of C42DFdI were grown by the standard methods as described previously (49, 50). However, the protein appeared to denature quickly in drops that contained higher concentrations of ammonium sulfate. Touchseeding and attempts to macroseed did not work. Macroseeds quickly denatured when placed in prequenched drops. A few crystals grew spontaneously after several months’ incubation at 4 °C and appeared to be small but regular. These crystals grew in only two of all the drops seeded.

Attempts to cryoprotect these crystals did not work. The crystals quickly melted when placed in ammonium sulfate saturated drop buffer supplemented with 20% or 25% glycerol. As a result, after several failed attempts to mount a crystal, the crystal used for data collection was scooped directly out of the drop conditions and frozen in the N2 cryostream without cryoprotectant.

**Structure Determination**—The structure of native FdI was modeled with a glycerine at position 42, and refined by rigid body and positional refinement against all of the data to 2.3 Å resolution (Table I) using Xplor version 3.8 (51). The unbiased 2Fo − Fc and Fc − Fo electron density maps clearly revealed the position of the Asp42 side chain with very little other perturbation in the structure. The Asp42 side chain was modeled into the density with Xfit/Xtalview (52), and the structure was refined with isotropic B-factors and a bulk solvent correction using Xplor version 3.8. The Asp42 side chain was restrained to form a 2.1 Å bond to Fe of the [4Fe-4S] cluster, as indicated by the density. The refined model was checked and adjusted against a 2.3-Å resolution 2Fo − Fc and Fc − Fo map. A Fo − Fc and Fc − Fo map was used to locate well ordered H2O molecules, which were included in the model and refined. Statistics for the final model are summarized in Table I. Coordinates have been deposited with the Protein Data Bank with accession number 1FF2.

**RESULTS AND DISCUSSION**

**Cell Growth and Protein Purification**—A. vinelandii is a nitrogen-fixing soil bacterium that appears to synthesize at least 12 different ferredoxins (44). FdI is the only seven-iron ferredoxin that has been identified in that organism. Previous studies have established that FdI has a regulatory function as part of an oxidative stress response system in A. vinelandii (53–58) and a metabolic function unrelated to nitrogen fixation that is important for cell growth (59). Mutagenesis experiments have shown that even subtle alterations in the environment of the [3Fe-4S] cluster have profound negative effects on the growth rate of the organism (42). In contrast, much more drastic alterations in the environment of the [4Fe-4S] cluster have no effect on cell growth (60). In keeping with these observations, the A. vinelandii strain expressing C42D FdI and the strain expressing native FdI exhibit identical lag phases and growth rates.

Previous studies have also shown that mutations involving FdI [Fe-S] cluster ligands always lead to proteins that accumulate to much lower levels in vivo than does the native protein (41, 61). This is also true for C42D FdI. Despite the low levels of accumulation of the C42D FdI in vivo, however, once the protein is purified it appears to be as stable as native FdI. What is particularly important for this study is the fact that the protein is completely stable toward oxygen.

**Like Native FdI, C42D FdI Is A Seven-Iron Protein**—Native AvFdI contains one [4Fe-4S]2+/0 cluster and one [3Fe-4S]−1 cluster (49, 62, 63). The C42D mutation in AvFdI converted the central cysteine of the [4Fe-4S]2+/0 binding motif, Cys38-X-X-Cys45, to aspartate. Naturally occurring ferredoxins that contain Cys-X-X-Asp-X-Cys motifs assemble [4Fe-4S]2+/0 clusters that are easily converted by air oxidation to [3Fe-4S]−1 clusters (10, 19–24); this was the expected outcome in this case. We were therefore surprised to observe that the UV-visible absorption spectrum of the air-oxidized C42D FdI was nearly identical to that of the native FdI (Fig. 3), suggesting that the protein retained its original cluster composition. Oxidized [3Fe-4S]−1 clusters exhibit characteristic g = 2.01 EPR signals that integrate to one spin per molecule. If air-oxidized C42D FdI contained two [3Fe-4S]−1 clusters, then we would expect the size of its EPR signal to double, integrating to two spins per molecule. In contrast, as shown in Fig. 4, the EPR spectrum of air oxidized C42D FdI exhibits a g = 2.01 signal, similar in shape and size to that of native FdI, which is again consistent with its retaining the original cluster composition. Iron analysis was used to confirm that C42D FdI had not lost an iron to become a 6Fe-containing protein, and a value of

