Spectroscopic Investigation of Selective Cluster Conversion of Archaeal Zinc-containing Ferredoxin from *Sulfolobus* sp. Strain 7*

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** ABSTRACT **

Archaeal zinc-containing ferredoxin from *Sulfolobus* sp. strain 7 contains one [3Fe-4S] cluster (cluster I), one [4Fe-4S] cluster (cluster II), and one isolated zinc center. Oxidative degradation of this ferredoxin led to the formation of a stable intermediate with 1 zinc and ~6 iron atoms. The metal centers of this intermediate were analyzed by electron paramagnetic resonance (EPR), low temperature resonance Raman, x-ray absorption, and 1H NMR spectroscopies. The spectroscopic data suggest that (i) cluster II was selectively converted to a cubane [3Fe-4S]+ cluster in the intermediate, without forming a stable radical species, and that (ii) the local metric environments of cluster I and the isolated zinc site did not change significantly in the intermediate. It is concluded that the initial step of oxidative degradation of the archaeal zinc-containing ferredoxin is selective conversion of cluster II, generating a novel intermediate containing two [3Fe-4S] clusters and an isolated zinc center. At this stage, significant structural rearrangement of the protein does not occur. We propose a new scheme for oxidative degradation of dicluster ferredoxins in which each cluster converts in a stepwise manner, prior to apoprotein formation, and discuss its structural and evolutionary implications.

Ferredoxins, small iron-sulfur (FeS) proteins in archaea, serve as water-soluble electron acceptors of acyl-coenzyme A forming 2-oxoacid:ferredoxin oxidoreductase, a key enzyme involved in the central archaeal metabolic pathways (1–5). The 2.0-Å resolution x-ray crystal structure (PDB1 entry 1XER; Ref. 6) of ferredoxin from *Sulfolobus* sp. strain 7 (JCM 10545; optimal growth conditions, pH 2.5–3 and 80 °C) showed the presence of an unexpected isolated zinc center, tetrahedrally coordinated by three nitrogen atoms from histidine residues in the N-terminal extension region (N61 of His16, Ne2 of His19, and N61 of His34) and one O61 atom from Asp76 in the FeS cluster-binding core fold (Fig. 1). A similar zinc site was also found in ferredoxin from *Thermoplasma acidophilum* strain HO-62 that also contained one [3Fe-4S]1+,0 cluster, and one [4Fe-4S]2+,1- cluster (7). This site was analyzed by zinc K-edge x-ray absorption spectroscopy (XAS) (8) and was found to be essentially identical to the zinc site in *Sulfolobus* sp. ferredoxin. Thus, these unusual ferredoxins contain both the conventional FeS clusters and a structurally conserved, isolated zinc center. This new class of bacterial type ferredoxin, isolated from phylogenetically diverse members of several aerobic and thermocladophila archaea, are thus called “zinc-containing ferredoxins” (2, 6–10).

Another unexpected result of the crystal structure of air-oxidized *Sulfolobus* sp. zinc-containing ferredoxin was the presence of two [3Fe-4S] clusters beside one isolated zinc center (9) (Fig. 1). The number and type of FeS clusters in this structure are inconsistent with our previous spectroscopic analysis of the purified protein, which suggested one [3Fe-4S]1+,0 cluster (cluster I; $E_{1/2} = -290$ mV) and one [4Fe-4S]2+,1- cluster (cluster II; $E_{1/2} = -530$ mV) (5). Other archaeal zinc-containing ferredoxins from *T. acidophilum* (7, 8), *Sulfolobus acidocaldarius* (11), and *Acidianus ambivalens* (formerly *Desulfurolobus ambivalens*) (12) also contain one [3Fe-4S] cluster and one [4Fe-4S] cluster, rather than two [3Fe-4S] clusters. The primary structure of *Sulfolobus* sp. ferredoxin showed the presence of seven cysteines that are arranged in two FeS cluster-binding motifs, one from the sequence, Cys45-Leu-Ala-Asp48-Gly-Ser-Cys51 and Cys58-Pro, and the other from the sequence, Cys55-Pro and Cys83-Ile-Phe-Cys86-Met-Ala-Cys89 (5, 13). The latter motif provides a typical binding site for a [4Fe-4S] cluster with complete cysteinyl ligation, although Cys86 does not serve as a ligand to the [3Fe-4S] cluster II in the crystal structure (Fig. 1). Because FeS clusters have a remarkable facility for interconversion under protein-bound conditions (reviewed in Ref. 14), the significant discrepancy of the types of FeS clusters of *Sulfolobus* sp. zinc-containing ferredoxin in the crystalline and as-isolated states may represent selective degradation of the cluster II to a [3Fe-4S] form *in vitro*. Although the [4Fe-4S] ↔ [3Fe-4S] cluster interconversion is well known in some bacterial type ferredoxins (14–25) and aconitase (26–29), there is no conclusive report demonstrating the selective cluster conversion at the cluster II site in bacterial type dicluster ferredoxins. The most extensive spectroscopic analyses of oxidative degra-
The reduction process in dicluster ferredoxins have been conducted with *Azotobacter vinelandii* ferredoxin I with one [3Fe-4S] cluster and one [4Fe-4S] cluster (30–35). In this instance, it has been proposed that ferricyanide oxidation results in complete destruction of the [4Fe-4S] cluster, to produce an intermediate containing only one [3Fe-4S] cluster (cluster I), en route to complete disruptions of both clusters and ultimately to unfolded protein (30, 31).

