A stable intermediate product of the archaeal zinc-containing 7Fe ferredoxin from *Sulfolobus* sp. strain 7 by artificial oxidative conversion

Toshio Iwasaki[a],*, Tairo Oshima[b]

[a]*Department of Biochemistry and Molecular Biology, Nippon Medical School, Sendagi, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113, Japan*
[b]*Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Horinouchi, Tokyo 192-03, Japan*

Abstract Irreversible conversion of the purified zinc-containing 7Fe ferredoxin from the thermoacidophilic archaeon *Sulfolobus* sp. strain 7 was found to occur under aerobic conditions at pH 5.0 and at 4°C. This process accompanied a substantial increase of the electron paramagnetic resonance signal attributed to a $[3Fe-4S]^{1+}$ cluster, and the converted form containing ~ 6 Fe/Zn (mol/mol) had a net charge different from that of the native 7Fe ferredoxin. These data provide evidence for the formation of a stable intermediate product of the archaeal ferredoxin having two [3Fe-4S] clusters and a zinc center by artificial oxidative degradation. This further explains the discrepancy that a zinc center and two [3Fe-4S] clusters (but not a zinc center and one [3Fe-4S] cluster plus one [4Fe-4S] cluster) are observed in the crystal structure at pH 5.0.
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1. Introduction

The selective oxidative degradation of [4Fe-4S] clusters into the corresponding cubane [3Fe-4S] clusters has been studied with several bacterial-type ferredoxins from *Azotobacter vinelandii*, *Clostridium pasteurianum*, and *Pyrococcus furiosus* [1–7] with interest of chemistry of the biological iron-sulfur (FeS) clusters. Unlike the cases of monocluster-type ferredoxins, the intermediate products of 7Fe-containing dicluster ferredoxins by oxidative degradation remain incompletely characterized. It has been reported that ferricyanide oxidation of *A. vinelandii* ferredoxin I proceeds initially at a $[4Fe-4S]^{2+}$ cluster to form an electron paramagnetic resonance (EPR)-active intermediate identified as a 3-electron oxidation product in which a putative cysteinyldisulfide radical is formed, and that no 6Fe intermediate containing two [3Fe-4S] clusters is formed [1,2,4,5,7]. In the case of a related 7Fe ferredoxin from *Thermus thermophilus* [8], no EPR-active intermediates have been detected presumably because of its marked stability against heat and oxidative degradation by ferricyanide [9]. On the other hand, Imai et al. [10] have recently reported that a novel 6Fe form of *Mycobacterium smegmatis* ferredoxin can be purified under certain aerobic conditions together with the native 7Fe form. Although the authors were unable to convert the 7Fe form of *M. smegmatis* ferredoxin into the corresponding 6Fe form, evidence for their identity at the apoprotein level was reported [10].

More recently, another example of 6Fe ferredoxin was structurally characterized by X-ray crystallography. This ferredoxin has been purified as the 7Fe form (containing one $[3Fe-4S]^{1+,0}$ cluster and one $[4Fe-4S]^{2+,1+}$ cluster) from the thermoacidophilic archaeon *Sulfolobus* sp. strain 7, while its crystal structure showed the presence of two structurally dissimilar [3Fe-4S] clusters in addition to an isolated zinc center which is in tetragonal coordination by three histidine residues (His-16, His-19, and His-34) in the N-terminal extension region and one aspartate residue (Asp-76) in the FeS cluster-binding core domain [11–13]. The *Sulfolobus* ferredoxin represents one example of the recently recognized 'zinc-containing ferredoxin' family [14], which seems to be widely distributed among phylogenetically distantly related thermoacidophilic archaea including *Thermoplasma acidophilum* strain HO-62 [14] and may be found in some other archaea. In addition to the significant discrepancy of the types of FeS clusters in the as-isolated and crystalline forms of the *Sulfolobus* zinc-containing ferredoxin, we are aware of the presence of a minor form of the archaeal ferredoxin, namely Fd-B, found in a large-scale preparation [15]. Initial analyses suggested that purified Fd-B is identical to the 7Fe form at the apoprotein level but has a different charge density which could not be interpreted conclusively; artificial conversion from one form to another was unsuccessful [15].

