[4Fe–4S]-cluster-depleted *Azotobacter vinelandii* ferredoxin I: A new 3Fe iron–sulfur protein

(iron-sulfur clusters/magnetic CD/iron x-ray absorption spectroscopy)

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ABSTRACT $\text{Fe}(\text{CN})_6^{3-}$ oxidation of the aerobically isolated 7Fe *Azotobacter vinelandii* ferredoxin I, (7Fe)FdI, is a degradative reaction. Destruction of the [4Fe-4S] cluster occurs first, followed by destruction of the [3Fe-3S] cluster. At a $\text{Fe}(\text{CN})_6^{3-}$ /(7Fe)FdI concentration ratio of 20, the product is a mixture of apoprotein and protein containing only a [3Fe-3S] cluster, (3Fe)FdI. This protein mixture, after partial purification, has been characterized by absorption, CD, magnetic CD, and EPR and Fe x-ray absorption spectroscopies. EPR and magnetic CD spectra provide strong evidence that the [3Fe-3S] cluster in (3Fe)FdI is essentially identical in structure to that in (7Fe)FdI. Analysis of the extended x-ray absorption fine structure (EXAFS) of (3Fe)FdI finds Fe scattering at an average Fe...Fe distance of $\approx 2.7 \text{ \AA}$. The structure of the oxidized [3Fe-3S] cluster in solutions of oxidized (3Fe)FdI, and, by extension, of oxidized (7Fe)FdI, is thus different from that obtained by x-ray crystallography on oxidized (7Fe)FdI. Possible interpretations of this result are discussed.

X-ray crystallography has recently shown that *Azotobacter vinelandii* ferredoxin I [(7Fe)FdI] as isolated aerobically contains one [4Fe-4S] cluster and one [3Fe-3S] cluster (1, 2). The former possesses a $[\text{Fe}_4\text{S}_4(\text{Cys})_4]$ structure (where S is sulfide, and cysteine is bonded through its sulfur to Fe) essentially identical to those found in *Peptococcus aerogenes* ferredoxin (ref. 3 and references therein) and *Chromatium vinosum* high-potential iron protein (HIPIP) (ref. 4 and references therein). The latter is found to have a novel $[\text{Fe}_3\text{S}_3(\text{Cys})_5\text{X}]$ structure, where X is unidentified. The presence of clusters containing 4Fe and 3Fe was independently deduced by Mössbauer spectroscopy (5), which also showed that the [3Fe-3S] cluster is paramagnetic in both oxidized (as isolated) and dithionite-reduced states, while the [4Fe-4S] cluster is diamagnetic in both states. Thus, the oxidized [3Fe-3S] cluster is responsible for the $g \approx 2.01$ EPR signal of (7Fe)FdI as isolated (6). Its Mössbauer and EPR parameters have been rationalized by assuming all Fe to be (formally) Fe^{3+} (7). The oxidized and reduced [3Fe-3S] clusters are then in the 3+ and 2+ oxidation levels, respectively. The [4Fe-4S] cluster is in the 2+ oxidation level. The oxidation states of (7Fe)FdI and its component clusters are thus:

Fe-S clusters containing 3Fe have been detected in several other proteins by Mössbauer spectroscopy (8–10). However, in *Desulfovibrio gigas* ferredoxin II (*D. gigas* FdII) (11) and

beef heart aconitase (12), Fe EXAFS (extended x-ray absorption fine structure) measurements have found an average Fe...Fe distance of ≈ 2.7 Å, in marked contrast to the ≈ 4.1 -Å distances found in (7Fe)Fdl by x-ray crystallography (1, 2). In addition, an $[\text{Fe}_3\text{S}_4]$ core stoichiometry has been reported in beef heart aconitase (12, 13), differing from that found in (7Fe)Fdl by x-ray crystallography. Therefore, it appears that considerable heterogeneity of structure must exist in 3Fe clusters.