We have recently found conditions where the native 7Fe form of *Sulfolobus* sp. ferredoxin (5) is slowly and irreversibly converted to a stable intermediate species under aerobic conditions (10). Herein, we report comparisons of EPR, resonance Raman, XAS, and 1H nuclear magnetic resonance (NMR) analyses of the metal centers in the native 7Fe and the intermediate forms of *Sulfolobus* sp. ferredoxin. Our spectroscopic data demonstrate the oxidative degradation of cluster II to form a [3Fe-4S] cluster, yielding a 6Fe-containing intermediate of zinc-containing ferredoxin. This will also be discussed with respect to the structure and evolution of a ferredoxin core fold module.

**EXPERIMENTAL PROCEDURES**

DEAE-Sepharose Fast Flow and Sephadex G-50 gels were purchased from Amersham Pharmacia Biotech, and NMR-grade D 2O was from Wako Pure Chemicals (Tokyo, Japan). Water was purified by the Milli-Q purification system (Millipore). Other chemicals used in this study were of analytical grade.

*Sulfolobus* sp. strain 7 cells (JCM 10545), originally isolated from Beppu Hot Springs, Japan, were cultivated aerobically and chemoheterotrophically at pH 2.5–3 and 75–80 °C, and the 7Fe form of the archaeal ferredoxin was routinely purified as described previously (5, 10). The intermediate form of *Sulfolobus* sp. ferredoxin was obtained by artificial conversion at pH 5.0 as described previously (10), after removal of the unconverted 7Fe form by a DEAE-Sepharose Fast Flow column chromatography (Amersham Pharmacia Biotech) followed by a Sephadex G-50 column chromatography (Amersham Pharmacia Bio-tech). 2-Oxoacid:ferredoxin oxidoreductase of *Sulfolobus* sp. strain 7 was purified as described previously (5, 36). 2-Oxoacid:ferredoxin oxidoreductase activity was monitored with a horse heart cytochrome c reduction assay at 50 °C using purified ferredoxin an intermediate electron acceptor (2, 5). Enzymatic reduction of *Sulfolobus* sp. ferredoxin with the cognate 2-oxoacid:ferredoxin oxidoreductase was conducted under anaerobic conditions at 55 °C as described previously (5).

Absorption spectra were recorded using a Hitachi U3210 spectrophotometer or a Beckman DU-7400 spectrophotometer. Matrix-assisted laser desorption ionization-time of flight mass spectrometry of purified apoferredoxin (made in distilled water) was performed by a Finnigan MAT VISION 2000 instrument at an accelerating potential of 5.0 kV, using a 2,5-dihydroxybenzoic acid matrix. Electron paramagnetic resonance (EPR) measurements were performed using a JEOIL JES-PE3XG spectrometer equipped with an Air Products model LTR-3-110 Helitran cryostat system and a Scientific Instruments series 5500 temperature indicator/controller. Spin concentrations were estimated by double integration, with 0.1 and 1 mM Cu-EDTA as standards. The spectral data were processed using KaleidaGraph version 3.05 (Abelbeck Software).

Low temperature resonance Raman spectra were recorded at 77 K.
using 488.0-nm and 457.9-nm Ar laser excitation (500 mW) as described previously (7, 37). The sample was immersed into a liquid nitrogen reservoir and the scattered light was collected at 45 degrees to the incident beam. The spectral slit width was 4 cm⁻¹, and a multiscan averaging technique was employed.

**Tables**

| Table I X-ray absorption spectroscopic data collection for iron and zinc analysis |
|-----------------|-----------------|
| Iron EXAFS | Zinc EXAFS |
| SR facility | SSRL | SSRL |
| Beamline | 7–3 | 7–3 |
| Current in storage ring | 80–100 mA | 50–60 mA |
| Monochromator crystal | Si(220) | Si(220) |
| Detection method | Fluorescence | Fluorescence |
| Detector type | Solid-state array | Solid-state array |
| Scan length, min | 28 | 25 |
| Scans in average | 16 | 10 |
| Temperature (K) | 10 | 10 |
| Energy standard | Iron foil, first inflection | Zinc foil, first inflection |
| Energy calibration (eV) | 7111.3 | 9660.7 |
| \(E_\text{c} (\text{eV})\) | 7120 | 9670 |
| Pre-edge background | | |
| Energy range (eV) | 6789–7075 | 8657–9625 |
| Gaussian center (eV) | 6403 | 8638 |
| Width (eV) | 750 | 750 |
| Spline background | | |
| Energy range (eV) | 7120–7354 (4) | 9333–9902 (4) |
| (Polynomial order) | 7354–7589 (4) | 9902–10134 (4) |
| | 7589–7822 (4) | 10134–10366 (4) |

The 13-element Ge solid-state x-ray fluorescence detector at Stanford Synchrotron Radiation Laboratory (SSRL) is provided by the NIH Biotechnology Research Resource.

