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Expression, Purification, and Characterization of Iron-Sulfur Cluster Assembly Regulator IscR from *Acidithiobacillus ferrooxidans*

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IscR (iron-sulfur cluster regulator) has been reported to be a repressor of the *iscRSUA* operon, and *in vitro* transcription reactions have revealed that IscR has a repressive effect on the *iscR* promoter in the case of $[Fe_2S_2]$ cluster loading. In the present study, the *iscR* gene from *A. ferrooxidans* ATCC 23270 was cloned and successfully expressed in *Escherichia coli*, and then purified by one-step affinity chromatography to homogeneity. The molecular mass of the IscR was 18 kDa by SDS-PAGE. The optical and EPR spectra results for the recombinant IscR confirmed that an iron-sulfur cluster was correctly inserted into the active site of the protein. However, no $[Fe_2S_2]$ cluster was assembled in apoIscR with ferrous iron and sulfide *in vitro*. Therefore, the $[Fe_2S_2]$ cluster assembly in IscR *in vivo* would appear to require scaffold proteins and follow the Isc "AUS" pathway.

Keywords: *Acidithiobacillus ferrooxidans*, His-tag, iron-sulfur cluster, assembly, regulator

Iron-sulfur proteins are present in all living organisms and exhibit diverse functions, including electron transport, redox and nonredox catalysis, stabilization of proteins, DNA synthesis and repair, and sensing for regulatory processes [2, 7, 15]. Recent studies have revealed that a highly conserved gene cluster, *iscSUA-hscBA-fdx*, is essential for the general biogenesis of iron-sulfur proteins in bacteria [20, 21, 26], which has made the three *isc* genes, *iscS*, *iscU*, and *iscA*, a focus for research [1, 5, 8, 11, 13, 16].

However, although various reports have focused on the iron-sulfur cluster assembly, relatively few studies have investigated the regulation mechanism of the iron-sulfur cluster assembly, and especially how the Fe-S cluster assembly proteins are controlled *in vivo*. It has already been reported that the genes encoding the Fe-S cluster assembly proteins from *E. coli* may be regulated by the iron-sulfur protein, IscR, which is similar to the well-characterized transcriptional regulator MarA (multiple antibiotic resistant) from *E. coli* [17], as shown in Fig. 1. MarA is a gene regulator induced by antibiotics, and a member of a subfamily of AraC-like transcriptional regulators, called the Mar/Sox/Rob family [12]. IscR functions as a repressor of the *iscRSUA* operon, as strains with a deleted *iscR* increase their expression of this operon. An analysis of the IscR from *E. coli* using electron paramagnetic resonance also revealed that the protein contains an $[Fe_2S_2]$ cluster [17].

Acidithiobacillus ferrooxidans is one of the most studied bacteria that thrives in acidic mine drainage, and obtains energy through the oxidation of ferrous ions into ferric ions with molecular oxygen as the terminal electron acceptor [6, 14]. It has also been reported that the genome sequence of *A. ferrooxidans* ATCC 23270 (http://www.tigr.org) contains the *iscRSUA* operon, making *A. ferrooxidans* an interesting focus for studying the mechanism of iron-sulfur cluster assembly and regulation.

Accordingly, in this study, the *iscR* gene from *A. ferrooxidans* ATCC 23270 was cloned and successfully

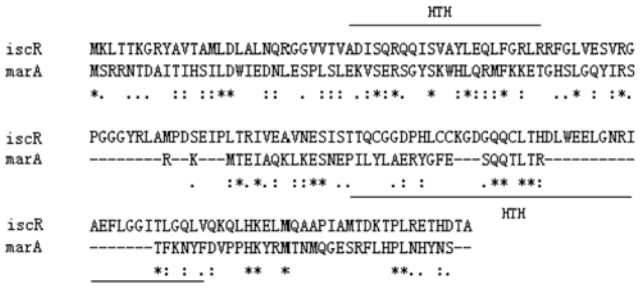

Fig. 1. Similarity of *Acidithiobacillus ferrooxidans* ATCC 23270 IscR to *Escherichia coli* K12 MarA.
Amino acid sequence alignment of IscR and MarA showed 20% identical residues and 47% similar residues. Solid lines indicate the two helix-turn-helix DNA-binding domains of MarA.