The existence of the 6Fe forms of *M. smegmatis* ferredoxin and *Sulfolobus* sp. zinc-containing ferredoxin raises the interesting possibility that a 6Fe form may represent an intermediate product of a 7Fe ferredoxin by oxidative degradation. However, straightforward experimental evidence is lacking to support this hypothesis because no successful artificial conversion of the 7Fe form to the corresponding 6Fe form has been achieved so far. Here we report spectroscopic characterization of the *Sulfolobus* zinc-containing ferredoxin treated under aerobic conditions similar to those used for crystallization [16], and discuss the multiple forms of the archaeal ferredoxin with respect to the artificial oxidative degradation of the protein.

2. Materials and methods

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, originally isolated from Beppu hot springs, Japan, were cultivated aerobically and chemoheterotrophically at pH 2.5–3 and 75–80°C, and the two different forms of the archaeal ferredoxin, Fd-A and Fd-B, were routinely purified as described previously [11,15]. Each of the purified proteins showed a single band on analytical polyacrylamide gel electrophoresis in the absence of sodium dodecylsulfate (native PAGE) on 20% resolving gels performed as described previously [15].

The 7Fe form of the purified *Sulfolobus* ferredoxin (Fd-A), with a purity index $A_{408\,nm}/A_{281\,nm}$ of 0.70 [11], was passed through a disk filter with a pore size of 0.2 μm and divided into three parts, each of which was treated and stored under different conditions as follows.

*Corresponding author. Fax: (81) (3) 5685-3054.

The 'control sample' refers to purified Fd-A made in 80 mM potassium phosphate buffer, pH 6.8, and this was stored at −80°C without further treatment until use; 'sample 1' was made in 0.5 M Tris-maleate buffer, pH 5.0, and stored at 4°C under aerobic conditions until use; and 'sample 2' was made in 0.5 M Tris-maleate buffer, pH 5.0, containing 1.9 M ammonium sulfate and stored at 4°C under aerobic conditions until use (the conditions of sample 2 are essentially identical to those used for crystallization of the *Sulfolobus* ferredoxin [16], except that the protein concentration was kept lower ($\sim$70–100 μM) to avoid crystallization or precipitation). Samples 1 and 2 were then placed at 4°C for 2 months, the period typically required for crystallization prior to X-ray diffraction studies [16] (crystals of salts formed during storage at 4°C were redissolved by gentle agitation at room temperature).

When required, the samples treated at pH 5.0 were extensively dialyzed against 40 mM Tris-HCl buffer, pH 7.4, and further purified by Pharmacia DEAE-Sepharose Fast Flow column chromatography essentially as reported previously (see Fig. 1 in [15]).

Absorption spectra were recorded as described previously [11]. Circular dichroism (CD) spectra were recorded with a Jasco J720 spectropolarimeter with 0.5-cm and 0.1-cm cells. EPR measurements were carried out using a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system, in which temperature was monitored with 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. All spectral data were processed using KaleidaGraph software ver. 3.05 (Abelbeck Software).

Purified 7Fe ferredoxin (Fd-A) was measured by the BCA assay (Pierce Chemical) with bovine serum albumin as a standard and by dividing the results by 1.48 for calibration [11]. Calorimetric estimation of the converted form (Fd-B) after separation by DEAE column chromatography was judged inaccurate because the sample contained an unknown portion of apoferredoxin; the concentration of the converted ferredoxin was therefore estimated assuming an absorption coefficient around 14.5 mM^{-1} cm^{-1} for each cluster at 413 nm. Metal content analyses were carried out by inductively coupled plasma atomic emission spectrometry with a Seiko SPS 1500 VR instrument at the Tokyo Institute of Technology and a Jobin-Yvon JY 38S instrument at Rigaku Ltd.