**Degradation Intermediate of Zinc-containing Ferredoxin**—The 7Fe form of *Sulfolobus sp.* ferredoxin elicited a sharp \(g = 2.02\) EPR signal (0.9–1.0 spin/mol) attributed to a [3Fe-4S]\(^{1+}\) cluster as reported previously (5, 8) (Fig. 2, trace A). The intermediate form also elicited a sharp EPR signal at \(g = 2.02\), detectable up to 20 K, but with different lineshapes and relaxation behavior. The microwave power saturation behavior of the EPR signal at 8 K, of the intermediate form \((P_\% \approx 36 \text{ mW assuming a single component; } P_\% \approx 0.7 \text{ mW and 110 mW assuming two components; open squares in Fig. 2B), was also different from that of the native 7Fe form (P\% of 60 mW; closed squares in Fig. 2B). Under non-saturation conditions, the spin concentration of the \(g = 2.02\) EPR signal of the intermediate was estimated to be \(-1.7\) spin/mol, indicating the presence of approximately two \(S = 1/2\) [3Fe-4S]\(^{1+}\) clusters. No EPR signals were detected at temperatures above 35 K, suggesting the absence of a stable radical species in the air-oxidized intermediate (of Refs. 30, 33, and 94).

**Results**

Upon reduction of the native 7Fe form, the sharp \(g = 2.02\) EPR signal attributed to that of a [3Fe-4S]\(^{1+}\) cluster was fully reduced, thus giving rise to a very broad low field resonance at \(g = 12\), which is characteristic of the reduced \(S = 2\) [3Fe-4S]\(^{0}\) cluster (Fig. 3, trace A). A rhombic EPR signal at \(g = 2.06\), 1.94, and 1.90, attributed to the reduced \(S = 1/2\) [4Fe-4S]\(^{1+}\) cluster, was detected. This signal had additional wings on the high and low field sides of the main EPR signal \((g = 2.11 \text{ and } 1.85)\), resulting from magnetic interactions with the reduced \(S = 2\) [3Fe-4S]\(^{0}\) cluster. These EPR signals could be detected up to 30 K (data not shown), and no evidence for the presence of a high multiplicity \(S = 3/2\) [4Fe-4S]\(^{1+}\) cluster was obtained (Fig. 3, trace A).

Reduction of the intermediate under the same conditions resulted in the disappearance of most of the sharp \(g = 2.02\) EPR signal attributed to the \(S = 1/2\) [3Fe-4S]\(^{1+}\) clusters, and the appearance of the broad low field resonance at \(g = 12\) characteristic of the \(S = 2\) [3Fe-4S]\(^{0}\) cluster, as the predominant species (Fig. 3, traces B, C, and D). Several weak and minor resonances, mainly consisting of the remaining \(S = 1/2\) [3Fe-4S]\(^{1+}\) cluster at \(g = 2.02\) and a very weak radical feature at \(g = 2\) of unknown origin, were also reproducibly detected in the \(g = 2\) region (<0.1 spin/mol). All these minor species existed in a stoichiometric amount (<0.1 spin/mol), indicating minor heterogeneity in the dithionite-reduced intermediate form.

**Resonance Raman Spectroscopy**—Low temperature resonance Raman spectroscopy has been utilized as a probe for...
Degradation Intermediate of Zinc-containing Ferredoxin

![Image of EPR spectra from Fig. 2 and Fig. 3]

**Fig. 2.** EPR spectra of the native 7Fe form of zinc-containing ferredoxin from *Sulfolobus* sp. ferredoxin and its intermediate form in the air-oxidized state (A); the power saturation curves of the oxidized protein at 8 K are shown in B. The power saturation curves were fitted with $P_{1/2}$ of 36 mW assuming a single component (dashed trace) or of 0.7 and 110 mW, assuming two components (solid trace) for the intermediate form (open squares), and $P_{1/2}$ of 60 mW for the native 7Fe form (solid trace and closed squares). The samples were dissolved in 600 mM CAPS buffer, pH 9.3, and adjusted at the same concentrations. Modulation amplitude of the instrument is 0.63 millitesla, and the $g$ values and other conditions are indicated in the figure.

**Fig. 3.** EPR spectra of the native 7Fe form of zinc-containing ferredoxin from *Sulfolobus* sp. ferredoxin (A) and its intermediate form (B–D) in the dithionite-reduced state at pH 9.3. The samples used for the measurement are the same as in Fig. 2.

band was shifted to a higher frequency (16). It has been reported that the resonance Raman spectra for biological [4Fe-4S]$^{2+}$ clusters are normally much less intense than those for biological [3Fe-4S]$^{1+}$ clusters (reviewed in Refs. 45 and 47). Relative intensity of the Fe-S bridging mode of a [4Fe-4S]$^{2+}$ cluster at 335-cm$^{-1}$ in the native 7Fe form of *Sulfolobus* sp. ferredoxin (Fig. 4A) is very similar to that reported for *Thermus thermophilus* 7Fe ferredoxin (45, 47). A very weak Fe-S terminal mode at -249 cm$^{-1}$ is normally seen for [4Fe-4S]$^{2+}$ clusters; however, the signal in this region, for the 7Fe form of *Sulfolobus* sp. ferredoxin, is too weak to analyze. In conjunction with other spectroscopic data reported in this paper (Figs. 2 and 3), the resonance Raman data of the native 7Fe form indicate the presence of a [4Fe-4S]$^{2+}$ cluster, in addition to a [3Fe-4S]$^{1+}$ cluster.