We recently have studied the $\text{Fe}(\text{CN})_6^{3-}$ oxidation of (7Fe)Fdl_{ox} (14, 15) and have shown that (i) it is a destructive oxidation process but that (ii) two intermediate forms of Fdl exist *en route* to apoprotein:

In the first stage of the reaction, oxidation of the $[4\text{Fe}-4\text{S}]^{2+}$ cluster leads to a paramagnetic product $[4\text{Fe}-4\text{S}]'$, whose nature is discussed elsewhere (14). Subsequently, the $[4\text{Fe}-4\text{S}]$ cluster is fully destroyed, leaving $(3\text{Fe})\text{FdI}_{\text{ox}}$, in which the $[3\text{Fe}-3\text{S}]^{3+}$ cluster remains intact (15). Finally, the $[3\text{Fe}-3\text{S}]$ cluster is destroyed, creating apoprotein. Therefore, it is feasible by controlled oxidation of $(7\text{Fe})\text{FdI}_{\text{ox}}$ to generate a mixture of $(3\text{Fe})\text{FdI}_{\text{ox}}$ and apo-FdI, from which $(3\text{Fe})\text{FdI}_{\text{ox}}$ should be purifiable.

We report here preparation and partial purification of (3Fe)FdI and its preliminary characterization by absorption, CD, EPR, magnetic CD (MCD), and x-ray absorption spectroscopies. The EPR and MCD results confirm that the [3Fe-3S] cluster is essentially unchanged in structure upon removal of the [4Fe-4S] cluster. An ≈ 2.7 Å per Fe...Fe average distance is found for (3Fe)FdI_{ox} by analysis of its EXAFS, in contrast to the ≈ 4.1 Å distances obtained by x-ray crystallography of (7Fe)FdI_{ox}.

MATERIALS AND METHODS

All methods and materials pertaining to purification of (7Fe)FdI_{ox}, to reactions with K₃Fe(CN)₆, sodium dithionite, and dithiothreitol, to G-25 Sephadex and DE-52 DEAE-cellulose chromatographies, and to absorption, CD, EPR,

Abbreviations: (7Fe)Fdl, 7Fe ferredoxin I; (3Fe)Fdl, ferredoxin I containing only a [3Fe-3S] cluster; MCD, magnetic circular dichroism; EXAFS, extended x-ray absorption fine structure; HIPIP, high-potential iron protein; Fdl and FdII, ferredoxins I and II; FT, Fourier transform.

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and MCD spectroscopies were as detailed (14–16). Tiron (the disodium salt of 4,5-dihydroxy-1,3-benzenedisulfonic acid) was from Aldrich. Protein Fe content was determined as described (17). Potassium phosphate buffer (pH 7.5) at 0.1 M was used throughout, with the exception of the Fe analysis, which required exchange into 0.1 M Tris-HCl buffer (pH 7.4). All experiments subsequent to purification, with the exception of Fe analysis, were executed anaerobically.

X-ray absorption data were collected by fluorescence excitation at the Stanford Synchrotron Radiation Laboratory on focused beam line II-2 during dedicated running at 3.0 GeV and ≈ 40 mA using Si[220] monochromator crystals. Protein data were collected on a sample mounted under helium gas and at 0–5°C and consisted of 16 30-min scans. Each scan was internally calibrated by using an Fe foil as described (18).

The EXAFS data were extracted by first subtracting a polynomial designed to mimic the scattering background. This polynomial was calculated by fitting the data in the EXAFS region (7250–7930 eV) with a quadratic equation and then subtracting a constant from it so that it passed through the data just before the edge. Then a third-order polynomial spline was fit to the same range of data, with spline points at 7330 and 7630 eV. This spline was subtracted, and the data were normalized using a Victoreen (19) to model the atomic fall-off, giving the final EXAFS.