3. Results and discussion

Fig. 1 shows the analytical native PAGE on 20% resolving gels of the ferredoxin samples 1 (lane 2) and 2 (lane 3) (see Section 2) with purified Fd-A (lane 1) and Fd-B (lane 4) as references. Under the experimental conditions, Fd-B is distinguished from Fd-A because of the difference in net charge that is not due to proteolytic cleavage [15]. Sample 1, which had been treated at pH 5.0, exhibited two bands corresponding to those of Fd-A and Fd-B (lane 2), whether or not it was dialyzed against 20 mM potassium buffer, pH 6.8, just prior to the PAGE analysis. Sample 2 also exhibited these two bands on the native PAGE (lane 3). These data suggest that Fd-B is a main product of Fd-A by irreversible conversion in 0.5 M Tris-maleate buffer, pH 5.0, either in the presence or in the absence of 1.9 M ammonium sulfate, and that the apparent efficiency of conversion is $\sim$50% under these conditions. In contrast, Fd-A stored in 80 mM potassium phosphate buffer, pH 6.8, for 2 months at 4°C showed formation of a small amount of apoprotein (as indicated by a lower purity index $A_{408\,nm}/A_{281\,nm}$ of $\sim$0.65) but virtually no Fd-B (data not shown). Previous native PAGE analysis suggested that no conversion of Fd-A to Fd-B was detected at pH 4–9 in 36 h [15], indicating that this process proceeds gradually under the applied conditions.

In view of the inherent stability of the FeS clusters in the archaeal ferredoxin toward oxidative damage [11], the slow and irreversible conversion of Fd-A to Fd-B under aerobic
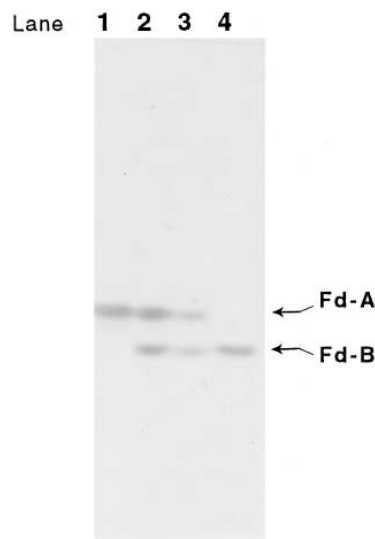

Fig. 1. Native PAGE analysis in the absence of denaturants on 20% resolving gels of purified Fd-A (lane 1), sample 1 (lane 2), sample 2 (lane 3) and purified Fd-B (lane 4). Samples 1 and 2 were prepared from the same batches of purified Fd-A (lane 1) as described in Section 2, and all samples were dialyzed against 20 mM potassium phosphate buffer, pH 6.8, at 4°C for 24 h prior to the native PAGE analysis. Proteins were visualized by Coomassie brilliant blue staining for clarity, although all bands in the figure could be detected by brown color without staining due to the presence of bound FeS clusters (data not shown).