The low temperature resonance Raman spectrum at 488.0-nm Ar$^+$ ion laser excitation of the intermediate form showed that the [3Fe-4S]$^{1+}$ cluster also exhibited three Fe-S bridging modes at 260, 285, and 347 cm$^{-1}$, and at least two Fe-S terminal modes at 369 and 385 cm$^{-1}$ (Fig. 4D). Detection of these bands in both the native 7Fe (Fig. 4B) and intermediate 6Fe forms (Fig. 4D) suggests that the [3Fe-4S]$^{1+}$ core structure is structurally not very different in these two forms. On the other hand, the weak band primarily associated with the Fe-S bridging mode at 335 cm$^{-1}$ of the [4Fe-4S]$^{2+}$ cluster of the native 7Fe form, resonantly enhanced upon 457.9-nm Ar$^+$ ion laser excitation (Fig. 4A), was not detected in the intermediate form (Fig. 4C). These results are consistent with the absence of a [4Fe-4S]$^{2+}$ cluster in the intermediate form. It should be noted that the band at 359 cm$^{-1}$, resonantly enhanced upon 457.9-nm Ar$^+$ ion laser excitation (Fig. 4A and C), was detected in both forms of *Sulfolobus* sp. ferredoxin, whereas the equivalent band was observed in the 7Fe, but not the 6Fe, form of *Mycobacterium smegmatis* ferredoxin (37, 50). This indicates that a weak band at -359 cm$^{-1}$ cannot be used diagnostically for the presence of a conventional biological [4Fe-4S]$^{2+}$ cluster.

X-ray Absorption Spectroscopy—The zinc K-edge x-ray absorption spectra of the purified 7Fe and 6Fe forms of *Sulfolobus*
The presence of a carboxylate ligand results in destructive interference with the EXAFS multiple-scattering contributions from outer shell atoms of histidine imidazoles. This interference can be visualized in the Fourier transform of the data as a decrease in the ~3-Å peak relative to the ~4-Å peak. Previously, we reported that the 7Fe form of Sulfolobus sp. ferredoxin was best fit assuming a Zn(imid)₃(COO⁻)₄ coordination environment with a Zn-O-C bond angle of ~126° (Fit 4, Table II) (3). Similarly, the 6Fe form can also be fit assuming the same coordination geometry (cf. Fits 4 and 8, Table II; Fig. 6). The zinc XAS results clearly show that the zinc site found in the intermediate is an isolated center having very similar coordination environment to the native 7Fe form. The XAS-determined bond distances and bond angles of the intermediate are also in agreement with the crystallographically determined Zn-N and Zn-O bond distances (1.96 and 1.90 Å, respectively) and Zn-O-C angle (~126°) (6).

The iron K-edge x-ray absorption spectra for the two forms of Sulfolobus sp. ferredoxin were almost identical (Fig. 5). Curve-fitting analysis of the native 7Fe form and the stable intermediate reveals the presence of a 2.25–2.26-Å Fe-S and a 2.71–2.72-Å Fe-Fe interaction (8). The best fit (by goodness-of-fit values) is obtained from calculated EXAFS for Fe₅S₇Fe₂ (Fit 10, Table II). However, the data can also be fit assuming Fe₅S₇Fe₂,₅ (Fit 11, Table II), as expected for one [3Fe-4S] and one [4Fe-4S] cluster (8). EXAFS for the 6Fe form (Fig. 6) is best fit assuming Fe₅S₇Fe₂ (Fit 13, Table II), as expected for two [3Fe-4S] clusters. The goodness-of-fit value is lower for Fe₅S₇Fe₁ (Fit 12, Table II); however, the Debye-Waller factor for the Fe-Fe interaction is physically unreasonable. Similar to the 7Fe form, the data can also be fit assuming Fe₅S₇Fe₂,₅ (Fit 14, Table II). However, the Debye-Waller factors indicate that there are on average fewer (or more disordered) Fe-Fe interactions in the 6Fe form than in the 7Fe form, as would be expected given the [4Fe-4S] ↔ [3Fe-4S] cluster conversion. It should be noted that the EXAFS analysis of the oxidatively degraded 3Fe-containing intermediate and a single crystal of the native 7Fe form of A. vinelandii ferredoxin I showed the average Fe-Fe distance of ~2.7 Å (32, 35, 53), similar to the results obtained here.