The resultant EXAFS data were fit by using a hybrid theoretical/empirical approach which will be described in detail elsewhere. Empirical amplitude and phase functions were generated by complex backtransformation (20) of the appropriate shells (Fe–S, Fe–Fe) of model compounds, and these functions were adjusted (by variation of E_0) to have the same k scale as the theoretical functions of Teo and Lee (21). The Debye–Waller σ_{as} (where σ_{as} = relative root mean square deviation in distance) for the models was estimated by assuming that the overall shape (but not the magnitude) of the theoretical amplitude functions was correct. For the protein data, two-shell curve-fitting was performed on Fourier-filtered EXAFS consisting of the two main peaks (Fe–S and Fe–Fe) in the Fourier transform. R_{as} (the distance) and σ_{as} were varied for a few integer values of N_s (the number of scatterers) for each shell, and the derived σ_{as} values were compared with the values estimated for the model compounds as one measure of the validity of the fits. In generating the phase functions from the model compound data, it was found that the appropriate E_0 for an Fe–S interaction was ≈ 7135 eV, whereas that for Fe–Fe interaction was ≈ 7130 eV. Thus, in the fits of the protein data, the EXAFS was extracted with $E_0 = 7130$ eV, and ΔE_0 was varied for the Fe–S shell.

The absorption, CD, and EPR of samples used to obtain x-ray absorption data were compared before and after data collection. The absence of any significant difference showed that radiation damage was negligible.

RESULTS AND DISCUSSION

Anaerobic $\text{Fe}(\text{CN})_6^{3-}$ oxidation of (7Fe)Fdl_{ox} at ambient temperature and with $\approx 60 \mu\text{M}$ (7Fe)Fdl_{ox} and $\text{Fe}(\text{CN})_6^{3-}$ /(7Fe)Fdl_{ox} concentration ratio ≈ 20 caused loss of UV-visible absorption and CD (Figs. 1 and 2). The progress of the reaction was conveniently monitored by absorption and CD spectroscopies. Completion required 2–3 hr, after which time no absorption attributable to $\text{Fe}(\text{CN})_6^{3-}$ was observable at 420 nm. Anaerobic chromatography on G-25 Sephadex and then DE-52 DEAE-cellulose did not change the shape of the visible absorption and CD but led to loss of near-UV absorption ($\lambda \lesssim 300$ nm). The ratio A_{400}/A_{280} of the protein after G-25 and DE-52 chromatography, henceforth termed Fdl(20FCN), was ≈ 0.3 . No precipitation—brown, white, or blue—was observed during $\text{Fe}(\text{CN})_6^{3-}$ oxidation of (7Fe)-

FIG. 1. Absorption of $\text{Fe}(\text{CN})_6^{3-}$ -oxidized (7Fe)Fdl_{ox} at $\text{Fe}(\text{CN})_6^{3-}$ /(7Fe)Fdl_{ox} concentration ratio of 20. Scan 0 was prior to addition of $\text{Fe}(\text{CN})_6^{3-}$; (7Fe)Fdl_{ox} was 0.0596 mM in 0.1 M potassium phosphate buffer (pH 7.5). Scans 1–8 were initiated 5, 20, 40, 60, 80, 100, 115, and 130 min after addition of $\text{Fe}(\text{CN})_6^{3-}$, when (7Fe)Fdl_{ox} was 0.0590 mM. Scan time was ≈ 10 min. Scan 9 was the buffer baseline. Cell path length was 0.517 cm.

Fdl_{ox}. No blue residue was observed on G-25 or DE-52 columns after chromatography.

Although (7Fe)Fdl_{ox} is stable to air, exposure of

FIG. 2. CD of $\text{Fe}(\text{CN})_6^{3-}$ -oxidized (7Fe)Fdl_{ox} at $\text{Fe}(\text{CN})_6^{3-}$ /(7Fe)Fdl_{ox} concentration ratio of 20. The sample was that described in the caption to Fig. 1. Scan 0 was prior to addition of $\text{Fe}(\text{CN})_6^{3-}$. Scans 1–7 were initiated 5, 20, 40, 60, 80, 100, and 115 min after addition of $\text{Fe}(\text{CN})_6^{3-}$. Scan 8 was the buffer baseline. Scan time was ≈ 15 min. Spectral bandwidth and time constant were 2 nm and 4 sec, respectively. Cell path length was 0.514 cm.