conditions at pH 5.0 implies that Fd-B may represent a stable intermediate product of Fd-A by oxidative degradation. Because a [3Fe-4S]$^{1+}$ cluster of the *Sulfolobus* ferredoxin elicits a characteristic EPR signal in the oxidized state [11], the EPR signal intensity is expected to increase considerably if the 7Fe-containing Fd-A is converted to a 6Fe form under the applied conditions. Fig. 2 depicts the EPR spectral changes at 7.4 K accompanied by artificial conversion of Fd-A to Fd-B at pH 5.0, using sample 1 (trace A) and sample 2 (trace C) prepared from the same batches of purified Fd-A (solid trace in Fig. 2B,D). Although all these ferredoxin samples elicited the sharp $g = 2.02$ EPR signal attributed to a [3Fe-4S]$^{1+}$ cluster, the relative EPR signal intensities at 7.4 K of the sample 1 (trace A) and sample 2 (trace C) clearly increased compared to that of Fd-A ($\sim$0.9–1.0 spin/mol; solid trace in Fig. 2B,D). Assuming the presence of $\sim$50% each of Fd-A and Fd-B in samples 1 and 2 (as estimated from the PAGE analysis in Fig. 1), the calculated difference EPR spectra suggest that the converted species (Fd-B) in both samples 1 and 2 contains approximately two [3Fe-4S]$^{1+}$ clusters at $g = 2.02$ ($\sim$1.5–1.7 spin/mol; dashed traces in Fig. 2B,D). Because Fd-A has been shown to contain a single [3Fe-4S]$^{1+,0}$ cluster [11], these data suggest that the artificial conversion of Fd-A to Fd-B at pH 5.0 accompanies the formation of an additional [3Fe-4S]$^{1+}$ cluster in Fd-B. Reexamination of the metal contents of purified Fd-A and Fd-B by inductively coupled plasma atomic emission spectrometry suggested the presence of tightly bound Zn and Fe atoms in both forms, but in a ratio of $\sim$6.9 Fe/Zn (mol/mol) for Fd-A and 5.8–6.1 Fe/Zn (mol/mol) for Fd-B (data not shown). The differences in the stoichiometry of these metals suggest that Fd-A and Fd-B are the 7Fe and 6Fe forms of the archaeal zinc-containing ferredoxin, respectively. In conjunction with the EPR analysis presented in Fig. 2, it is

concluded that the additional $[3Fe-4S]^{1+}$ cluster formed in Fd-B originates from a $[4Fe-4S]^{2+}$ cluster in Fd-A by oxidative degradation. Thus, under aerobic conditions similar to those used previously for crystallization at pH 5.0 [16], the gradual oxidative degradation of the *Sulfolobus* zinc-containing 7Fe ferredoxin (Fd-A) initially leads to the formation of a stable 6Fe-containing intermediate (Fd-B) without loss of a zinc center. This observation is substantially different from the case reported for *A. vinelandii* ferredoxin I where initial oxidative degradation at the $[4Fe-4S]^{2+}$ cluster leads to formation of an EPR-active intermediate identified as a 3-electron oxidation product in which a putative cysteinyldisulfide radical (but not a $[3Fe-4S]^{1+}$ cluster) is formed [1,2,4,5,7]. This may reflect the structural differences of the microenvironments around the [4Fe-4S] cluster in *Sulfolobus* sp. and *A. vinelandii* 7Fe ferredoxins. Although the existence of other transient intermediate products in *Sulfolobus* sp. ferredoxin might be possible, no such species could be detected under the applied conditions presumably because of their instability.