1H NMR Spectroscopy—1H NMR spectroscopy has been used to detect and assign the β-protons of cysteine ligand residues coordinated to the FeS centers in some 7Fe-containing ferredoxins (54–57). Bentrop et al. (55) have performed detailed paramagnetic NMR analysis of the native 7Fe form of another zinc-containing ferredoxin from A. ambivalens, which is 95% identical to Sulfolobus sp. ferredoxin at the primary structural level (only 5 out of 103 amino acids are different) (7, 12, 58). Interestingly, the 1H NMR spectrum of A. ambivalens ferredoxin shows eight major and eight minor hyperfine-shifted resonances, arising from its ligand protons. This unique pattern apparently results from the superposition of typical spectra from a 3Fe- and a 4Fe-containing cluster (55). This pattern has not been reported for other regular bacterial 7Fe-containing ferredoxins, all of which show only five hyperfine-shifted resonances in the downfield region (54, 56, 57, 59–62). It has been reported that eight major and eight minor hyperfine-shifted resonances likely reflect a heterogeneity of one of the two clusters of A. ambivalens ferredoxin, which does not simply result from sample impurity (55).

The overall spectral features, chemical shift values, and temperature dependences of the hyperfine-shifted resonances in the downfield 1H NMR spectrum of native Sulfolobus sp. ferredoxin (Fig. 7, A, C, and D) are essentially identical to those of the closely related A. ambivalens ferredoxin. A. ambivalens has complete cysteinyl coordination to both the [3Fe-4S]¹⁺ cluster and [4Fe-4S]²⁺ cluster (55). Eight major signals (C, G, H, J, K, N, O, P) and eight minor signals (A, B, D, E, F, I, L, M) were

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2 Edge position energies were calculated by determining the maxima of the first derivative of the absorption edge.

3 The number of imidazoles from this analysis is not absolute and probably depends on the exact geometry enforced on the carboxylate ligand.
but did not vary more than 1.5 eV. Numbers in square brackets were constrained to be either a multiple of the above value (\(s\)) or to maintain a constant difference from the above value (\(R_m\)). \(f\) is a normalized error (chi-squared):

\[
f' = \frac{\chi^2}{(\chi^2)^{1/2}} = \frac{(\sum (k^x \chi_{obs} - k^x \chi_{calc})^2)\chi^2_{max}}{(\sum (k^x \chi_{min})^2 \chi^2_{min})^{1/2}}
\]

### Table II

| Sample filename (\(\Delta k^2 x\)) | Fit | Shell | \(R_m\) | \(\sigma_m^2\) | \(f'\) |
|----------------------------------|-----|-------|---------|---------------|------|
| \(7Fe\) Zn-ferredoxin 1 | 4 Zn-N(O) | 2.00 | 0.0016 | 0.085 | 6Fe Zn-ferredoxin 5 | 4 Zn-N(O) | 1.99 | 0.0022 | 0.088 |
| \(ZSFDA (2–13 \text{ Å}^{-1})\) | 2 | 1 Zn-C | 2.99 | 0.0054 | \(\Delta k^2 x = 13.05\) | 3 Zn-C | 2.97 | 0.0063 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-C | [3.05] | 0.0054 | | 3 Zn-C | [3.06] | 0.0054 |
| \(\Delta k^2 x = 13.05\) | 2 Zn-N | [4.14] | 0.0054 | | 3 Zn-C | [4.14] | 0.0054 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-N | [4.18] | 0.0054 | | 3 Zn-N | [4.18] | 0.0054 |
| \(\Delta k^2 x = 13.05\) | 4 Zn-C | [4.12] | 0.0054 | | 3 Zn-C | [4.12] | 0.0054 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-N | [4.16] | 0.0054 | | 3 Zn-N | [4.16] | 0.0054 |
| \(\Delta k^2 x = 13.05\) | 2 Zn-O | 1.91 | 0.0007 | 0.069 | 7 Zn-O | 1.91 | 0.0010 | 0.076 |
| \(\Delta k^2 x = 13.05\) | 1 Zn-C | [2.82] | 0.0010 | | 1 Zn-C | [2.83] | 0.0015 |
| \(\Delta k^2 x = 13.05\) | 1 Zn-O | [3.08] | 0.0011 | | 1 Zn-O | [3.09] | 0.0016 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-N(O) | 2.01 | -0.0002 | | 3 Zn-N(O) | 2.01 | 0.0009 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-C | 2.94 | -0.0002 | | 3 Zn-C | 2.94 | 0.0002 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-N | [3.07] | -0.0003 | | 3 Zn-N | [3.06] | 0.0003 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-C | [4.10] | -0.0004 | | 3 Zn-C | [4.11] | 0.0004 |
| \(\Delta k^2 x = 13.05\) | 3 Zn-N | [4.18] | -0.0004 | | 3 Zn-N | [4.19] | 0.0004 |
| \(\Delta k^2 x = 13.05\) | 4 Zn-C | | 0.0017 | 0.067 | | 4 Zn-C | | 0.0014 | 0.069 |

### Degradation Intermediate of Zinc-containing Ferredoxin

...the degradation intermediate of zinc-containing ferredoxin (Fig. 1). This process was sluggish and did...
FIG. 7. One-dimensional $^1$H NMR spectrum at 303 K of the native 7Fe form (A) and the partially converted sample by oxidative degradation (B) of Sulfolobus sp. zinc-containing ferredoxin. The hyperfine-shifted resonances were labeled according to the $^1$H NMR data of Bentrop et al. (55). The spectrum of samples in B was measured after storage of sample A in NMR tubes for 7 months under auxogenic conditions at 5 °C. Concomitant with the decrease of major signals, J, N, O, and P, several hyperfine-shifted resonances appeared at around 16.0 ppm (signal b), 21.4 ppm (not labeled), and 24.0–24.6 ppm (signal a) in sample B. The temperature dependences of the hyperfine-shifted resonances (in a chemical shift versus $1/T$ plot) of the major species (C), the minor species (D) of the native 7Fe form (spectrum A), and the newly developed species (E) of the partially converted sample (spectrum B) are also shown in the figure.