FdI(20FCN) to air caused slow continual changes in absorption and CD. Consequently anaerobic conditions continued to be used throughout this work. Addition of dithiothreitol caused immediate significant change in the CD without much change in absorption. However, in addition, slow, continuous changes ensued without well-defined termination. Addition of excess Tiron changed neither absorption nor CD, showing that adventitious Fe^{3+} was absent. [Absorption at ≈ 500 nm attributable to Fe^{3+} -Tiron complexation was observed on addition of Tiron directly after $\text{Fe}(\text{CN})_6^{3-}$ oxidation of (7Fe)FdI_{ox}.]

Further change in A_{400}/A_{280} could be created by additional chromatography. However, as a result of the limited quantities of protein available, all experiments reported here used FdI(20FCN) further treated by diafiltration with excess Tiron. Fe analysis of this FdI(20FCN) found 1.2 ± 0.3 Fe per molecule of FdI.

The EPR at ≈ 11 K and MCD at ≈ 2 K of both (7Fe)FdI_{ox} and FdI(20FCN) are shown in Figs. 3 and 4. The EPR after $\text{Fe}(\text{CN})_6^{3-}$ oxidation remained essentially isotropic with $g \approx 2.01$ and exhibited comparable temperature dependence to that of (7Fe)FdI_{ox} (14). No EPR signal was observed at 50 K. At 11 K slight broadening was observed relative to (7Fe)FdI_{ox}. Integration of the 11 K EPR together with the Fe analysis led to 0.9 spin per 3Fe. The MCD of FdI(20FCN) at ≈ 2 K was indistinguishable in shape from that of (7Fe)FdI_{ox}.

The loss of visible-near-UV absorption and CD on $\text{Fe}(\text{CN})_6^{3-}$ oxidation of (7Fe)FdI_{ox} at $\text{Fe}(\text{CN})_6^{3-}/(7\text{Fe})\text{FdI}_{\text{ox}}$ concentration ratio ≈ 20 is consistent with substantial destruction of Fe-S clusters. The increase in absorption at $\lambda \leq 300$ nm is attributable to $\text{Fe}(\text{CN})_6^{3-}$ and other inorganic products of destructive oxidation of Fe-S clusters. "Free" Fe^{3+} was apparently present, as gauged by reaction with Tiron. After chromatography on G-25 and DE-52, however, there was no longer any indication of absorption from $\text{Fe}(\text{CN})_6^{3-}$ or Fe^{3+} ; the latter was not detectable on addition of Tiron. At no stage was precipitation attributable to either aggregated protein or Prussian blue observed.

According to previous studies (15), after oxidation of (7Fe)FdI_{ox} by $\text{Fe}(\text{CN})_6^{3-}$ at $\text{Fe}(\text{CN})_6^{3-}/(7\text{Fe})\text{FdI}_{\text{ox}} \approx 20$, the product is a mixture of apo-FdI and (3Fe)FdI_{ox}. Direct Fe analysis was consistent with this expectation and, on the basis of 1.2 Fe per molecule, led to 60% apo-FdI and 40% (3Fe)FdI_{ox}. By assuming that the $[\text{3Fe-3S}]^{3+}$ and $[\text{4Fe-4S}]^{2+}$ clusters require 11 and 14 equivalents of $\text{Fe}(\text{CN})_6^{3-}$ for complete destruction (14, 15) and that oxidation of the two clusters is sequential, the Fe content is predicted to be 1.4 Fe per molecule after oxidation by 20 equivalents of $\text{Fe}(\text{CN})_6^{3-}$.