| Property | Value |
|----------|-------|
| Space group | P4_12_2 |
| Unit cell (Å) | a = 55.30; c = 90.59 |
| Data collection | All data (last shell) |
| Resolution (Å) | 40.0–2.30 (23.4–2.30) |
| Total observations | 48,416 |
| Unique reflections | 6,640 |
| Completeness (%) | 98.8 (99.7) |
| Rsym (%) | 0.171 (0.78) |
| I/σ(I) | 11.6 (3.1) |
| Refinement | Resolution (Å) 23.8–2.30 |
| Reflections | 6,320 |
| R-factor | 0.217 |
| Rfree (5% of reflections) | 0.269 |
| Geometry | Root-mean-square deviations |
| Angles (degrees) | 2.64 |
| Average B-factors (Å²) | 24.7 (843) |
| Protein (atoms) | 23.9 (15) |
| [Fe-S] clusters (atoms) | 29.1 (48) |
| Solvent (H₂O molecules) | 29.1 (48) |

**FIG. 3. UV-visible absorption spectra of air-oxidized (A) and reduced (B) C42D and native FdI.** Thin line, native FdI; thick line, C42D FdI. The samples were reduced with 2 mM sodium dithionite for 1 h in 0.05 M Tris-HCl, pH 8.0, and 0.1 mM NaCl.
7.5 ± 0.4 iron atoms/molecule for C42D FdI was obtained. That the cluster composition of air oxidized C42D FdI is identical to that of native FdI was subsequently confirmed by x-ray crystallography.

The [4Fe-4S]^{2+/+} Cluster of C42D FdI Has Only Three Cysteine Ligands—For reference, Fig. 5 shows the [4Fe-4S]^{2+/+} region of native FdI. In general, [4Fe-4S]^{2+/+} clusters like the one in native FdI obtain three of their four cysteine ligands from a Cys-XX-Cys-XX-Cys sequence, whereas the fourth cysteine ligand comes from a remote part of the protein, in this case Cys24. As shown in Fig. 5 there is also a fifth free cysteine (Cys24) in van der Waals contact with the [4Fe-4S]^{2+/+} cluster of native FdI. Previous studies have shown that both C20A (64) and C20S (50) variants of FdI assemble [4Fe-4S] clusters with four cysteine ligands by recruiting Cys24. In order to use that cysteine as a new ligand, polypeptide rearrangements were necessary in both cases (50, 64). For the C42D mutant, we wanted to address the possibility that the [4Fe-4S]^{2+/+} cluster might ignore the introduced aspartate and assemble instead using the four cysteine residues Cys20, Cys24, Cys39, and Cys45.

In general, visible region CD spectroscopy is very sensitive to the type of [Fe-S] cluster a protein contains. As shown in Fig. 6, oxidized native FdI exhibits a distinctive CD spectrum with contributions from both clusters. Previous studies have shown that both the wavelength dependence and form of this spectrum is dramatically changed for both the C20A and C20S variants, each of which undergo ligand exchange and structural rearrangement (50, 64). In contrast, as shown in Fig. 6, the CD spectrum of oxidized C42D FdI is similar in wavelength dependence and form to that of native FdI. These data argue against a ligand exchange with an accompanied structural rearrangement.

Further support for the conclusion that [4Fe-4S]^{2+/+} cluster of C42D FdI does not recruit the free cysteine at position 24 (Fig. 5) as a new ligand comes from studies of the oxidation of the protein by ferricyanide. Previous studies have established that native FdI reacts with Fe(CN)$_6^{3-}$ in a three-step degradation process in which the first step is a three-electron oxidation, involving the [4Fe-4S]^{2+/+} cluster and giving rise to a paramagnetic species (39, 40). As shown in Fig. 7, this exhibits EPR up to quite high temperature and is most easily monitored in the range 40–60 K (c and d), where the [3Fe-4S]$^{+}$ cluster EPR is not detectable (a and b) (39, 40). It has been established that this reaction absolutely requires a free cysteine at position 24 and the paramagnetic species is not observed for a C24A variant or for the C20A variant that has rearranged to utilize C24 as a new ligand (61). Fig. 7 shows that the behavior of C42D FdI is indistinguishable from that of native FdI in this reaction, confirming that C24 remains free in this variant. Taken together, the CD and the ferricyanide oxidation experiments lead to the conclusion that in solution the [4Fe-4S]^{2+/+} cluster of C42D FdI is ligated by only three cysteine residues.