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expressed in *Escherichia coli*, and the protein was then finally purified by one-step affinity chromatography to homogeneity. Optical and EPR spectra results confirmed that an iron-sulfur cluster was correctly inserted into the active site of the protein. Additionally, the $[Fe_2S_2]$ cluster assembly in IscR *in vivo* was found to require scaffold proteins and may follow the Isc "AUS" pathway.

MATERIALS AND METHODS

Materials

The *Acidithiobacillus ferrooxidans* ATCC 23270 was obtained from the American Type Culture Collection. The HiTrap chelating metal affinity column was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). The TOP10 competent cells and *E. coli* strain BL21(DE3) competent cells came from Invitrogen Life Technologies (Carlsbad, U.S.A.). The Plasmid Mini kit, gel extraction kit, and synthesized oligonucleotides were all obtained from the Sangon Company (Shanghai, China). The *Taq* DNA polymerase, T4 DNA ligase, and restriction enzymes came from MBI Fermentas (Burlington, Canada). All the other reagents were of research grade and obtained from commercial sources.

Cloning of *iscR* Gene from *A. ferrooxidans* ATCC 23270

The genomic DNA from *A. ferrooxidans* ATCC 23270 was prepared using an EZ-10 spin column genomic DNA isolation kit from Bio Basic Inc., according to the manufacturer's instructions for bacterial DNA extraction. This genomic DNA was then used as the template for a PCR reaction. The gene was amplified by a PCR using primers designed to add six continuous histidine codons to the 5' primer. The sequence of the forward primer was 5'-CGCGCG-AATTCAGGAGGAATTTAAAATGAGAGGATCGCATCACCATC ACCATCACAAACTGACGACAAAAGGACGCTACGCGGTGAC CGCC-3', containing an EcoRI site (GAATTC), ribosome binding site (AGGAGGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 2-12 in the mature IscA. The sequence of the reverse primer was 5'-CTGCAGGTCGACTTAAGCGGTGTCATGCGTCTCGCGCAGTG GTGT-3', containing a SalI site (GTCGAC), stop anticodon (TTA), and anticodons for the last eight amino acids in the mature Iro protein. The PCR amplification was performed using *Taq* DNA polymerase, and the samples were subjected to 25 cycles of 45 sec of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of elongation at 72°C in a Mastercycler Personal of Eppendorf Model made in Germany. The amplification products were analyzed by electrophoresis on a 0.9% agarose gel and stained with ethidium bromide. The resulting PCR product was gel purified, double digested, and ligated into a pLM1 expression vector, resulting in the pLM1::ISCR plasmid [19]. The constructed pLM1::ISCR plasmid was then transformed into TOP10 competent cells for screening purposes. A positive colony with a gene insert in the plasmid was identified by single restriction digestion of the plasmid with EcoRI and double restriction digestion of the plasmid with EcoRI and SalI, followed by an agarose gel analysis. The identified positive colony was grown in an LB medium containing ampicillin (50 mg/l), and the plasmid pLM1::ISCR isolated from harvested bacteria cells using a plasmid extraction kit. The isolated pLM1::ISCR plasmid was then transformed into *E. coli* strain BL21(DE3) competent cells for expression purposes. DNA sequencing of the cloned IscR protein gene was performed and the inserted gene sequence identified as previously reported without any mutation.

Expression of Recombinant Protein of IscR from *A. ferrooxidans*

The following procedure was used for the expression of a soluble IscR protein. The *E. coli* strain BL21(DE3) cells with the pLM1::ISCR 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. At this point, the cells were incubated overnight at room temperature with the addition of 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) with shaking at 180 rpm. Thereafter, the cells were harvested by centrifugation and the cell pellet was washed with an equal volume of sterile water. The cells were then reharvested by centrifugation, suspended in a start buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl), incubated with 5 mg of lysozyme at room temperature for 0.5 h, and stored at –80°C for purification.

Purification of IscR from *A. ferrooxidans*

The cells were lysed by sonication four times for 30 sec each time using a 150-Watt Autotune Series High Intensity Ultrasonic sonicator equipped with an 8-mm-diameter tip. The insoluble debris was removed by centrifugation and the clear supernatant used for the protein purification. The Hi-Trap column was first equilibrated with 0.1 M nickel sulfate to charge the column with nickel ions, followed by 5 column volumes of MiliQ water to remove any unbound nickel ions from the column, and then 5 column volumes of a start buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl) to equilibrate the column. The clarified sample was applied to the Hi-Trap column after being filtering through a 0.45-μm filter. Next, the column was washed with 5 column volumes of the start buffer, followed by 5 column volumes of a wash buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, 50 mM imidazole), and then the protein was eluted with an elution buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl, 500 mM imidazole). The Bradford method was used to determine the protein content with bovine serum albumin as the standard [3]. The eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% of acrylamide according to Laemmli [9]. The gels were stained with Coomassie Brilliant Blue R-250. The purified enzyme fractions were combined and dialyzed against a 20 mM potassium phosphate buffer, pH 7.4, 5% glycerol, and 5 mM β-mercaptoethanol, and then stored in a freezer at –80°C.