FIG. 3. EPR of (7Fe)FdI_{ox} (curve a) and FdI(20FCN) (curve b) at 11 K. (7Fe)FdI_{ox} was 0.059 mM in potassium phosphate buffer (pH 7.5). FdI(20FCN) was 0.107 mM (in Fe) in 0.1 M potassium phosphate buffer (pH 7.5). Power, frequency, and modulation were 2 mW, 9.355 GHz, and 1 G, respectively. The gain was 10^4 and 2×10^4 for curves a and b, respectively.

FIG. 4. Low-temperature MCD of (7Fe)FdI_{ox} (curve a) and FdI(20FCN) (curve b). (7Fe)FdI_{ox} was 0.553 mM in 0.1 M potassium phosphate buffer (pH 7.5) prior to addition of glycerol and 0.289 mM after addition of glycerol. Field and temperature were +0.938 T and 1.78 K, respectively. Path length was 1.02 mm. FdI(20FCN) was 0.33 mM (in Fe) in 0.1 M potassium phosphate buffer (pH 7.5) prior to addition of glycerol and 0.14 mM (in Fe) after addition of glycerol. Field and temperature were 3.00 T and 1.77 K. The MCD of FdI(20FCN) was normalized in intensity to that of (7Fe)FdI_{ox} by using the difference in MCD from 470 to 515 nm. Spectral bandwidth was 2 nm; time constants were 1 and 4 sec in curves a and b, respectively.

Therefore, it appears that G-25 and DE-52 did not achieve substantial separation of apo-FdI and (3Fe)FdI_{ox}.

The 11 K EPR spectrum of FdI(20FCN) is very similar in g value, shape, and temperature-dependence to that of (7Fe)FdI_{ox}. It is attributable to the $[\text{3Fe-3S}]^{3+}$ cluster and indicates that the latter is very similar in structure in (3Fe)FdI_{ox} and (7Fe)FdI_{ox}. No EPR was observed at 50 K, showing that the initial product of oxidation of the $[\text{4Fe-4S}]^{2+}$ cluster, $[\text{4Fe-4S}]'$, was fully destroyed. No other EPR signals, including one at $g \approx 4.3$, were observed.

Integration of the 11 K EPR spectrum led to 0.9 spin per 3Fe, which is in excellent agreement with the expectation that the Fe-S cluster remaining in FdI is in the form of $[\text{3Fe-3S}]^{3+}$ cluster.

The 2 K MCD spectrum of FdI(20FCN) was superposable on that of (7Fe)FdI_{ox}, confirming the presence of the $[\text{3Fe-3S}]^{3+}$ cluster and the conclusion that no change in its structure occurred during $\text{Fe}(\text{CN})_6^{3-}$ oxidation of the $[\text{4Fe-4S}]$ cluster.

The Fourier transform (FT) of the Fe EXAFS data for a sample of FdI(20FCN), concentrated to 4.4 mM Fe, is shown in Fig. 5a. By using the filter window indicated by the dashed line, the Fourier-filtered EXAFS illustrated by the solid line in Fig. 5b was generated. The larger FT peak (at 1.69 Å) under the filter window in Fig. 5a is assigned to an Fe-S interaction, while the second peak (at 2.29 Å) is assigned to an Fe-Fe interaction. No FT peak is visible at ≈ 4 Å, the distance obtained by x-ray crystallography for the $[\text{3Fe-3S}]$ cluster of (7Fe)FdI_{ox}. This is not surprising since the data were collected at $\approx 0^\circ\text{C}$, and any Fe-Fe interaction at such a long distance would be expected to yield a broadened FT peak due to thermal motion.

The results of curve-fitting analysis of the Fe EXAFS data are summarized in Table 1. Also shown in Table 1 are the results of analysis of the EXAFS data on the Fe-S compounds used as models for Fe-S and Fe-Fe interactions, as well as curve-fitting results for beef heart aconitase. The σ_{as} values for the two model compounds were estimated by curve-fitting during the generation of the phase and amplitude functions used in the protein fits. It is these σ_{as} values that should be used for comparison with the best-fit protein σ_{as} values.