Aspartate Ligation Is Confirmed by X-ray Crystallography—Prior to this report, there were no x-ray structures available for any [4Fe-4S] clusters with aspartate ligation. After 2 years of trying, and only after the original version of this paper had been submitted, we were finally successful in obtaining diffra-
The EPR measurements were made in either 30 K (a–d) or 10 K (e and f). The rest condition of measurements: microwave frequency, 9.59 GHz; modulation amplitude, 0.64 mT; microwave power, 1 mW; receiver gain, $2 \times 10^4$ for a–d, $5 \times 10^3$ for e and f.

FIG. 7. X-band EPR spectra of native (a, c, and e) and C42D (b, d, and f) FdI either not treated (a and b) or treated (c–f) with 3 equivalents of K$_2$Fe(CN)$_4$. The samples were 40 μM in 0.050 M Tris-HCl, pH 8.0, 0.1 M NaCl. The EPR measurements were made in either 30 K (a–d) or 10 K (e and f). The rest condition of measurements: microwave frequency, 9.59 GHz; modulation amplitude, 0.64 mT; microwave power, 1 mW; receiver gain, $2 \times 10^4$ for a–d, $5 \times 10^3$ for e and f.

The wavelength dependence and form of the visible region CD spectra exhibited by different [Fe-S] proteins is not only sensitive to cluster type and protein environment but also changes substantially upon oxidation and reduction. Fig. 6B compares the CD spectra exhibited by dithionite-reduced C42D and native FdI. Again, the [4Fe–4S]$^{2+}$ cluster remains oxidized under these conditions. The similarity of the wavelength dependence and overall shape of the spectrum confirm that there is no major structural change occurring upon reduction of the [3Fe–4S]$^{2+}$ cluster. The small changes in form of the reduced spectrum likely arise from the Cys to Asp change in ligation for the [4Fe–4S]$^{2+}$ cluster.

The $^1$H NMR spectrum of oxidized native AvFdI shows several well resolved, paramagnetically shifted resonances (66). The five most downfield resonances are shown in Fig. 9. Three of these, designated A–C, have previously been assigned to the methylene protons of cysteine ligands to the [3Fe–4S]$^{2+}$ cluster, while the other two are believed to arise from methylene pro-

FIG. 8. X-ray crystal structure of [4Fe–4S] cluster regions in C42D FdI. The view is similar to that in Fig. 5. Atoms are colored as follows: Fe, orange; S, yellow; O, red; N, blue; C, gray. A, all atoms of residues 19–22 and 40–45, and the [4Fe–4S] cluster, are shown. Dotted lines indicate short hydrophobic contacts of Asp 42 with amino acid residues Pro21, Ile40, and Leu 44, and hydrogen bonds between O

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Fig. 9. 500-MHz 1H NMR spectra of the oxidized native and C42D FdI. The spectra were obtained at the room temperature using a 90° pulse of 8 μs with 0.5-s delay times. About 6000 scans were accumulated for each spectrum over the 2.5-h. The samples were exchanged into a 50 mM Tris-buffered D2O solution, pH 7.8, by passage through a Sephadex G-25 column (1.5 × 15 cm) and then concentrated to ~0.6–1.0 mM.

Fig. 10. Bulk solution voltammetry of native and C42D AsFdI. Protein samples were suspended in 60 mM mixed buffer, 0.1 mM NaCl, and 4 mM neomycin at 0 °C, pH 7.0, scan rate 5 mV s−1. Voltammograms measured on the third cycle. The concentration of C42D was lower than that of native FdI; hence, the currents are smaller.

