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Assembly Mechanism of $[Fe_2S_2]$ Cluster in Ferredoxin from *Acidithiobacillus ferrooxidans*

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Ferredoxin is a typical iron-sulfur protein that is ubiquitous in biological redox systems. This study investigates the *in vitro* assembly of a $[Fe_2S_2]$ cluster in the ferredoxin from *Acidithiobacillus ferrooxidans* in the presence of three scaffold proteins: IscA, IscS, and IscU. The spectra and MALDI–TOF MS results for the reconstituted ferredoxin confirm that the iron-sulfur cluster was correctly assembled in the protein. The inactivation of cysteine desulfurase by L-allylglycine completely blocked any $[Fe_2S_2]$ cluster assembly in the ferredoxin in *E. coli*, confirming that cysteine desulfurase is an essential component for iron-sulfur cluster assembly. The present results also provide strong evidence that $[Fe_2S_2]$ cluster assembly in ferredoxin follows the AUS pathway.

Keywords: *Acidithiobacillus ferrooxidans*, ferredoxin, iron-sulfur cluster, assembly

Iron-sulfur clusters are one of the primordial and ubiquitous redox centers in nature, and are involved in diverse physiological processes, including respiratory electron transfer, nitrogen fixation, photosynthesis, biosynthesis of amino acids, and DNA synthesis and repair [1, 5]. However, the mechanism underlying iron-sulfur cluster assembly is still not fully understood. Recent studies have revealed that iron-sulfur cluster assembly *in vivo* mainly follows the "AUS" pathway, which requires scaffold proteins, and three important proteins [IscA (iron-binding protein), IscS (cysteine desulfurase), and IscU (scaffold protein)] are involved in iron-sulfur cluster assembly [12, 14, 22]. Yet, the exact mechanism of iron-sulfur cluster assembly remains unclear.

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Although various reports have already been published on the iron donor or scaffold protein involved in iron-sulfur cluster assembly [3, 7, 13], very little research has focused on the sulfur donor. L-Allylglycine has been reported to be a suicide inhibitor of the cysteine desulfurase IscS/NifS by forming a covalent adduct with the active-site cysteine of IscS/NifS, thereby inactivating the enzyme [21] and blocking the AUS-dependent assembly pathway. Thus, selective inactivation of cysteine desulfurase can provide an efficient way to study the mechanism of iron-sulfur cluster assembly *in vivo*.

Ferredoxins are small, acidic, electron transfer proteins that are ubiquitous in biological redox systems. In particular, ferredoxins with one $[Fe_2S_2]$ cluster per molecule are present in plants, animals, and bacteria and form a distinct family of $[Fe_2S_2]$ cluster-containing proteins [8].

Accordingly, this study reports on the *in vitro* assembly of a $[Fe_2S_2]$ cluster in the ferredoxin from *A. ferrooxidans* in the presence of three scaffold proteins: IscA, IscS, and IscU. The spectra and MALDI–TOF MS results for the holo-ferredoxin confirm that the iron-sulfur cluster was correctly assembled in the protein. The inactivation of cysteine desulfurase completely blocked any iron-sulfur cluster assembly in *E. coli*.

Materials and Methods

Materials

The *A. ferrooxidans* ATCC 23270 was obtained from the American Type Culture Collection. A HiTrap chelating metal affinity column was purchased from GE Healthcare Ltd. The *E. coli* TOP10 and BL21(DE3) competent cells came from Invitrogen Life Technologies. The Plasmid Mini kit, gel extraction kit, and synthesized oligonucleotides were obtained from Sangon in Shanghai, China. The *Taq* DNA polymerase, T4 DNA ligase, and restriction enzymes came from MBI Fermentas in Germany. The L-allylglycine was

purchased from Shanghai Hengbai Chemical Company, China. All the other reagents were of research grade and obtained from commercial sources. The recombinant IscA, IscS, and IscU from *A. ferrooxidans* were prepared as described previously [17–19].