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

The UV-visible spectra scanning of the IscR was carried out at 25°C on a Techcomp UV-2300 spectrophotometer. The protein sample (20 μM) was prepared in a 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4. The X-band EPR spectra were recorded at 100 K on 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, and 4 mW incident microwave power, with a sweep time of 2 min. The samples were diluted to 5 μM in a 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4. Reduced IscR samples were then obtained by incubating with 5 mM sodium dithionite for 30 min.

Determination of Iron and Sulfur Contents of IscR
The iron assay was performed using the colorimetric method [10], and the sulfide content in the purified IscR was determined according to Siegel [18]. The samples were prepared in a 20 mM phosphate buffer containing 0.5 M NaCl, pH 7.4.

Preparation of apoIscR
ApoIscR was prepared by boiling purified holoIscR 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 [Fe_2S_2] cluster ligation were reduced to free sulfhydryl groups. After boiling for 30 sec, apoIscR 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.

Results and Discussion

Cloning of *iscR* Gene from *A. ferrooxidans* ATCC 23270
The PCR technique successfully added six continuous histidine residues to the *N*-terminal of the IscR from *A. ferrooxidans* ATCC 23270, which greatly accelerated the protein purification process. The initial transformation was carried out with Top10 competent cells for screening purposes, as the transformation efficiency with Top10 competent cells is very high. The identified positive colony was grown in an LB medium and the corresponding plasmid isolated and re-transformed into *E. coli* BL21(DE3).

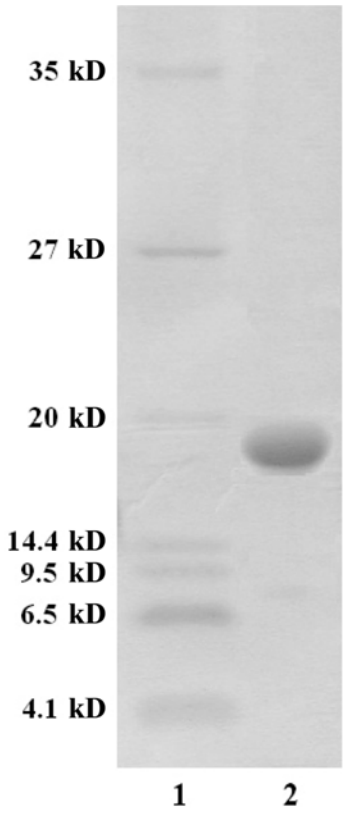

Fig. 2. Coomassie blue-stained SDS-PAGE of purified IscR from *A. ferrooxidans* ATCC 23270.
Lane 1, molecular mass standards; lane 2, purified IscR.

Expression and Purification of IscR from *A. ferrooxidans*
The expression of the IscR protein from *A. ferrooxidans* ATCC 23270 in *E. coli* BL21(DE3) was carried out at different temperatures and IPTG concentrations, and proteins were obtained under all the tested conditions. The protein expression was better with a 0.5 mM IPTG concentration, and a more soluble protein was produced at room temperature (25°C) than at 37°C.

A nickel metal-affinity resin column was used for the single-step purification of the His-tagged IscR protein. The final protein yield after affinity chromatography was 13.2%, and the result also suggested that the T7 polymerase promoter/BL21(DE3) expression system was an ideal system for expressing a high yield of IscR, where the leader peptide and hexahistag in the *N*-terminal may have been responsible for the efficient protein expression. The protein fractions were dialyzed against a 20 mM potassium phosphate buffer, pH 7.4, 5% glycerol, and 5 mM β-mercaptoethanol as soon as possible after the purification.

The purity of the enzymes was further examined by SDS-PAGE, and a single band corresponding to a 18 kDa protein was observed with >95% purity (Fig. 2). The stability of the purified IscR was tested on the basis of its [Fe_2S_2] cluster stabilization, and the His-tagged protein proved to be highly stable under anaerobic conditions. The protein could be stored at 4°C for one month without any significant change in activity.