The 4Fe-4S2 cluster and the 3Fe-4S cluster (67). A sequence-specific assignment of resonances D and E has not clearly been made for A. vinelandii FdI; however, by analogy to a homologous ferredoxin from Bacillus schlegelii (68, 69), it is probable that resonance D arises from β-CH2 protons from Cys42, the residue of interest in this study, and that resonance E arises from β-CH2 protons from Cys45. This assignment is supported by the observation of weak dipole contacts between the β-CH2 protons of Cys45 in the [3Fe-4S]+ cluster and the β-CH2 protons of Cys45 that ligates [4Fe-4S]2+; together with the observation in the 1H NMR structure of a nuclear Overhauser effect between signals C and E (66, 67). As shown in Fig. 9, the spectrum of C42D indicates that replacement of ligand Cys42 by Asp perturbs the structures of both the Cys42 site and the Cys45 site, which results in one new signal appearing at 14.4 ppm. This perturbation at Cys45 is observed in the x-ray structure described above (Fig. 8, A and B). This perturbation also gives rise to a splitting of the Cys45 signal into two equal components with half-intensities. The substitution, however, does not change the other β-CH2 ligand resonances for the [3Fe-4S]+ cluster, as seen in the same chemical shifts for signals A and B.

Reduction of the [4Fe-4S]2+ Cluster of C42D Versus Native FdI—The [4Fe-4S]2+/+ cluster of native FdI has an unusually low reduction potential and cannot be reduced by dithionite at neutral pH. Previous studies have established, however, that it can be reduced using direct electrochemical methods (47). Fig. 10 shows bulk solution voltammetry of C42D AsFdI at pH 7.0. Couple A arises from [3Fe-4S]2+/+, whereas couple B arises from [4Fe-4S]2+/+, confirming again that the cluster composition of native and C42D FdI are the same. Examination of the data in Fig. 10 shows a shift of +30 mV in the reduction potential of the [4Fe-4S]2+/+ couple; from −0.620 V (for native FdI) to −0.590 V (for C42D FdI) versus standard hydrogen electrode. As expected, there was no change in the reduction potential of the [3Fe-4S]2+/+ cluster, which is remote from the site of the mutation (60). The observation that the reduction potential of the [4Fe-4S]2+/+ cluster of C42D FdI is similar to that in native FdI is not unexpected. Thus, as shown in Table II, mutagenesis of other ferredoxins where a naturally occurring Cys-X-Asp-X-Cys motif is changed to Cys-X-X-Cys-X-Cys also resulted in only minor changes in reduction potential (36, 71).

In most cases, reduced [4Fe-4S]+ clusters that have four cysteine ligands exhibit gav ≈ 1.94 signals that arise from an S = 1/2 ground state. This is true not only for naturally occurring ferredoxins but also for variants that have been constructed by mutagenesis where naturally occurring Cys-X-X-Asp-X-Cys motifs have been converted to Cys-X-X-Cys-X-X-Cys motifs (36, 71). In contrast, where information is available, naturally occurring reduced ferredoxins that contain [4Fe-4S]2+ cluster ligated by three cysteines and one aspartate ligands exhibit a g ≈ 5.3 signal that arises from the ground state m = ±3/2. In some cases, these clusters exhibit only the S = 3/2 spin state (for example, DaFdIII (Refs. 25, 72, and 73) and an A33Y mutant of PfFd (Ref. 74)), whereas, in others, they exhibit a mixture of S = 1/2 and S = 3/2 states (23, 71). In order to compare the spectra of the reduced [4Fe-4S]+ clusters in C42D and native FdI, a low reduction potential system was employed, utilizing the photoreduction of 5′-deazariboflavin with EDTA as sacrificial electron donor. As shown in Fig. 11A, the reduced [4Fe-4S]2+ cluster of C42D FdI exhibits an S = 1/2 EPR signal with resonances, g = 2.14, 1.86, and 1.79. The signal is much broader than that observed for the native protein. The same extent of signal broadness has been reported previously for other oxygen-ligated proteins, substrate-complexed aconitase2 (75), aspartate-ligated PfFd (76), and probably OH−. In substrate-bound aconitase, both H2O and the carboxylate group of substrate are also bound to one iron of [4Fe-4S] cluster. Unlike C42D FdI, the fourth iron is easily lost upon exposure to air. In addition to signal broadening, there is a distinct shift in g-values when substrate binds to reduced aconitase with bound g-values of 2.04, 1.85, and 1.78, which compares with reduced C42D FdI with 2.14, 1.86, and 1.79. Aconitase without substrate-bound is 2.06, 1.93, and 1.86.
mixed $S = 1/2$ and $S = 3/2$ spin state despite the fact that it has four cysteine ligands rather than three cysteine and one aspartate (78). There are several reports in the literature showing that spin crossover is solvent-induced (23, 79–85). For the nitrogenase Fe protein, the addition of a denaturing agent such as $0.4 \, \text{M}$ urea stabilizes the $S = 3/2$ state, whereas both $50\%$ (v/v) ethylene glycol and glycerol favor the $S = 1/2$ spin state (79). In $P/Fd$, $50\%$ (v/v) glycerol, but not $0.4 \, \text{M}$ urea affects the relative populations of the $S = 1/2$ and $S = 3/2$ states and results in a shift in favor of the $S = 1/2$ species (23). Solvents such as acetonitrile or dimethyl sulfoxide also induce spin mixtures in the synthetic analog $\left[\text{Fe}_4\text{S}_4(S\text{R})_4\right]^{3-} (\text{R} = \text{alkyl or aryl})$ (80). Finally, the $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ $\text{PsaC}$ protein mutants, $C14D$ and $C51D$, are also known to be easily converted from $S = 3/2$ to $S = 1/2$ at the modified cluster upon binding to the photosystem I core complex, a situation that is likely to affect solvent accessibility (81–85).

