Identification of an Intermediate Form of Ferredoxin That Binds Only Iron Suggests That Conversion to Holo-Ferredoxin Is Independent of the ISC System in *Escherichia coli*

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ABSTRACT *Escherichia coli* [2Fe-2S]-ferredoxin and other ISC proteins encoded by the *iscRSUA-hscBA-fdx-iscX* (*isc*) operon are responsible for the assembly of iron-sulfur clusters. It is proposed that ferredoxin (Fdx) donates electrons from its reduced [2Fe-2S] center to iron-sulfur cluster biogenesis reactions. However, the underlying mechanisms of the [2Fe-2S] cluster assembly in Fdx remain elusive. Here, we report that Fdx preferentially binds iron, but not the [2Fe-2S] cluster, under cold stress conditions ($\leq 16^{\circ}\text{C}$). The iron binding in Fdx is characterized by a unique absorption peak at 320 nm based on UV-visible spectroscopy. In addition, the iron-binding form of Fdx could be converted to the [2Fe-2S] cluster-bound form after transferring cold-stressed cells to normal cultivation temperatures above 25°C . *In vitro* experiments also revealed that Fdx could utilize bound iron to assemble the [2Fe-2S] cluster by itself. Furthermore, inactivation of the genes encoding *IscS*, *IscU*, and *IscA* did not limit [2Fe-2S] cluster assembly in Fdx, which was also observed by inactivating the *isc* or *suf* operon, indicating that iron-sulfur cluster biogenesis in Fdx arose from a unique pathway in *E. coli*. Our results suggest that the intracellular assembly of [2Fe-2S] clusters in Fdx is susceptible to environmental temperatures. The iron binding form of Fdx (Fe-Fdx) is a precursor during its maturation to a cluster binding form ([2Fe-2S]-Fdx), and reassembly of the [2Fe-2S] clusters during temperature increases is not strictly reliant on other specific iron donors and scaffold proteins within the *Isc* or *Suf* system.

IMPORTANCE Fdx is an electron carrier that is required for the maturation of many other iron-sulfur proteins. Its function strictly depends on its [2Fe-2S] center that bonds with the cysteinyl S atoms of four cysteine residues within Fdx. However, the assembly mechanism of the [2Fe-2S] clusters in Fdx remains controversial. This study reports that Fdx fails to form its [2Fe-2S] cluster under cold stress conditions but instead binds a single Fe atom at the cluster binding site. Moreover, when temperatures increase, Fdx can assemble clusters by itself from its iron-only binding form in *E. coli* cells. The possibility remains that Fdx can effectively accept clusters from multiple sources. Nevertheless, our results suggest that Fdx has a strong iron binding activity that contributes to the assembly of its own [2Fe-2S] cluster and that Fdx acts as a temperature sensor to regulate *Isc* system-mediated iron-sulfur cluster biogenesis.

KEYWORDS ferredoxin, iron-sulfur cluster, ISC machinery, iron, iron binding activity

Iron-sulfur clusters are small, inorganic prosthetic groups that serve as essential cofactors for iron-sulfur proteins that participate in a variety of biochemical processes, including transcriptional regulation, DNA/RNA synthesis and repair, electron transport,