UV Scanning and EPR Spectra of IscR from *A. ferrooxidans*
The UV-visible spectra of the recombinant IscR, shown in Fig. 3A, revealed two major absorption peaks between 300 and 500 nm, corresponding to 315 nm and 410 nm, respectively, which is typical for proteins containing an [Fe_2S_2] cluster. An [Fe_2S_2] cluster containing the IscA, IscU, and ferredoxin proteins also showed similar spectra [1, 22, 23].

The EPR spectra of the recombinant IscR in a reduced state, shown in Fig. 3B, exhibited a typical S= 1/2 EPR signal, indicating the presence of an $[Fe_2S_2]^{1+}$ cluster. The results further indicated that the [Fe_2S_2] cluster was successfully incorporated into the IscR. The spectra was also similar to that previously reported for the IscR from *E. coli* [17] and other iron-sulfur proteins [1, 22, 23]. The recombinant IscR from *A. ferrooxidans* was successfully produced in *E. coli* with a correctly incorporated [Fe_2S_2] cluster inserted in the cytoplasm. However, previous reports have noted that the cofactor insertion for recombinant

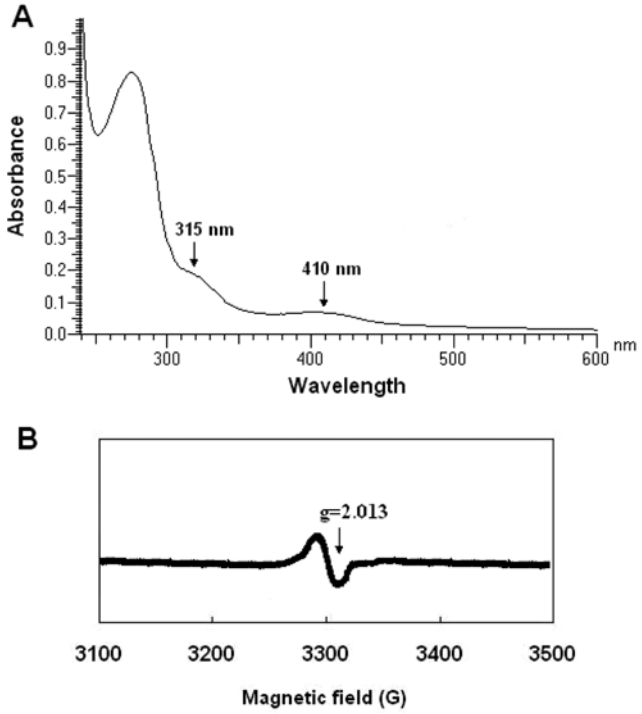

Fig. 3. A. UV-vis scanning of recombinant IscR from *A. ferrooxidans* ATCC 23270; **B**. EPR spectra of recombinant IscR from *A. ferrooxidans* ATCC 23270.

rusticyanin, cytochrome *c*, and cytochrome *c* oxidase was always in the periplasm, meaning the cofactors were not incorporated *in vivo* and required reconstitution *in vitro* [4].

$[Fe_2S_2]$ Cluster in IscR was Sensitive to Molecular Oxygen

The purified IscR was found to be sensitive to molecular oxygen, as its $[Fe_2S_2]$ cluster decomposed quickly when exposed to air, as shown in Fig. 4, plus the A_{415} of the protein sample decreased rapidly under aerobic conditions, indicating the loss of the iron-sulfur cluster. The protein sample also deposited quickly without the $[Fe_2S_2]$ cluster in the IscR, meaning the $[Fe_2S_2]$ cluster was crucial for the stability of the protein. Under anaerobic conditions with argon, the $[Fe_2S_2]$ cluster in the protein remained stable, allowing storage at 4°C for one month without any significant change in activity. The IscR from *E. coli* was also reported to show similar results [17].

$[Fe_2S_2]$ Cluster Assembly in IscR *In Vivo* Required Scaffold Proteins

The purified apoIscR (20 μM) was incubated anaerobically at 25°C with 5-fold molar excess of both Na_2S and $Fe(NH_4)_2(SO_4)_2$ for 3 h in the presence of 5 mM dithiothreitol in 0.1 M Tris-HCl, pH 8.0, and then desalted on a Sephadex G-25 column, resulting in a colorless protein. The UV-visible spectra of the apoIscR treated with ferrous ion and sulfide, as shown in Fig. 5, exhibited no significant absorption between 350 and 500 nm, indicating the absence of an iron-sulfur cluster. Therefore, these results suggest that the $[Fe_2S_2]$ cluster could not be incorporated into the apoIscR *in vitro* in the presence of ferrous ion and sulfide, implying that the iron-sulfur cluster assembly *in vivo* is not a spontaneous process, but requires scaffold proteins.