Preparation of Apo-Ferredoxin

Purified ferredoxin from *A. ferrooxidans* was prepared according to a method described previously [20]. Apo-ferredoxin was then prepared by boiling the purified holo-ferredoxin in the presence of 100 mM EDTA and 500 mM dithiothreitol to trap the iron atoms liberated from the holoprotein and ensure that the side chains of the four cysteines previously participating in the $[Fe_2S_2]$ cluster ligation were reduced to free sulfhydryl groups. After boiling for 30 s, the apo-ferredoxin was purified by gel filtration column chromatography using a Fast-desalting column (Amersham Pharmacia Biotech) that had been equilibrated with 20 mM potassium phosphate, pH 7.4, and 0.5 M NaCl [10].

***In Vitro* Assembly of Iron-Sulfur Cluster in Ferredoxin**

The $[Fe_2S_2]$ cluster was loaded in the apo-ferredoxin in the presence of the scaffold proteins IscA, IscS, and IscU. The apo-ferredoxin (20 μM) was incubated anaerobically at 25°C with the purified IscA (0.5 μM), IscS (0.5 μM), and IscU (1 μM) in the presence of $Fe(NH_4)_2(SO_4)_2$ (100 μM) and dithiothreitol (2 mM). L-Cysteine (1 mM) was then added and the reaction mixture incubated anaerobically at 37°C. Finally, the reaction mixture was desalted on a Sephadex G-25 column, the protein samples were analyzed using 20% nondenaturing PAGE, and the gel was stained based on iron-staining [4].

***In Vivo* Assembly of Iron-Sulfur Cluster in Ferredoxin in Presence of L-Allylglycin**

The *E. coli* strain BL21(DE3) cells and pLM1::FDX plasmid were grown at 37°C in 500 ml of an LB medium containing ampicillin (100 mg/l) to an OD_{600} of 0.6. Next, 50 mM L-allylglycine and 0.5 mM IPTG were added and the cells incubated at room temperature overnight with shaking at 180 rpm. The expressed ferredoxin was then purified according to a method described previously [20].

UV–Vis Scanning and Electronic Paramagnetic Resonance (EPR) Spectra

The UV–visible spectra scanning was carried out at 25°C using a Shimadzu UV-1800 spectrophotometer. Samples of the apo- and holo-ferredoxins (10 μM) were prepared in a 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4. The X-band EPR spectra were recorded at 70K using a JEOL JES-FE1XG spectrometer. The parameters used to record the EPR spectra were typically a 15–30 mT/min sweep rate, 0.63 mT modulation amplitude, 9.153 GHz frequency, 4 mW incident microwave power, and 2 min sweep time. The samples were diluted to 5 μM in a 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4.

Determination of Iron and Sulfide Contents in Reconstituted Ferredoxin

The iron assays were performed using a colorimetric method [6], whereas the sulfide content was determined according to Siegel [11]. The samples were prepared in a 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4.

MALDI–TOF MS of Reconstituted Ferredoxin

The molecular mass of the reconstituted ferredoxin was determined using an Ultraflex TOF/TOF spectrometer (Bruker Daltonics) equipped with a nitrogen laser (337 nm) and operated in the reflector/delay extraction mode. An accelerating voltage of 25 kV was used in this study. The MALDI–TOF MS results were obtained in a linear positive mode using α-cyano-4-hydroxy-cinnamic acid (saturated solution in 50% acetonitrile with 0.1% trifluoroacetic acid) as the UV-absorbing matrix. Prior to the MALDI–MS analysis, the protein samples were diluted to a concentration of approximately 1 μM in a 20 mM potassium phosphate buffer containing 0.5 M NaCl, pH 7.2. The theoretical molecular mass was calculated using the Compute pI/Mw tool from the Expasy Proteomics Server of the Swiss Institute of Bioinformatics (SIB).