Although the FeS cluster conversion of the *Sulfolobus* ferredoxin proceeds very slowly even under aerobic conditions and is unlikely to be of physiological significance, it is of considerable interest as a route to preparation of the 6Fe form (Fd-B) which has been obtained in a small amount from the soluble fraction of *Sulfolobus* sp. strain 7 ($\sim$1–2 mg protein from 150 g (wet weight) of cells, representing roughly 2–3% of the total *Sulfolobus* zinc-containing ferredoxin) [15]. Using the sample 1 dialyzed against 40 mM Tris-HCl buffer, pH 7.4, as a starting material, Fd-B was readily separated from residual Fd-A in a single step on anion-exchange column chromatography. Partially purified Fd-B thus obtained usually contained a small portion of apoprotein as indicated by a lower purity index $A_{413\,nm}/A_{281\,nm}$ of $\sim$0.59–0.63 (presumably due to further oxidative degradation of Fd-B), but its visible absorption (data not shown) and CD spectra (Fig. 3A) are characteristic of those previously reported for Fd-B obtained from the archaeal soluble fraction [15]. The overall intensities of these spectra of Fd-B are virtually similar
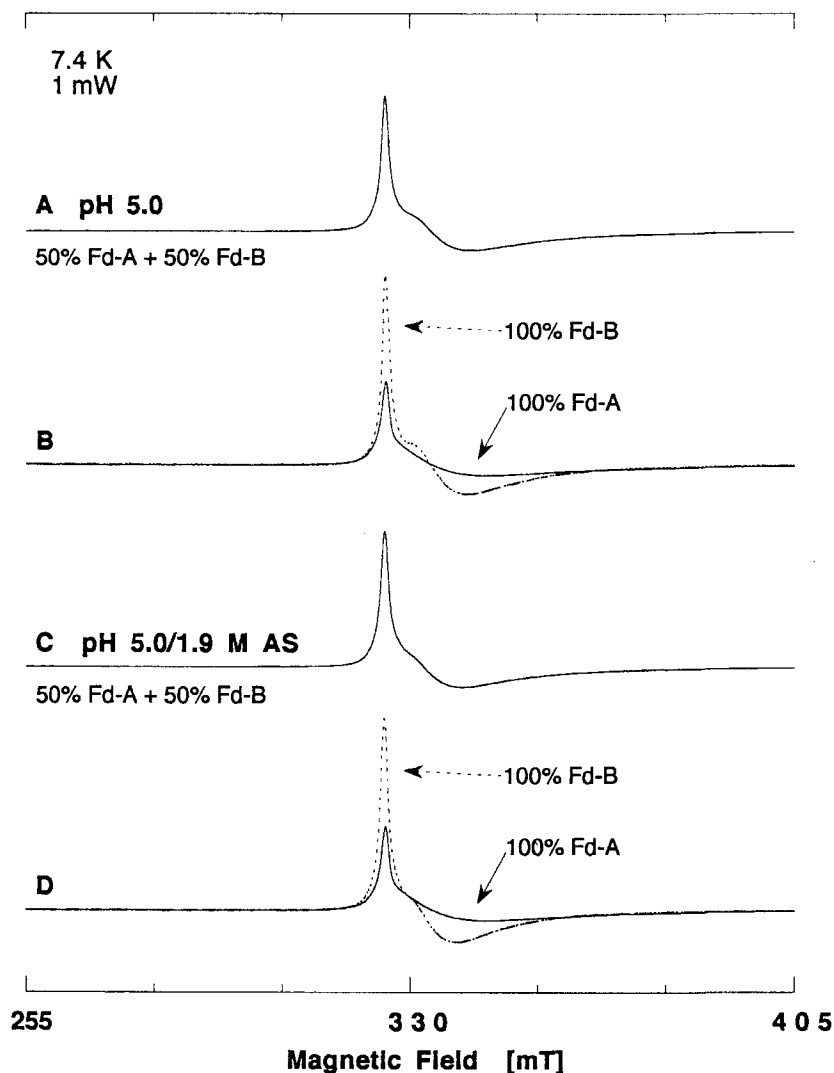