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iron and heme metabolism, and oxygen sensing (1, 2). Despite the simple compositions and structures of iron-sulfur clusters, their biogenesis in most prokaryotes is believed to require complex multiprotein iron-sulfur cluster (ISC) assembly proteins encoded by the *isc* operon, *iscRSUA-hscBA-fdx-IsC* (3). However, molecular-level studies of the ISC system proteins are needed to further understand the details of iron-sulfur cluster assembly. Eight proteins exist in the *Escherichia coli* ISC system, including a pyridoxal phosphate-dependent cysteine desulfurase (IsC) that produces sulfide from cysteine; an iron-sulfur cluster assembly scaffold, IscU; an iron donor or iron-sulfur cluster carrier protein, IscA; an ATP-dependent cochaperone system, HscB/HscA; a [2Fe-2S] type ferredoxin (Fdx); a possible iron donor, IscX; and a transcriptional regulator, IscR (4–9). Likewise, higher eukaryotes have evolved orthologues of iron-sulfur cluster assembly proteins for iron-sulfur cluster biosynthesis within mitochondria (10). While extensive biochemical and genetic studies have elucidated well-defined roles for the core proteins IscS, IscU, and IscA, such roles have not been determined for the HscB, HscA, Fdx, and IscX proteins. The ISC-specific HscA/HscB cochaperones are believed to facilitate the assembly of clusters on IscU or the transfer of clusters from IscU to acceptor proteins (11, 12). IscX (also referred to as ORF3 or YfhJ) is a small acidic protein encoded by the last gene in the *isc* operon and is structurally capable of binding to iron (13). Furthermore, *in vitro* studies have shown that IscX acts as a regulator of iron-sulfur cluster assembly by forming different complexes with IscU and/or IscS (7, 14).

Genetic knockout of Fdx has also led to its identification as a central component in the iron-sulfur cluster biosynthesis pathway via observed disruption of iron-sulfur cluster assembly (15). Furthermore, Fdx is considered an electron transfer protein due to its importance for ISC-mediated iron-sulfur cluster biogenesis and the redox properties of its [2Fe-2S] cluster. Fdx, together with ferredoxin reductase, directly mediates electron transfer between intermediates such as NADPH and the iron-sulfur cluster assembly proteins IscU and/or IscS. These observations have led to the proposal of an electron transfer model from NADPH/ferredoxin reductase to oxidized Fdx (16). Recent studies have shown that Fdx binds directly to cysteine desulfurase, IscS, and supplies electrons to reduce S^0 to S^{2-} for iron-sulfur cluster assembly (17, 18). Furthermore, Fdx is involved in the reductive coupling of two $[2Fe-2S]^{2+}$ clusters on IscU to form a single $[4Fe-4S]^{2+}$ cluster (19, 20). Investigations of *E. coli* Fdx crystal structure have also revealed that the amino acid sequences of Fdx are rich in conserved cysteine and histidine residues. Specifically, four cysteine residues (C42, C48, C51, and C87) coordinate the [2Fe-2S] cluster, wherein the cysteinyl S atoms of Cys42 and Cys48 bind the iron atom that is close to the molecular surface, while those of Cys51 and Cys87 bind the other iron atom buried in the molecule. Tertiary structure analysis of Fdx also has shown that His40, Cys46, and His52 are located close to the [2Fe-2S] cluster. Among these, Cys46 is noteworthy because of its reactive sulfhydryl group on the molecular surface, leading to speculated roles in the binding and reduction of S^0 and/or Fe^{3+} . Moreover, all of those conserved cysteine and histidine residues have been suspected of binding additional iron and/or sulfur atoms (6).

Multiple [2Fe-2S] Fdx isoforms are present in plants and algae and are involved in electron transfer in chloroplast photosynthesis (21). Mitochondria from vertebrates also contain a [2Fe-2S] Fdx homolog, termed adrenodoxin, that transfers electrons from NADPH-dependent reductase to cytochrome P450 to synthesize steroid hormones (22). In addition, the yeast Fdx, Yah1p, has also been demonstrated to be an electron transfer protein involved in mitochondrial iron-sulfur cluster biosynthesis (19, 23). Functionally disruptive mutations in *FDX1L*, which encodes FDX2, are associated with a human mitochondrial muscle myopathy, consistent with iron-sulfur defects (24). Despite the fact that many studies have explored the physiological role of Fdx, the detailed biophysical characterization of Fdx is still lacking.