***In Vitro* Inactivation of IscS by L-Allylglycine**

Purified IscS (1 μM in 20 mM potassium phosphate, pH 7.4) was incubated with 0–50 μM of L-allylglycin for different times at 25°C. DTT (1 mM) and 0.5 mM substrate cysteine were then added to the mixture to initiate the reaction. IscS activity was based on the formation of hydrogen sulfide, as described previously [23].

RESULTS AND DISCUSSION

***In Vitro* Assembly of Iron-Sulfur Cluster in Ferredoxin**

The incubation of apo-ferredoxin anaerobically with purified IscA, IscS, IscU (1 μM), and $Fe(NH_4)_2(SO_4)_2$ in the presence of L-cysteine, followed by desalting on a Sephadex G-25 column resulted in a brownish protein containing an iron-sulfur cluster. The protein samples were analyzed by 20% nondenaturing PAGE with iron-staining, as shown in Fig. 1. The apo-ferredoxin showed no band in the PAGE; however, the reconstituted holo-ferredoxin and native holo-ferredoxin both showed iron-stained bands, indicating the presence of iron in the proteins.

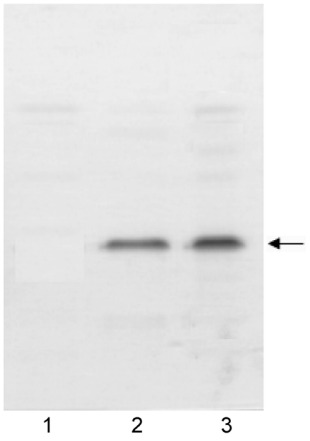

Fig. 1. Nondenaturing PAGE of purified apo- and holo-ferredoxins with iron-staining.
Lane 1, apo-ferredoxin; lane 2, reconstituted holo-ferredoxin with assembled iron-sulfur cluster; lane 3, native ferredoxin.

Table 1. Iron and sulfur contents in apo- and reconstituted ferredoxin.

	Iron content (μmol/(μmol protein)	Sulfur content (μmol/μmol protein)	Iron/sulfur
Apo-ferredoxin	<0.1	<0.1	NA
Reconstituted ferredoxin	1.77±0.07	1.91±0.10	0.93

The total iron content in the reconstituted ferredoxin was estimated to be 1.77 μmol/μmol protein based on a colorimetric method, whereas the total sulfide content was determined to be 1.91 μmol/μmol protein, as shown in Table 1. Thus, the reconstituted holo-ferredoxin from *A. ferrooxidans* contained a single $[Fe_2S_2]$ cluster, which agreed well with the native ferredoxin (Table 1).

UV-Scanning and EPR Spectra of Reconstituted Ferredoxin

The UV–visible spectra for the apo-ferredoxin and holo-ferredoxin with the assembled iron-sulfur cluster are shown in Fig. 2. The apo-ferredoxin showed no significant absorption between 350–450 nm, whereas the holo-ferredoxin with the assembled iron-sulfur cluster exhibited two major absorption peaks located at 415 nm and 459 nm, respectively, which is typical for proteins containing a $[Fe_2S_2]$ cluster. Moreover, the spectra were similar to those previously reported for ferredoxins from various sources [2, 10, 15].

The EPR spectra for the apo- and holo-ferredoxins are shown in Fig. 3. The apo-ferredoxin exhibited no EPR activity, whereas the iron-sulfur-loaded holo-ferredoxin showed a typical S=1/2 EPR signal, indicating the presence of a $[Fe_2S_2]^+$ cluster, and the g value was estimated to be 1.997. These results also agreed well with those for ferredoxins from other sources [2, 10, 15].