For the two-shell fits of protein data, it was assumed that each Fe would be surrounded by four sulfur atoms at approximately the same distance. The calculated Debye-Waller σ_{as} values are comparable to that calculated for the tetraethylammonium salt of the Fe(III) tetrathiolate complex $[\text{Fe}(\text{SC}_2\text{H}_4\text{S})_4]$, indicating this to be a reasonable

FIG. 5. Fe EXAFS of FdI(20FCN), 4.4 mM in Fe, in 0.1 M potassium phosphate buffer (pH 7.5). (a) k^3 -weighted FT over range $k = 3.5$ – $11.5 \text{ \AA}^{-1}$. The dashed line represents the filter window used to generate the filtered data in b. The heavy line is the product of the filter window and the FT data. (b) Fourier-filtered EXAFS data (—) and best fit (---). R and α are the interatomic distance and phase shift, respectively; k and $\chi(k)$ are the photoelectron wave vector and EXAFS quantity, respectively.

assumption. For curve-fitting of FdI(20FCN) data, three separate fits were performed, each assuming a different (integer) number of Fe neighbors in the Fe–Fe shell. The results of these fits are shown in Table 1. The calculated Fe–S and Fe–Fe distances do not change from one fit to another. In comparing f values, the fit assuming one Fe neighbor is clearly worse than the others. In addition, the σ_{as} calculated for this fit is significantly lower than that observed for the [2Fe–2S] model compound. A much better fit is obtained when one assumes two or three Fe neighbors; however, the σ_{as} calculated for two Fe neighbors is much closer to the value calculated for the [2Fe–2S] model compound (some increase due to static disorder in a 3Fe cluster is to be expected). Thus, the best fit, which is shown in comparison to the observed (Fourier-filtered) EXAFS in Fig. 5b, is for two Fe neighbors. The results on beef heart aconitase (Table 1) are very similar. This is not surprising because the EXAFS

and FT data for aconitase (12) are virtually superimposable on the data for FdI(20FCN).

Thus, the EXAFS analysis shows the presence in (3Fe)FdI_{ox} of average Fe...S and Fe...Fe distances of $\approx 2.3 \text{ \AA}$ and $\approx 2.7 \text{ \AA}$, respectively, and is consistent with a 3Fe cluster. The Fe...S distance is in good agreement with the average of Fe–S²⁻ and Fe–S_{Cys} distances, $\approx 2.3 \text{ \AA}$, found by x-ray crystallography on (7Fe)FdI_{ox} (1, 2). The Fe...Fe distance is substantially different, however, from the $\approx 4.1\text{-}\text{\AA}$ distance obtained by crystallography. The EXAFS data also suggest that the 3Fe cluster in (3Fe)FdI_{ox} is very similar to that in beef heart aconitase.

The possible interpretations of this result are: (i) the structure of the [3Fe–3S]³⁺ cluster in (3Fe)FdI_{ox} is indeed different from that in (7Fe)FdI_{ox}, despite the similarity in EPR and MCD; (ii) the structure of the [3Fe–3S]³⁺ cluster is different in crystalline (7Fe)FdI_{ox} and solutions of (7Fe)FdI_{ox}; and (iii) either EXAFS or x-ray crystallography data or analysis is in error. At this point we cannot differentiate definitively among these alternatives. However, it is clear that (i) crystallization and structural analysis by x-ray crystallography of (3Fe)FdI_{ox}, (ii) further refinement of the x-ray diffraction analysis of (7Fe)FdI_{ox}, and (iii) more careful study of possible differences between crystalline and non crystalline (7Fe)FdI_{ox} are important.