Numerous *in vitro* iron-sulfur cluster transfer studies (25–27) have used Fdx as the gold standard cluster recipient protein to receive iron-sulfur clusters. However, [2Fe-2S] cluster-containing Fdx appears to be involved in its own maturation *in vivo*, raising

an interesting question regarding the actual mechanism of iron-sulfur cluster assembly in Fdx. In the present study, we show that Fdx has a strong and specific iron binding activity in *E. coli* cells grown aerobically under cold stress conditions and *in vitro*. Purified Fdx from cold-stressed cells displayed a unique absorption peak at 320 nm, as shown by UV-visible spectroscopy, that is indicative of iron binding in the protein. The iron center in Fdx can be used as an iron source to assemble [2Fe-2S] clusters in proteins *in vitro*. Moreover, the directed mutational analysis demonstrated that the deletion of *IscS*, *IscU*, and *IscA* did not significantly affect [2Fe-2S] cluster assembly in Fdx. Furthermore, even the knocking out of *isc* or *suf* operons did not block cluster assembly in Fdx, suggesting that Fdx has a distinct pathway to obtain a [2Fe-2S] cluster. In addition, [2Fe-2S] cluster assembly in Fdx is extremely inhibited in *E. coli* cells under aerobic, cold stress conditions at 15°C but can be quickly restored upon temperature increases. The intact [2Fe-2S]-ferrodoxin is required for ISC-mediated iron-sulfur biogenesis. Consequently, these observations also introduce the possibility that Fdx plays a role in the regulation of iron-sulfur cluster biogenesis when *E. coli* cells are exposed to cold stress.

RESULTS

Fdx preferentially binds iron but not [2Fe-2S] clusters when expressed at low temperatures. We previously showed that the assembly of the [2Fe-2S] cluster in *E. coli* Fdx is independent of the physiological function of the proposed iron chaperones *IscA*/*SufA* (28). Furthermore, Fdx is not only an iron-sulfur assembly protein but also a [2Fe-2S] protein. Consequently, the assembly of Fdx clusters is theoretically different from that of other iron-sulfur proteins. To test this hypothesis and explore whether Fdx can bind iron *in vivo*, we constructed the pCold expression vector (suitable for low-temperature and normal-temperature expression) of the Fdx protein and then expressed the recombinant Fdx in *E. coli* BL21 cells grown aerobically in LB medium at different temperatures. Fdx purified from *E. coli* cells grown at 37°C had two typical absorption peaks at 415 nm and 459 nm due to the [2Fe-2S] cluster (Fig. 1A), while Fdx purified from *E. coli* cells grown at 15°C had inconspicuous absorption peaks at 415 nm and 459 nm but exhibited a new prominent absorption peak at 320 nm. Analysis of acid-labile iron and sulfide contents further revealed that Fdx purified from *E. coli* cells grown at 37°C contained approximately 1.02 ± 0.13 mol iron and 0.64 ± 0.05 mol sulfide per mol protein ($n=3$), indicating that, on average, approximately 50% of purified Fdx contained an intact [2Fe-2S] cluster. In contrast, Fdx purified from *E. coli* cells grown at 15°C contained approximately 0.19 ± 0.03 mol iron per mol protein and very small amounts of acid-labile sulfide (less than 0.06 mol sulfide per mol protein). The data indicated that the [2Fe-2S] cluster bound in Fdx that was purified from *E. coli* cells grown at 15°C almost disappeared, while the iron-binding form of Fdx appeared under these conditions.

To test the effect of temperature on iron-sulfur cluster or iron binding in Fdx, cells were grown at several temperatures between 15°C and 37°C. The iron-sulfur cluster binding peaks at 415 nm and 459 nm from Fdx remained unchanged between 25°C and 37°C; however, the peaks exhibited a linear decrease with temperature, decreasing between 25°C and 18°C, along with a slight decrease between 18°C and 15°C. Thus, lower temperatures (especially between 15°C and 25°C) led to a gradual decrease in the iron-sulfur cluster binding of Fdx until it disappeared, ultimately retaining the role of iron binding in the protein.