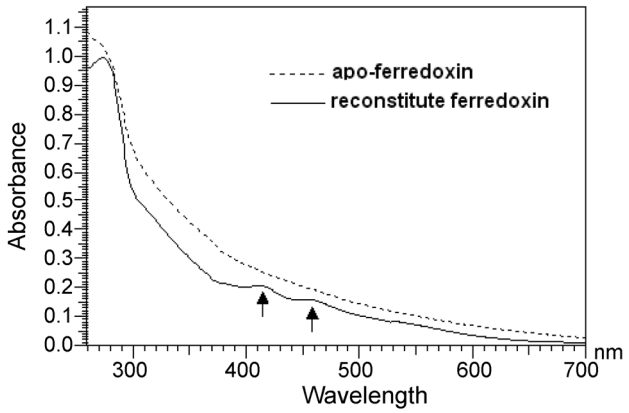

Fig. 2. UV–vis scanning of apo-ferredoxin and iron-sulfur-cluster-loaded holo-ferredoxin.
The dashed line indicates apo-ferredoxin; the solid line indicates reconstituted holo-ferredoxin in the presence of IscA, IscS, and IscU.

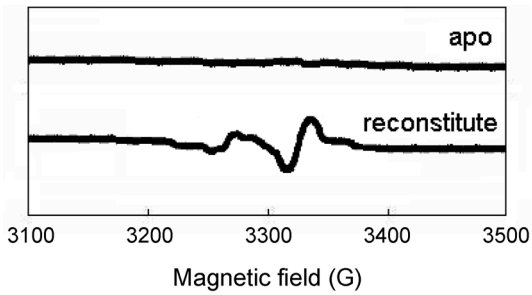

Fig. 3. EPR spectra of apo-ferredoxin and reconstituted holo-ferredoxin from *A. ferrooxidans*.

MALDI–TOF MS of Reconstituted Ferredoxin

The molecular mass obtained by MALDI–TOF MS for the reconstituted ferredoxin was 13,320.29 Da, as shown in Fig. 4. The protein sequence for ferredoxin with His-tags in the N-terminal plus a $[Fe_2S_2]$ cluster has a theoretical average molecular mass of 13,321.51 Da, which agreed well with the experimental results. Therefore, the results indicated that a $[Fe_2S_2]$ cluster was successfully assembled in the ferredoxin in the presence of scaffold proteins.

Effect of L-Allylglycin on Assembly of Iron-Sulfur Cluster in Ferredoxin in *E. coli*

It has been proposed that cysteine desulfurase plays a central role in Fe-S cluster formation in *E. coli*, as the sulfur in the iron-sulfur cluster is derived from L-cysteine *via* cysteine desulfurase. Thus, the absence of this protein *in vivo* would have a serious effect on the assembly of iron-sulfur clusters in *E. coli*, yet no experiments have been carried out to confirm this. L-Allylglycine is a suicide inhibitor of IscS/NifS and forms a covalent adduct with the active-site cysteine of the cysteine desulfurase IscS/NifS, resulting in enzyme inactivation. In this study, L-allylglycine was used to investigate the mechanism of IscS-catalyzed cysteine desulfurization systems. The incubation of IscS with L-allylglycine *in vitro* resulted in a time-dependent

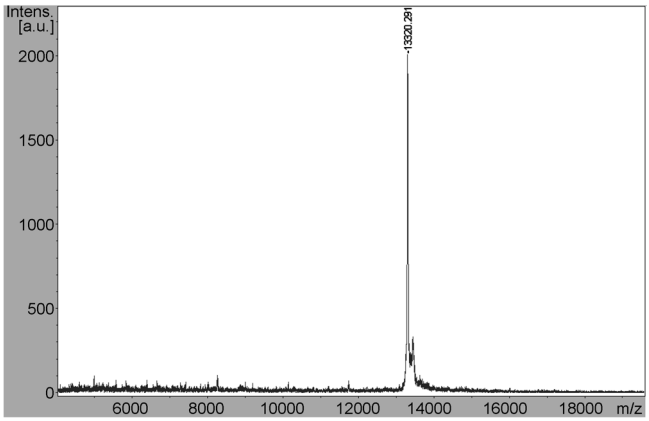

Fig. 4. MALDI–TOF MS of reconstituted ferredoxin.