The finding via EXAFS of a short Fe...Fe distance in (3Fe)FdI_{ox} permits speculation on alternative structures for the 3Fe cluster of (7Fe)FdI_{ox}. Given Fe...Fe and Fe...S distances of $\approx 2.7 \text{ \AA}$ and $\approx 2.3 \text{ \AA}$, it is natural to consider only structures closely related to the cubal [4Fe–4S] cluster structure. The common coordination of Fe by S in Fe–S clusters is 4. If one examines structures of the stoichiometry [Fe₃S_x(Cys)_y] ($x = 3$ or 4 and $y \leq 5$), in which Fe and S²⁻ are at corners of a cube, and selects those in which all Fe are 4-coordinate in S, then only the two structures shown in Fig. 6 and of stoichiometries Fe₃S₃(Cys)₅ and Fe₃S₄(Cys)₃ exist. Structure I has the stoichiometry obtained by x-ray crystallography for (7Fe)FdI_{ox} and can be obtained simply from the x-ray structure by (i) removing ligand X and (ii) folding the cluster to a more compact form in which Fe atoms are closer together. Therefore, it offers an alternative structure involving a minimal change from the x-ray structure, which is simultaneously consistent with the EXAFS result. Structure II is similar to the structure suggested for the aconitase cluster on the basis of its EXAFS and Fe/S²⁻ ratio (12), differing (by 1) in cysteine stoichiometry. However, this structure would require both the S²⁻ and the cysteine content of the (7Fe)FdI 3Fe cluster to be in error. Further, if less than five cysteines are involved in ligation of the cluster, (7Fe)FdI

Table 1. Curve-fitting analysis of Fe EXAFS data

Compound/protein	Fe–S				Fe–Fe			
	$R_{\text{as}}, \text{\AA}$	N_s	$\sigma_{\text{as}}, \text{\AA}$	$\Delta E_0, \text{eV}$	$R_{\text{as}}, \text{\AA}$	N_s	$\sigma_{\text{as}}, \text{\AA}$	f^*
(NEt ₄)[Fe(SC ₁₀ H ₁₃) ₄] [†]	(2.284) [‡]	(4)	0.046	(5)	—	—	—	0.455
(NEt ₄) ₂ [Fe ₂ S ₂ (S ₂ -o-xy ₁) ₂] [§]	—	—	—	—	(2.698) [‡]	(1)	0.065	0.142
Oxidized FdI(20FCN)	2.18	(4)	0.045	7.4	2.68	(1)	0.040	0.539
	2.19	(4)	0.043	6.3	2.68	(2)	0.078	0.328
	2.19	(4)	0.042	5.8	2.68	(3)	0.096	0.365
Oxidized beef heart aconitase [¶]	2.19	(4)	0.046	5.4	2.69	(1)	0.014	0.525
	2.20	(4)	0.040	3.6	2.69	(2)	0.067	0.226
	2.20	(4)	0.037	3.0	2.69	(3)	0.086	0.348

* $f = [\sum_{i=1}^N (k^3(\chi_{\text{obs}}^{(i)} - \chi_{\text{calc}}^{(i)})^2 / N)]^{1/2}$, where χ_{obs} and χ_{calc} are the observed and calculated EXAFS quantities, respectively.

The sum is taken over all N data points.

[†]The sample of tetraethylammonium Fe(III) tetrathiolate complex was a gift from S. A. Koch. Structural data were from ref. 22.

[‡]Numbers in parentheses were not optimized.

[§]The sample of tetraethylammonium binuclear Fe(III) dianion was a gift from K. S. Hagen and R. H. Holm. Structural data were from ref. 23.

[¶]EXAFS data taken from ref. 12.

FIG. 6. Possible structures for 3Fe clusters. Structures I and II have stoichiometries $\text{Fe}_3\text{S}_3(\text{Cys})_5$ and $\text{Fe}_3\text{S}_4(\text{Cys})_3$, respectively.

must possess free cysteine (or cysteine disulfide) residues. There is currently no evidence for such residues.