Interestingly, there are several examples of other proteins showing $S > 1/2$ spin states with cysteine-only ligated $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ clusters. For example, the Bacillus subtilis glutamine phosphoribosylpyrophosphate aminotransferase has the $S = 3/2$ $\left[\text{Fe}_4\text{S}_4\right]^{3+}$ as the dominant species and $S = 1/2$ and $5/2$ as minor components (86). In Thauera aromatica ferredoxin, which contains cysteine-only-ligated $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ clusters, a spin mixture of $S = 3/2$ and $5/2$ is observed for one cluster in the reduced state (87). Structures are not yet available for these clusters, and their solvent accessibility has not been reported.

The $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ Cluster of $C42D$ FdI Is Extremely Stable and Is Not Converted to a $\left[\text{Fe}_4\text{S}_4\right]^{3+/4+}$ Cluster by Conventional Methods—As shown above, like native FdI, $C42D$ FdI is a completely air-stable protein that contains one $\left[\text{Fe}_4\text{S}_4\right]^{3+/4+}$ cluster and one $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ cluster. This is the only known example of a $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ cluster with three cysteines and one water or aspartate ligand that is not converted to a $\left[\text{Fe}_4\text{S}_4\right]^{3+/4+}$ cluster upon exposure to oxygen. We therefore attempted to do the conversion using alternative methods that had been successful with other proteins. One of these methods involves addition of potassium ferricyanide (10, 88–91). However, in the case of $C42D$ FdI, addition of Fe($\text{CN})_6^{3-}$ did not result in cluster conversion, as monitored by quantitative examination of the $g = 2.01$ signal that arises from the $\left[\text{Fe}_4\text{S}_4\right]^{3+}$ clusters. A successful conversion should have doubled the size of that signal, but, as shown in Fig. 7, the $\left[\text{Fe}_4\text{S}_4\right]^{3+}$ signal exhibited by $C42D$ FdI was both qualitatively and quantitatively unchanged following the addition of ferricyanide.

In studies of $DdFdIII$, it was demonstrated that externally added thiocyanate could compete with the easily displaced aspartate ligand to form $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ clusters with three endogenous cysteine ligands and one exogenous thiolate ligand (92). The products could be easily identified using direct electrochemical methods because their reduction potentials were significantly different from that of the original cluster. For example, the transformed $(\text{O}_2$-sensitive) $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ cluster in $DdFdIII$ co-ordinates exogenous 2-mercaptoethanol as a ligand, whereupon its reduction potential becomes approximately $190 \, \text{mV}$ more negative. By contrast, no such shift in reduction potential was observed for $C42D$ FdI when an electrode coated with a protein film was placed in a buffered solution of $715 \, \text{mM}$ 2-mercaptoethanol at pH 8.0. Thus, all of our attempts to use conventional methods to replace the aspartate ligand of $C42D$ FdI were unsuccessful, thus demonstrating the extreme stability of this cluster.