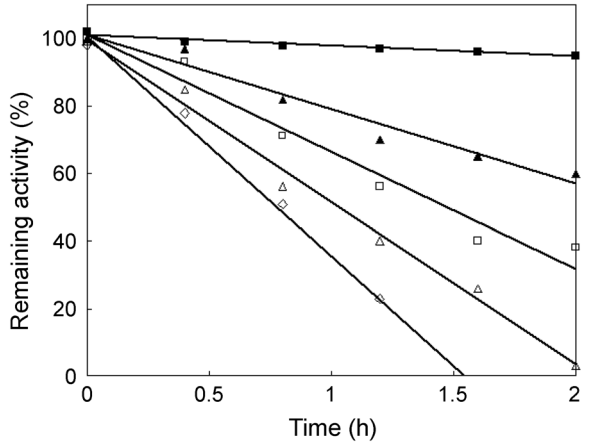

Fig. 5. Inhibition of IscS activity by L-allylglycine *in vitro*.
IscS (1 μM in 20 mM potassium phosphate, pH 7.4) was incubated with 0 μM (■), 5 μM(▲), 10 μM(□), 20 μM (△), and 50 μM (◇) L-allylglycine for different times at 25°C and the enzyme activities were assayed.

loss of cysteine desulfurase activity, as shown in Fig. 5. These results agreed well with a previous report [21].

The addition of L-allylglycine to the culture media had no effect on the expression of ferredoxin, which was expressed and purified in a soluble form, as shown in Fig. 6. The purified ferredoxin expressed in the presence of L-allylglycine was observed to be colorless compared with the brown color in the control experiment, indicating the absence of an iron-sulfur cluster.

UV-scanning of the purified ferredoxin indicated no absorption at 415 nm and 459 nm, the characteristic absorption for the holo-ferredoxin, as shown in Fig. 7. Additionally, no EPR spectrum signal was observed for the purified ferredoxin in the presence of L-allylglycine (data not shown). The molecular mass obtained by MALDI–TOF MS for the purified ferredoxin was 13,150.22 Da. The protein sequence for the ferredoxin without a $[Fe_2S_2]$ cluster has a theoretical average molecular mass of 13,149.90 Da, which agreed with the experimental result. Therefore, the results indicated that the ferredoxin was totally devoid of a $[Fe_2S_2]$ cluster in the absence of a cysteine desulfurase, confirming that the presence of cysteine desulfurase is crucial for the *in vivo* assembly of an iron-sulfur cluster in ferredoxin.

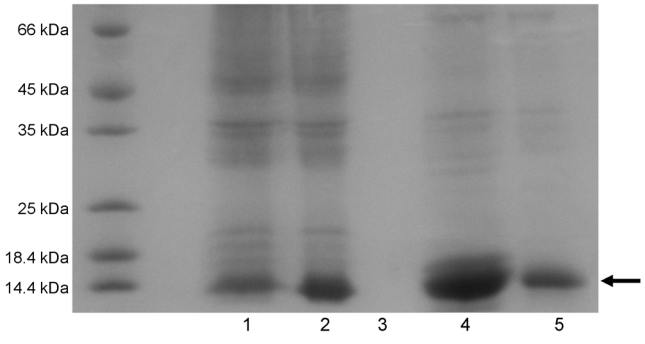

Fig. 6. SDS–PAGE of the expression and purification of ferredoxin in the absence or presence of L-allylglycine.
Lane 1, protein molecular markers; lane 2, cell lysate without addition of L-allylglycine to the medium; lane 3, cell lysate with addition of L-allylglycine to the medium; lane 4, purified ferredoxin without addition of L-allylglycine to the medium; lane 5, purified ferredoxin with addition of L-allylglycine to the medium.