The simplest explanation of our data would then appear to be that in solution the $[\text{3Fe-3S}]^{3+}$ cluster of $(7\text{Fe})\text{FdI}_{\text{ox}}$ is different in geometry (but not in S^{2-} and cysteine stoichiometry) from that in crystalline $(7\text{Fe})\text{FdI}_{\text{ox}}$, with the more compressed structure idealized above and with an average $\text{Fe}\cdots\text{Fe}$ distance of ≈ 2.7 Å. This conclusion would require heterogeneity of 3Fe cluster structure, if beef heart aconitase indeed contains a Fe_3S_4 core stoichiometry. Alternatively, if all 3Fe clusters are in fact identical in stoichiometry, it is possible that beef heart aconitase (and *D. gigas* FdII) possesses the $\text{Fe}_3\text{S}_3(\text{Cys})_5$ structure described above, and that the Fe/S^{2-} ratio of 3:4 reported in aconitase is in error.

Very little data exist for the 3Fe cluster in both crystals and solutions of $(7\text{Fe})\text{FdI}_{\text{ox}}$. Resonance Raman spectra have been reported to be indistinguishable (24). A single-crystal EXAFS study of $(7\text{Fe})\text{FdI}_{\text{ox}}$ has found polarization-dependent $\text{Fe}\cdots\text{Fe}$ scattering at ≈ 2.7 Å, which is more likely to arise from the more anisotropic 3Fe cluster than from the less anisotropic $[\text{4Fe-4S}]$ cluster but could not definitively separate the contributions to this effect of the 3Fe and $[\text{4Fe-4S}]$ clusters (25).

Evidence regarding hetero- vs. homogeneity of 3Fe cluster structure is not unanimous. Mössbauer spectra in both oxidized and reduced states are very similar in all proteins studied (8–10). Resonance Raman spectra of several oxidized 3Fe clusters have been reported to be identical (24), although this conclusion has been disputed (26). Different 3Fe cluster structures in $(7\text{Fe})\text{FdI}$ and *D. gigas* FdII have been deduced from NMR studies (27). The low-temperature MCD of $(7\text{Fe})\text{FdI}$ and beef heart aconitase exhibit considerable similarity in the oxidized state but not in the reduced state (unpublished results). The 3Fe cluster of $(7\text{Fe})\text{FdI}$ exhibits substantially different redox potential and $3\text{Fe} \leftrightarrow [\text{4Fe-4S}]$ cluster conversion behavior from beef heart aconitase and *D. gigas* FdII (unpublished results). At this time, therefore, a definitive conclusion cannot be drawn either that all 3Fe clusters are identical or that 3Fe clusters vary in structure.

According to our interpretation of the $\text{Fe}(\text{CN})_6^{3-}$ oxidation of $(7\text{Fe})\text{FdI}_{\text{ox}}$, which follows earlier work of Petering *et al.* (28), the cysteine and S^{2-} components of the Fe–S clusters suffer one- and two-electron oxidation respectively, and further, then form cysteinyl disulfide and cysteinyl trisulfide linkages. The latter could be intramolecular and/or intermolecular. We have no evidence currently regarding the disposition of cysteine and S^{2-} components of the $[\text{4Fe-4S}]$

cluster in $(3\text{Fe})\text{FdI}_{\text{ox}}$. The reaction occurring with dithiothreitol presumably involves reduction of di- and possibly tri-sulfide linkages. $(3\text{Fe})\text{FdI}_{\text{ox}}$ in $\text{FdI}(20\text{FCN})$ may be either monomeric or polymeric.

CONCLUSION

Our results further substantiate our earlier analysis of $\text{Fe}(\text{CN})_6^{3-}$ oxidation (14, 15) and clearly demonstrate the utility of selective destructive oxidation in the study of $(7\text{Fe})\text{FdI}$. It is likely that this technique will have widespread application to “complex” iron–sulfur proteins.

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