Recombinant Fdx expressed from *E. coli* cells grown at low temperature in LB medium clearly exhibited iron binding activity (Fig. 1A). However, the iron binding peak at 320 nm for Fdx was not very obvious under these conditions. To exclude the possibility that other metal ions were present in the LB medium that might attenuate the absorption peak at 320 nm, we also analyzed the iron binding of Fdx purified from *E. coli* cells grown in M9 minimal medium supplemented with exogenous iron (8 μM). While Fdx purified from *E. coli* cells grown in M9 medium at 37°C had distinct [2Fe-2S] cluster binding peaks at 415 nm and 459 nm, Fdx purified from *E. coli* cells grown in M9

FIG 1 Fdx exhibited iron binding activity by decreasing [2Fe-2S] cluster binding when expressed in *E. coli* cells at low temperature. (A) UV-visible absorption spectra of recombinant Fdx purified from *E. coli* cells grown in LB medium under a series of gradually decreasing temperatures (from 37°C to 15°C). The absorption peaks at 415 nm and 459 nm indicate [2Fe-2S] binding in Fdx. The absorption peak at 320 nm indicates iron binding in Fdx. (B) The amplitude of the [2Fe-2S] binding peaks at 415 nm and 459 nm of purified Fdx in panel A was plotted as a function of the gradually decreasing temperature. (C) UV-visible absorption spectra of recombinant Fdx purified from *E. coli* cells grown in M9 minimal medium supplemented with amino acids, thiamine, and 8 μM ferric citrate at 37°C and 15°C. The insets in panels A and C show photographs of the SDS-PAGE gel of purified Fdx proteins. Similar results were obtained from three independent experiments.

medium at 15°C appeared to lose the [2Fe-2S] cluster binding peaks but gained a more obvious iron-binding peak at 320 nm (Fig. 1C). This indicated that Fdx exhibited iron-binding ability when produced at low temperature. Altogether, these results show that [2Fe-2S] cluster assembly in Fdx is affected by environmental temperatures, and Fdx preferentially binds iron when expressed under cold stress.

The relative iron binding activity of Fdx *in vivo* and *in vitro*. To further elaborate the *in vivo* iron binding specificity of Fdx, Fdx was expressed in *E. coli* cells grown at low temperature (15°C) in M9 minimal medium supplemented with increasing concentrations of exogenous ferric citrate under aerobic growth conditions. The [2Fe-2S] cluster binding peaks (415 nm and 459 nm) in Fdx showed little discernible change under various ferric citrate concentrations (Fig. 2), while the iron binding peak (320 nm) of Fdx increased almost linearly as the exogenous iron concentration in the M9 minimal medium was increased from 0 to 1.0 μM, and this effect appeared to saturate above 1.0 μM ferric citrate (Fig. 2B).

To further determine if Fdx can bind iron *in vitro*, apo-Fdx was prepared as described in Materials and Methods. Apo-Fdx exhibited little absorption at 320 nm and contained no detectable iron. Increasing concentrations of ferrous iron were incubated with a fixed concentration of apo-Fdx in the presence of dithiothreitol (DTT) in microtubes at room temperature overnight, followed by passing the samples through a desalting column. Incubation of apo-Fdx with ferrous iron restored the protein

FIG 2 Relative iron binding activity of Fdx in *E. coli* cells and *in vitro*. (A) UV-visible absorption spectra of recombinant Fdx purified from *E. coli* cells grown aerobically at 15°C in M9 minimal medium supplemented with ferric citrate (0.0, 0.1, 0.5, 1.0, and 2.0 μM). The protein concentration of purified Fdx was approximately 40 μM. The inset shows a photograph of the SDS-PAGE gel of Fdx proteins purified from the *E. coli* cells grown in M9 minimal medium supplemented with 0.0 to 2.0 μM ferric citrate. (B) The apparent iron binding activity of Fdx in *E. coli* cells. The amplitude of the absorption peaks at 320 nm, 415 nm, and 459 nm of purified Fdx in panel A was plotted as a function of the exogenous iron concentration supplemented in the M9 minimal medium. (C) UV-visible absorption spectra of apo-Fdx after reconstitution with iron. Apo-Fdx (25 μM) was incubated with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0 to 15 μM) in the presence of dithiothreitol (2 mM) in microtubes at room temperature overnight. Protein was repurified by passing through a HiTrap desalting column. (D) Iron binding curve of Fdx. The amplitudes of the absorption peak at 320 nm of apo-Fdx were obtained from the spectra in panel C and plotted as a function of the ferrous iron concentration in the incubation solution. Similar results were obtained from three independent experiments.