Fig. 2. Comparative EPR spectra at 7.4 K of air-oxidized sample 1 (trace A), sample 2 (trace C), and purified Fd-A (solid traces in B and D), attributed to a $[3Fe-4S]^{1+}$ cluster. The concentrations of all samples were $\sim$0.1 mM, and each EPR spectrum was normalized to the identical protein concentration (see Section 2) and presented with the same gain. EPR spectra of Fd-B in samples 1 and 2 (dashed trace) were calculated assuming the presence of 50% Fd-A and 50% Fd-B in both samples 1 (trace B) and 2 (trace D) as estimated from the relative intensities of the corresponding bands in the native PAGE analysis (Fig. 1). Instrument setting for EPR spectroscopy: temperature 7.4 K; microwave power 1 mW; modulation amplitude 0.79 mT.
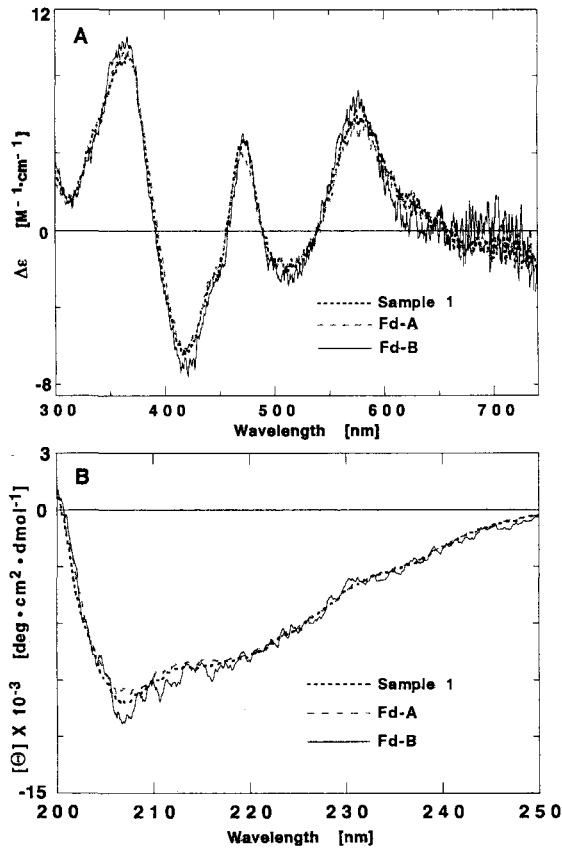
Fig. 3. Comparative near-UV visible (A) and far-UV (B) spectra of purified Fd-A (purity index $A_{408\,nm}/A_{281\,nm}$ 0.70; dashed trace), partially purified Fd-B (purity index $A_{413\,nm}/A_{281\,nm}$ ~0.5–0.63; solid trace), and sample 1 (dotted trace). All these samples had been dialyzed against 20 mM potassium phosphate buffer, pH 6.8, at 4°C for 24 h prior to recording the absorption and CD spectra, and the spectroscopic data were normalized to their specific absorption of FeS clusters at 408 nm for Fd-A and at 413 nm for Fd-B because of the presence of an unknown portion of apoferredoxin in partially purified Fd-B (see Section 2).

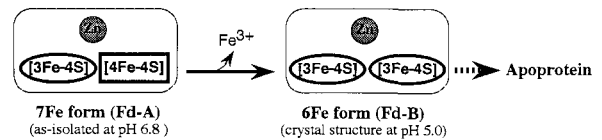Fig. 4. Schematic illustration of the initial FeS cluster degradation of the Sulfolobus zinc-containing ferredoxin by artificial oxidative conversion.

characterized structurally by X-ray crystallography [12,13], explaining the discrepancy of the types of FeS clusters in the as-isolated and crystalline forms of the Sulfolobus zinc-containing ferredoxin. Since another 6Fe form has been reported recently for M. smegmatis dicluster ferredoxin containing no bound zinc center [10], we suggest that a 6Fe form may represent one of the common intermediates produced by oxidative degradation of 7Fe ferredoxins and would also be found in other bacterial-type dicluster ferredoxins.

to those of Fd-A, except for small differences in the visible CD spectra in the 550–750 nm region (Fig. 3A). Far-UV CD spectra of Fd-A, Fd-B, and sample 1 suggest that the overall secondary structures did not change significantly before and after the oxidative conversion (Fig. 3B), indicating that conversion of the 7Fe form to the 6Fe form is not accompanied by major structural rearrangements. Thus, in spite of the loss of one Fe atom, the absorption and CD spectral changes before and after the oxidative conversion of the Sulfolobus ferredoxin are unexpectedly small (cf. [6]). Further spectroscopic characterization of the 7Fe and 6Fe forms of the archaeal zinc-containing ferredoxin is under way.

In conclusion, it is demonstrated herein that the archaeal zinc-containing 7Fe ferredoxin of Sulfolobus sp. strain 7 is gradually converted by artificial oxidative degradation at pH 5.0 to a stable intermediate, Fd-B, which is a 6Fe form probably containing two $[3Fe-4S]^{1+}$ clusters and a zinc center (Fig. 4). The properties of Fd-B match a protein molecule recently

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