FIG. 6. $k^3$-weighted zinc (left) and iron (right) EXAFS (insets, over $k = 2–12.5 \ \AA^{-1}$, for zinc; $k = 2–13.5 \ \AA^{-1}$, for iron) and Fourier transforms of native 7Fe (dashed line) and intermediate 6Fe (solid line) forms of Sulfolobus sp. ferredoxin.
one ligand (CysIV) of a [3Fe-4S] cluster domain (55) (Fig. 7, C and D). The details of the sequence-specific assignments of the new hyperfine-shifted resonances are the subject of future analysis, and their correlation with the minor peaks observed in the native 7Fe form remain unclear at this stage. However, some of these resonances (such as signal a) probably belong to a [3Fe-4S] cluster domain and are in line with the increase of the EPR signal intensity at g = 2.02 (data not shown; see Fig. 2).

Taken together, these data suggest that the [4Fe-4S] cluster II in the native 7Fe form is less stable to oxidative degradation than the [3Fe-4S] cluster I, and that the [4Fe-4S]2+ cluster II is gradually and selectively converted to a corresponding cubane [3Fe-4S]1+ cluster II, whereas the [3Fe-4S] cluster I remains essentially unchanged under the applied conditions. Further 1H NMR analysis is underway to reveal the sequence-specific assignments of the new hyperfine-shifted resonances and the electronic structure of the two [3Fe-4S] clusters in the 6Fe-containing intermediate form.

Biochemical Properties—Archaeal zinc-containing ferredoxins have been shown to serve as water-soluble electron acceptors of acyl-coenzyme A forming 2-oxoacid:ferredoxin oxidoreductase, a key enzyme involved in the central archaeal metabolic pathways (2, 3, 5, 7, 12). Previous enzymatic reduction of the native 7Fe form by Sulfolobus sp. 2-oxoacid:ferredoxin oxidoreductase reduced only the [3Fe-4S] cluster I, whereas the [4Fe-4S] cluster II with a lower midpoint redox potential remained in the oxidized state during the steady state (5). The specific activity of the cognate 2-oxoacid:ferredoxin oxidoreductase in the presence of 4 mM 2-oxoglutarate, 50 μM coenzyme A, and 23 μg of the 6Fe-containing intermediate form at 50 °C was 55 μmol/min/mg, only slightly lower than that measured with the native 7Fe form under the same conditions (60 μmol/min/mg) (5, 36). Thus, the reactivity with the cognate oxidoreductase was not lost after the [4Fe-4S] → [3Fe-4S] cluster conversion. Enzymatic reduction of the 6Fe form with a catalytic amount of the 2-oxoacid:ferredoxin oxidoreductase (8.6 μg/ml) in the presence of 4 mM 2-oxoglutarate and 0.5 mM coenzyme A during the steady-state turnover of the oxidoreductase under anaerobic conditions at 55 °C caused bleaching of the g = 2.02 EPR signal attributed to the [3Fe-4S]1+ clusters (see Fig. 3) by ~95% in 30 min, with concomitant formation of the broad low field resonance at g ~ 12, which is indicative of the S = 2 [3Fe-4S]0 clusters (data not shown). This indicates that the two [3Fe-4S] clusters, i.e. both clusters I and II, of the 6Fe form can be reduced enzymatically by the cognate oxidoreductase under the applied conditions, due to the change of electron distributions within the reduced ferredoxin molecule by relative redox potential upshift of the cluster II upon the selective cluster conversion.

**DISCUSSION**

The present spectroscopic investigation indicates that the initial step of oxidative degradation of the native 7Fe form of *Sulfolobus* sp. zinc-containing ferredoxin, at fairly acidic or neutral pH, is a selective conversion of the [4Fe-4S] cluster I to a [3Fe-4S] cluster. This degradation pathway results in a stable 6Fe-containing intermediate with one isolated zinc center and two [3Fe-4S] clusters, en route to complete protein unfolding. Although the selective interconversion of a [4Fe-4S] ↔ [3Fe-4S] cluster at the cluster I site has been well established in some bacterial ferredoxins (14–25), this novel intermediate is unique in that its formation is triggered by selective cluster conversion at the cluster II site, rather than complete destruction of the cluster. The local metric environments of the [3Fe-4S] cluster I and isolated zinc site in the intermediate form do not change significantly as compared with those in the native 7Fe form,
indicating that structural rearrangement of the whole molecule does not occur at the initial step of oxidative degradation. The ability of the intermediate to accept electrons transferred from the cognate 2-oxoacid:ferredoxin oxidoreductase is also maintained at this stage.