Therefore, this raises the question of how the recombinant IscR from *A. ferrooxidans* was produced in *E. coli* with a correctly incorporated $[Fe_2S_2]$ cluster. Recent studies have revealed that an *iscSUA-hscBA-fdx* gene cluster is essential for the general biogenesis of iron-sulfur proteins in bacteria, with the three key proteins of IscA, IscS, and IscU involved in the assembly of an iron-sulfur cluster [20, 21, 26]. Thus, experiments were conducted to determine whether the $[Fe_2S_2]$ cluster assembly in IscR also followed this mechanism.

As the cysteine desulfurase, IscS, plays a major role in Fe-S cluster formation in *E. coli*, the absence of this

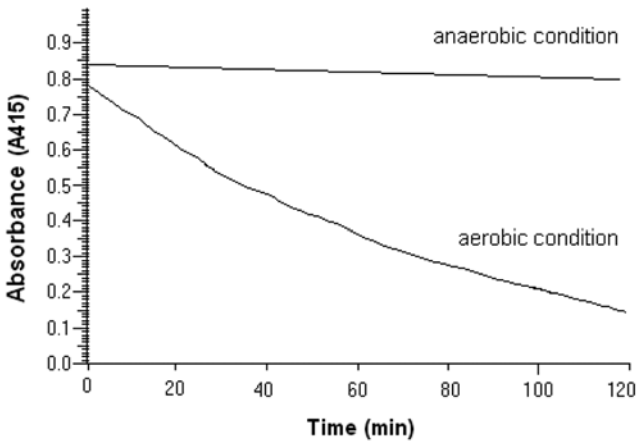

Fig. 4. Time scanning at 415 nm for IscR under areobic and anaerobic conditions.

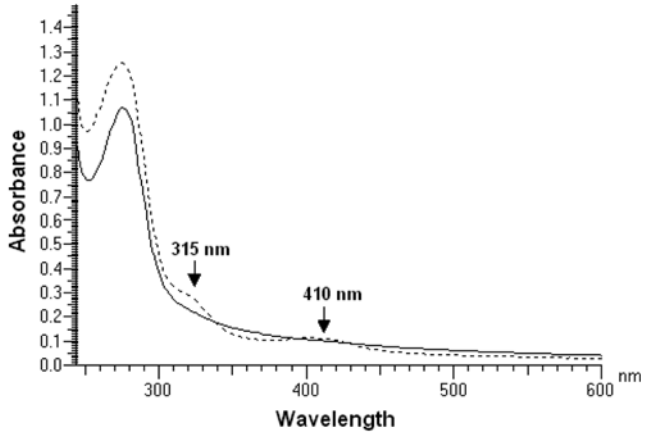

Fig. 5. UV-vis scanning of holoIscR and apoIscR treated with ferrous ion and sulfide.
Dashed line indicates native holoIscR; solid line indicates apoIscR.

protein *in vivo* significantly affects the iron-sulfur cluster assembly in *E. coli* [16, 26]. In a previous study, L-allylglycine was identified as a membrane-permeable suicide inhibitor of IscS that forms a covalent adduct with the active site cysteine of IscS in *E. coli*, resulting in enzyme inactivation [25]. Therefore, the present study investigated the effects of L-allylglycine on the iron-sulfur cluster assembly in IscR in *E. coli*. The IscR was expressed with the addition of 50 mM L-allylglycine in the culture, and then purified as described in Materials and Methods. The UV scanning and EPR results of the purified IscR indicated no iron-sulfur cluster assembly in the *E. coli* with the presence of 50 mM L-allylglycine in the culture, as shown in Figs. 6A and 6B. No iron-sulfur cluster was incorporated into the protein. Thus, the $[Fe_2S_2]$ cluster assembly in IscR clearly did require scaffold proteins, as the inactivation of IscS seriously affected the iron-sulfur cluster assembly in *E. coli*. Consequently, the *in vivo* assembly of an $[Fe_2S_2]$ cluster in IscR in *E. coli* appeared to follow the Isc "AUS" pathway, involving the three proteins of IscS (a cysteine desulfurase), IscU (a scaffold protein), and IscA (an iron chaperon) encoded by the operon *iscSUA*.