### Conversion of the C42D FdI $\left[\text{Fe}_4\text{S}_4\right]^{2+/3+}$ Cluster to a $\left[\text{Fe}_4\text{S}_4\right]^{3+/4+}$ Cluster Was Ultimately Accomplished Using a New Electrochemical Method—The mechanism of the 4Fe to 3Fe cluster conversion reaction in any protein has yet to be established but,
as shown in Reaction I, it has been suggested to involve transient oxidation of the [4Fe-4S]2+ cluster to the superoxidized HiPIP [4Fe-4S]3+ state prior to removal of iron (75, 93–95).

\[
[4Fe-4S]^{3+} \rightarrow ([4Fe-4S]^{5+}) \rightarrow [3Fe-4S]^{+}
\]

**REACTION I**

This possibility has recently been studied using direct electrochemical methods with *Clostridium pasteurianum* Fd, which contains two [4Fe-4S]2+/3+ clusters with complete cysteine ligation. The experiment, which is discussed in detail elsewhere (96), involves subjecting a protein film at an electrode surface to short pulses (e.g. 0.1 to 4 s) at various high potentials to create the superoxidized [4Fe-4S]3+ state. This species is usually unstable and breaks up, but it is possible to optimize conditions (pulse potential and duration) to control this oxidative fragmentation, so that just one iron atom is ejected, thus forming a [3Fe-4S] cluster. The success of the experiment is easily monitored by immediate cyclic voltammetry of the same film because the new [3Fe-4S] cluster exhibits two signals. One, designated A′, arises from the [3Fe-4S]−/0 transition; the other, designated C′, which is more prominent, arises from a further two-electron [3Fe-4S]0/−2 transition.

The above method was successful in converting both of the two [4Fe-4S]2+/3+ clusters of *C. pasteurianum* Fd to a [3Fe-4S] cluster, even though the original clusters each have four cysteine ligands. This method, however, has not so far been successful in converting the [4Fe-4S]2+/3+ cluster of native FdI. Although the [4Fe-4S]2+/3+ cluster of C42D FdI is extremely stable when compared with aspartate-ligated clusters in other proteins, we thought that it might be less stable than the cluster in native FdI. To test this, a variety of pulse times, pulse potentials, and other conditions were used. Optimum formation of new signals attributable to a second [3Fe-4S] cluster was observed when a film of C42D FdI was poised, at pH 8.0, at a potential of +0.643 V for 4 s, 3 s, and finally 2 s with voltammograms recorded between each pulse. The result is shown in Fig. 12. The 7Fe protein that is present at the start of the experiment (Fig. 12A, *top panel*) clearly shows three signals, A′, B′, and C′, corresponding to the [3Fe-4S]−/0, [4Fe-4S]2+/3+ and [3Fe-4S]0/−2 redox couples, respectively (97). As is commonly found in the film voltammetry of [3Fe-4S]-cluster-containing proteins, the C′ signal is kinetically complex and displays a large peak separation; thus, even at a relatively slow scan rate of 50 mV s−1, the C′ peak overlaps the B′ peak in the oxidative scan whereas they are clearly separated in the reducing direction. Fig. 12A (*bottom panel*) shows the voltammetry of the product; the B′ couple that arises from the indigenous [4Fe-4S]2+/3+ cluster has disappeared and has been replaced by two new signals labeled A″ and C″. Their appearance is fully consistent with the formation of a second [3Fe-4S] cluster.

The change in ratios A′/C′ after the pulse probably arises because application of high potentials also causes extensive desorption of the protein from the electrode surface (96). The desorbed ferredoxin molecules, many of which will escape without undergoing the cluster transformation, are initially still able to diffuse back to the electrode and undergo electron transfer, but without becoming adsorbed. The A′ and C′ signals in a diffusional voltammogram do not reflect the expected 1-to-2 stoichiometry, i.e., the C′ signal is rarely observed unless the protein is tightly bound to the electrode. (This is most likely due to the complexity of the cooperative two-electron redox reaction, which involves sequential electron-proton transfers that require a sufficiently long residence time at the electrode (Ref. 97).)