**Correlation with the X-ray Crystal Structure of the 6Fe Form of the Sulfolobus Feredoxin**—The spectroscopic properties of the stable intermediate form of Sulfolobus sp. ferredoxin are consistent with the structural features observed for the 2.0-Å resolution crystal structure of the 6Fe form (6, 9). In this structure, the [3Fe-4S] cluster I is coordinated by three cysteinylic ligands contributed from Cys55, Cys51, and Cys93, and the [3Fe-4S] cluster II by another three cysteinylic ligands contributed from Cys85, Cys83, and Cys89 (9) (Fig. 1). The two [3Fe-4S] clusters are separated by a crystallographic center-to-center distance of 12.0 Å, which is similar to that observed in some regular 7Fe- and 8Fe-containing dicluster ferredoxins. The second cysteine residue (Cys86) in the -Cys83-Xaa-Xaa-Cys86-Xaa-Xaa-Cys83-Pro-motif is located in the vicinity of the missing corner (Fe) of the cluster II cube, and its side chain is exposed to the solvent, away from cluster II (Fig. 1). A similar observation has been reported for the non-ligating cysteinyl residue (Cys11) in the D. gigas ferredoxin II structure with a single cubane [3Fe-4S] cluster (PDB entry 1FXD.pdb; Ref. 17). The equivalent residue (Cys15) of the closely related 4Fe-containing ferredoxin from Thermotoga maritima (PDB entry 1VJW.pdb; Ref. 66) serves as a ligand to the [4Fe-4S] cluster.

The electron density for Cys86 is lower than that of other cysteinyl ligand residues in the crystal structure. Additionally, the average temperature factors for the region surrounding cluster II (−24 Å², on average), especially Cys86 and Met87 (>30 Å²), are markedly higher than those for the region surrounding cluster I (−15 Å²) (Fig. 1). These observations are consistent with the present spectroscopic analyses indicating the structural similarity of the native 7Fe form and the stable intermediate, and suggest that the selective cluster conversion at the cluster II site is a local event. In the crystal structure, the polypeptide conformation in the vicinity of the cubane [3Fe-4S] cluster II and Cys86 was reported to be intermediate between the [3Fe-4S] and [4Fe-4S] cluster conformations (9). It is likely that the peptide backbone conformation in vicinity of Cys86 and Met87 may be somewhat perturbed upon formation of the 6Fe-containing intermediate by oxidative degradation.

Based on these considerations, we suggest that oxidative degradation of Sulfolobus sp. zinc-containing ferredoxin is initiated at the [4Fe-4S] cluster II site. As a result of this initial degradation step, the iron atom bound to Cys86 is released, forming a structurally interconvertible cubane [3Fe-4S] cluster II. Concomitant with the release of iron is a perturbation of the peptide backbone in the vicinity of Cys86-Met87 and rotation of the Cys86 side chain to solvent, away from the cluster (see Fig. 1). At this stage, no significant structural rearrangement of the entire molecule occurs. Further sluggish oxidative degradation of the two [3Fe-4S] clusters eventually leads to protein unfolding of the ferredoxin core fold and accumulation of apoprotein (10), and may involve other short-lived intermediates not yet characterized.

**Oxidative Degradation of Two FeS Clusters in Bacterial Type**

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5 Preliminary computer modeling analysis by using a SYBYL molecular modeling software (TRIPOS Associates, Inc.) also suggested that simple rotation of the Cys86 side chain did not allow appropriate coordination of this residue to the missing corner (Fe) of a structurally restrained biological [4Fe-4S] cluster introduced and superimposed to the [3Fe-4S] cluster II of Sulfolobus sp. ferredoxin (T. Iwasaki, K. Tomuro, Y. Hayashi-Iwasaki, and T. Oshima, unpublished results).

**Dicluster Ferredoxins**—The reaction of the wild-type A. vinelandii ferredoxin I with an excess of ferricyanide is a more complicated oxidative degradation, leading to formation of an unusual intermediate with a three-electron oxidation at sulfur sites (30, 31, 33, 34). Site-directed mutagenesis has suggested that formation of the latter species requires the presence of non-ligating Cys24 in the vicinity of a cluster sulfide (33). The difference between the oxidative degradation of ferredoxin from A. vinelandii and Sulfolobus sp. strain 7 is likely to reflect the differences in their cluster surroundings, i.e. the absence of the equivalent cysteine residue in the archaeal ferredoxin core fold domain (Fig. 1).

An analogous 6Fe-containing ferredoxin species has been isolated from *M. smegmatis* (37, 50) and similar hyperfine-shifted resonances appear in the downfield 1H NMR spectra of ferricyanide-treated ferredoxins from *M. smegmatis*, *Pseudomonas ovalis*, and *T. thermophilus* (59–61). These observations lend credence to our suggestion that a common step in the oxidative degradation pathway, of regular bacterial type dicluster ferredoxins, is the formation of an intermediate containing two [3Fe-4S] clusters, as illustrated in Fig. 9. In some less stable dicluster ferredoxins, such an intermediate form could be detected only transiently. In other words, detection of this intermediate might depend on the overall structural rigidity of the ferredoxin core fold and/or the cluster surroundings.