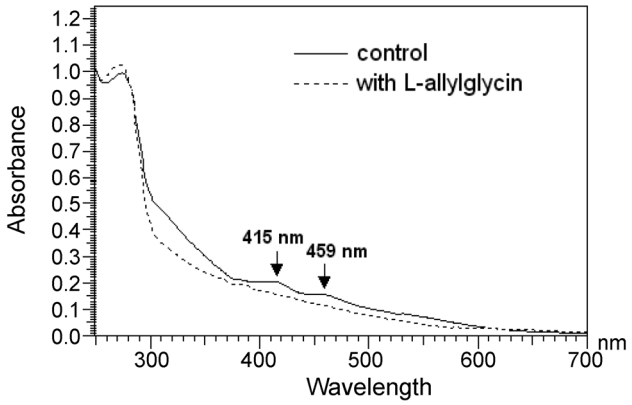

Fig. 7. UV–vis scanning of ferredoxin produced with or without addition of 50 mM L-allylglycine to the culture.
The solid line indicates no addition of L-allylglycine to the culture; the dashed line indicates addition of 50 mM L-allylglycine to the culture.

Proposed Mechanism for Iron-Sulfur Cluster Assembly in Ferredoxin

Recent studies have revealed that the *in vivo* assembly of an iron-sulfur cluster mainly follows the AUS pathway,

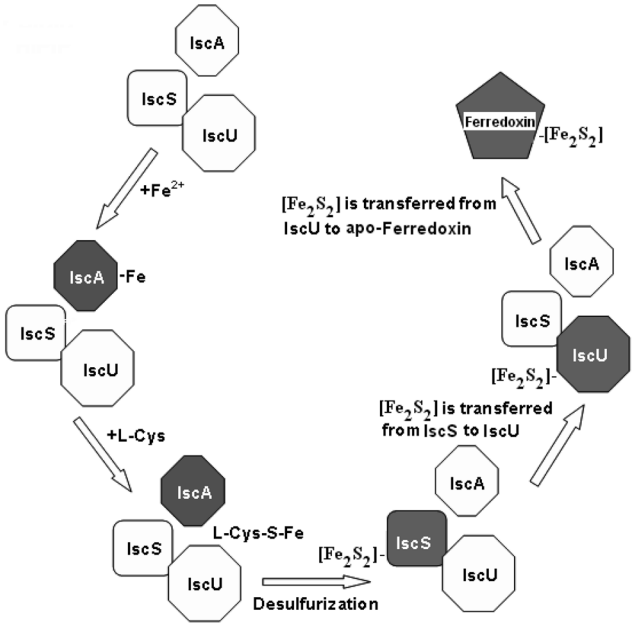

Fig. 8. Proposed mechanism of iron-sulfur cluster assembly in ferredoxin from *A. ferrooxidans*.

requiring scaffold proteins, and three important proteins (IscA, IscS, and IscU) are involved in the case of iron-sulfur cluster assembly [9, 12, 22].

The *in vitro* and *in vivo* results for [Fe_2S_2] cluster assembly in ferredoxin were clearly found to follow the AUS pathway, as shown in Fig. 8, which was first proposed by Ding's group [16]. First, IscA binds with intracellular ferrous iron to form iron-loaded IscA, and then binds with L-cysteine to form a transient L-cysteine–Fe complex. Next, IscS catalyzes the desulfurization of the L-cysteine–Fe complex and transfers the Fe-S to IscU for iron-sulfur cluster assembly in IscU *via* protein–protein interaction. Finally, the [Fe_2S_2] cluster assembled in IscU is transferred to the apo-ferredoxin.

In summary, this study investigated the assembly of a [Fe_2S_2] cluster in the ferredoxin from *A. ferrooxidans in vitro* in the presence of three scaffold proteins: IscA, IscS, and IscU. Cysteine desulfurase was shown to be an essential component for the assembly of a [Fe_2S_2] cluster in ferredoxin, and the iron-sulfur cluster assembly in ferredoxin was confirmed to follow the AUS pathway.

Acknowledgments

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