**Fig. 12.** A, base-line subtracted film voltammograms of C42D Ac-FdI measured before (*top panel*) and after (*bottom panel*) poised at a potential of +0.643 V for 4, 3 and then 2 s, at pH 8.0, 0 °C. B, plots of $E^*/E^0$ versus pH for the new (A) and indigenous (B) [3Fe-4S] cluster A′ and C′ couples. Film solution contains approximately 100 μM protein in 60 mM mixed buffer, 0.1 M NaCl, and 200 μg/ml polymyxin at pH 7.0. Cell solutions were similar to the film solution, but contained no protein and was at the required pH.

C42D FdI. The slopes for both redox couples and their p$K_a$ values are similar for both clusters, with the new A′ couple having p$K_a = 7.7$ and $E^0_{A′} = -0.341 V$ The p$K_a$ arises from coupled proton-electron transfer reactions that are well characterized for the native protein (97). The reduction potential of the new [3Fe-4S]−/0 cluster is about 100 mV more positive than that of the indigenous cluster. This is not surprising since [3Fe-4S]−/0 clusters in different proteins vary greatly in their reduction potentials.

Even though we were successful in converting the 4Fe cluster of C42D FdI to a [3Fe-4S] cluster by electrochemical pulsing, the product could not be readily converted back to a 4Fe cluster. Thus, unlike the situation with easily converted clusters (70, 98), when films of the 6Fe form of C42D FdI were placed in 5 mM solutions of Fe2+, Zn2+, and Tl1+, no signs of metal uptake were observed.

**Why Can’t the C42D FdI [4Fe-4S]2+/3+ Cluster Be Easily Converted to a [3Fe-4S]−/0 Cluster?**—Taken together, the above data demonstrate that it is not the presence of an aspartate ligand in the central position per se that allows the facile conversion of a [4Fe-4S]2+/3+ cluster to a [3Fe-4S]−/0 cluster upon oxygen and ferricyanide addition. Fig. 8 (A and B) shows the structure for the [4Fe-4S]2+/3+ cluster region of C42D FdI. The aspartate fits tightly and is held in place and restrained by van der Waals contacts with the three hydrophobic residues Ile40, Leu44, and Pro21. These interactions would be expected to
limit access of solvent to the [4Fe-4S]_{2+} cluster at the site of Asp_{42} ligation, and at the same time prevent dissociation of the aspartate from the cluster. Although the carbohydrate carries the same negative charge as a cysteine ligand, its oxygen atoms are less polarizable than sulfur. The negative charge, within the hydrophobic cavity formed by Pro_{21}, Ile_{40}, and Leu_{44}, is compensated not only by the [4Fe-4S]_{2+} cluster, but also by two electrostatic interactions, one involving OEt with the amide of Leu_{44} and the other OEt with Pro_{21} (Fig. 8a). The former interaction has good geometry for a hydrogen bond, and the latter, justified by the positions of the residues in the electron density, may represent a CH...O hydrogen bond. Alternatively, OEt of Asp_{42} may be protonated. Together, these hydrophobic contacts and electrostatic interactions help account for the stability of Asp_{42} as a ligand.

Examination of the sequences of the easily convertible proteins (Fig. 2) with a central aspartate shows that in most cases the residue at position 44 is much smaller and/or not hydrophobic, which may facilitate the movement of the aspartate. An exception is PfFdh, which does easily convert, albeit at a slower rate than for DafIII (70), and has a hydrophobic Ile at the position homologous to the Fdh residue number 44. Thus, although the residue 44 may be important in facilitating the cluster conversion, it cannot be the controlling factor. In order for the cluster conversion to occur, the backbone must be able to move to allow the aspartate to move away from the cluster. Examination of the sequences of the easily convertible proteins (Fig. 2) shows that, without exception, those proteins that have a central aspartate have a glycine on one or both sides of the aspartate to facilitate this backbone movement. In contrast, the Asp_{42} residue of C42D Fdh is flanked by aspartate and alanine, which restrict the corresponding backbone movement. Future experiments could therefore test the hypothesis that construction of a D41G/C42D double mutant in Fdh ought therefore to lead to an easily convertible cluster or that conversion of the homologous Gly_{22} position in Fdh/PF to an aspartate would lead to a [4Fe-4S]_{2+} cluster that could no longer be converted to [3Fe-4S]_{0}.

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