**Structural and Evolutionary Implications**—The polypeptide backbone structure of a bacterial type ferredoxin exhibits a pseudo two-fold symmetry regardless of the number of bound FeS clusters. It has been proposed that the distorted two-fold symmetrical structure has been derived from a putative common ancestor as a result of early gene duplication event, and that the cluster I is strictly conserved in all bacterial type monocluster and dicluster ferredoxins reported so far (5, 22, 67). There is no monocluster ferredoxin with a cluster bound only to the cluster II site, implying that the two cluster binding sites in a ferredoxin core fold are evolutionary not equivalent (5, 67). The selective interconversion of a [4Fe-4S] ← [3Fe-4S] cluster at the cluster I site has been well established in some bacterial ferredoxins (14–25), but this was not demonstrated at the cluster II site. Our results indicate that oxidative degradation of the cluster II site in dicluster ferredoxins probably follow the same chemistry with a general structural rule: The missing corner (Fe) of a biological [4Fe-4S] cube is associated with either replacement (e.g. Cys11, Asp), or tilting away to solvent, of the second cysteine residue (Cys11) in the -Cys1-Xaa-Xaa-Cys11-Xaa-Xaa-Cys11-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Cys11-Pro- motif (see Figs. 1 and 9). Although it is currently unclear why this particular site is the most sensitive target for oxidative damage, we expect that increased stability of oxygen-labile bacterial type ferredoxins against oxidant (such as molecular oxygen or ferricyanide) could be conferred by introducing appropriate mutations to the polypeptide region in the vicinity of Cys11.

A ferredoxin core fold of a bacterial type ferredoxin represents a [3Fe-4S]/[4Fe-4S] cluster-binding module in biological systems, and can be found in various simple and complex electron transfer proteins as a cluster-binding subunit and/or domain (14, 22). To the best of our knowledge, there are only a few instances of electron transfer proteins which have a [3Fe-4S] cluster at the cluster II site of the module. These include an unusual 7Fe-containing ferredoxin from a hyperthermophilic archaean, *Pyrococcus islandicum* (68) and the C-terminal domain of the cluster-binding subunit (subunit B) of some respiratory fumarate reductase and succinate dehydrogenase complexes (69–71). In all cases, the second cysteine residue (Cys11) in the -Cys1-Xaa-Xaa-Cys11-Xaa-Xaa-Cys11-Xaa-Xaa-Xaa-Xaa-Cys11-Pro- motif is replaced by a non-cysteine residue.
such as Asp, Val, Ile, and Ala. In *Escherichia coli* respiratory fumarate complex, this might have afforded reduction of the [3Fe-4S] cluster II directly by proximal menaquinol (69). In the native 7Fe form of archaeal zinc-containing ferredoxins, the [4Fe-4S] cluster II is not reduced by the cognate 2-oxoacid: ferredoxin oxidoreductase (5, 7, 12), although the [3Fe-4S] cluster II of the 6Fe intermediate form can be reduced enzymatically by the oxidoreductase. Hence, the apparent effect of these amino acid replacement at the cluster II site is to modulate electron distributions within a reduced ferredoxin module by upshift of the relative redox potential of the cluster, because a [3Fe-4S]$^{1+/-0}$ cluster generally has a higher midpoint redox potential than a [4Fe-4S]$^{2+/-1}$ cluster in biological systems (22). Nevertheless, the cluster conversion at the cluster II site by a single amino acid replacement seems to be not very favored in the course of molecular evolution of a ferredoxin core fold module, which may be related to the overall stability of the polypeptide backbone.

It is known that a ferredoxin core fold module of a bacterial type ferredoxin contains only a few secondary structural elements (5, 22) (see Fig. 1). The FeS cluster binding apparently contributes to the overall protein stability of the module, but the details of structural and thermodynamical roles of a metal cofactor in protein folding and unfolding reactions are little known (72, 73). Recent multidimensional heteronuclear NMR analysis of a partially unfolded high potential iron-sulfur protein under severe denaturing conditions indicated that its [4Fe-4S] cluster plays a decisive role in determining the resulting ordered structure, and that the backbone mobility increases with the structural indetermination (74). All the present results are consistent with the primary role of an FeS cluster in retaining the native-like ordered structure of the ferredoxin core fold module (Figs. 1 and 9). We therefore suggest that the cluster I site of a ferredoxin core fold module is strictly conserved in biological systems not only because of the functional importance in electron transfer (5), but also the structural importance as a possible nucleation site of the folding of the module that shifts equilibrium toward a native-like ordered structure presumably by forming the Fe-S(Cys) bonds in vivo.

The cluster II site is less conserved in the ferredoxin core module (5, 22, 67), but our results indicate that the native-like ordered structure of the cluster II site is maintained regardless of the type of an FeS cluster ([4Fe-4S] versus [3Fe-4S]) whenever a protein-bound cluster is present (Fig. 9).

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