The proposed "AUS" model for iron-sulfur cluster assembly in the IscR in *A. ferrooxidans* is shown in Fig. 7, as first proposed by Ding's group [24]. The IscA first binds intracellular ferrous iron to form an iron-loaded IscA, which subsequently binds an L-cysteine and forms a transient

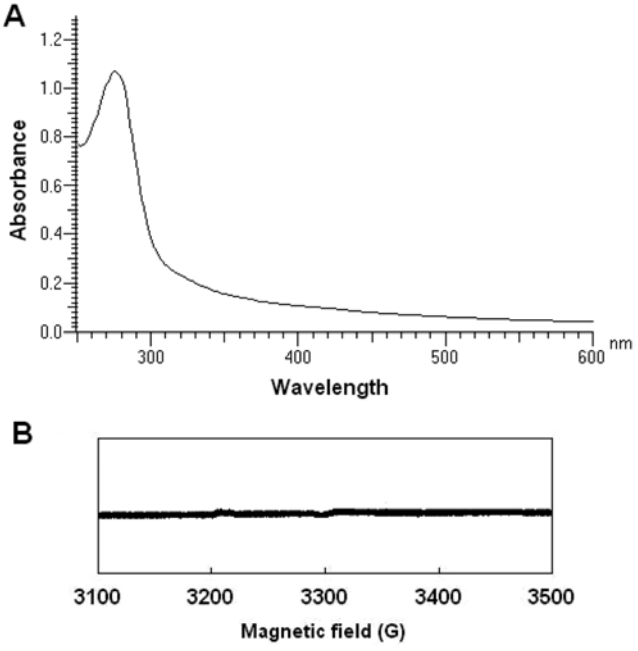

Fig. 6. Assembly of the $[Fe_2S_2]$ cluster in IscR *in vivo* required scaffold proteins.
A. UV-vis scanning of IscR produced with addition of 50 mM L-allylglycine in culture; **B**. EPR spectra of IscR produced with addition of 50 mM L-allylglycine in culture.

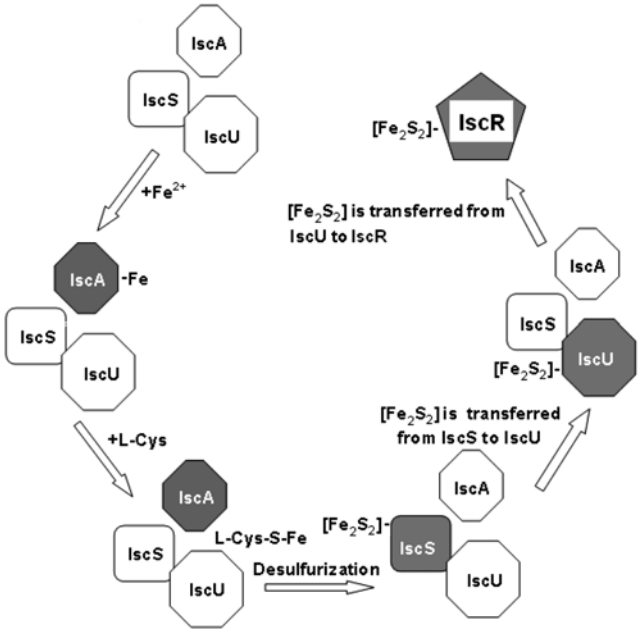

Fig. 7. Proposed mechanism for *in vivo* assembly of the iron-sulfur cluster in IscR.

L-cysteine-Fe complex. The IscS then catalyzes desulfurization of the L-cysteine-Fe complex and transfers the Fe-S to the IscU for $[Fe_2S_2]$ assembly in the IscU *via* a protein-protein interaction. The assembled $[Fe_2S_2]$ cluster in the IscU is then transferred to the IscR to form an active holoprotein.

In summary, this is the first report of the expression of an His-tagged IscR from *A. ferrooxidans* in *E. coli*. The properties of the recombinant protein all support the successful assembly of an iron-sulfur cluster in this protein in *E. coli*. The $[Fe_2S_2]$ cluster assembly in IscR *in vivo* was shown to require scaffold proteins, which may follow the Isc "AUS" pathway. Therefore, the successful expression of the IscR from *A. ferrooxidans* will greatly facilitate further investigation of this protein, including protein-DNA interaction and crystal structure studies.

Acknowledgments

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