absorption peak at 320 nm (Fig. 2C). As iron concentrations increased, the amplitude of the Fdx absorption peak at 320 nm increased almost linearly until the molar ratio of iron to Fdx was ~ 0.4 (Fig. 2D). The relative amplitude of the absorption peak at 320 nm of iron-loaded Fdx (Fig. 2C) was much higher than those observed for purified Fdx (Fig. 2A), suggesting that purified Fdx was not fully saturated with iron. It is possible that the intracellular iron content did not meet the requirements of overproduced recombinant Fdx in *E. coli* cells (29). Electron paramagnetic resonance (EPR) measurements further indicated that the iron-bound Fdx displayed a signal at $g = 4.3$ (Fig. 3A), reflecting a ferric iron center in the protein. Furthermore, the addition of sodium dithionite almost eliminated the EPR signal (Fig. 3B), confirming that the iron center of Fdx can be reduced with dithionite.

To further explore the properties of iron-bound Fdx, purified Fdx proteins from *E. coli* cells were analyzed using an anion exchange Mono Q column to separate proteins based on their net charges and protein conformations. Iron or iron-sulfur cluster binding of Fdx shifted the protein elution profile from fraction 23 (apo-Fdx) to fractions 25 and 35 (iron-bound Fdx) and fraction 30 (iron-sulfur-cluster-bound Fdx) (Fig. 4), indicating that iron binding significantly changed the conformation of Fdx. Nevertheless, further experiments are needed to evaluate the differences in protein conformation

FIG 3 EPR spectra of Fdx. (A) Iron-loaded Fdx ($500\ \mu\text{M}$) was dissolved in protein buffer containing 20 mM Tris (pH 8.0) and 0.5 M NaCl. (B) The same sample as that in panel A, except that freshly prepared sodium dithionite (2 mM) was added. (C) Apo-Fdx ($500\ \mu\text{M}$). The spectra shown were the net spectra obtained by subtracting the baseline (containing buffer only). Three sets of independently prepared samples were measured by EPR, and similar spectra were obtained.

or net charges of iron-bound Fdx from fractions 25 and 35. One explanation for these shifts is that Fdx exhibits two iron binding states due to different binding sites. The protein separation experiments also showed that the purified protein mixture mainly containing iron-bound Fdx from cold-stressed *E. coli* cells still contained small amounts of apo-Fdx and iron-sulfur cluster binding forms. Altogether, these data demonstrate that Fdx has strong iron binding activity *in vivo* and *in vitro*, and an absorption peak at 320 nm was a characteristic of iron-bound Fdx.

Iron-bound Fdx can be converted to the iron-sulfur cluster-bound form in *E. coli* cells and *in vitro*. Given that Fdx exhibited iron binding activity *in vivo* and *in vitro*, a subsequent question was whether the [2Fe-2S] cluster could be assembled in Fdx by precombining the iron source with Fdx. To answer this question, iron-bound Fdx was first induced in *E. coli* cells at low temperature, followed by transfer of cells to 37°C for further incubation after the addition of chloramphenicol to inhibit new protein synthesis. Fdx purified from *E. coli* cells grown at 15°C still had very minimal [2Fe-2S] cluster binding peaks at 415 nm and 459 nm but a prominent iron binding peak at 320 nm. In contrast, Fdx purified from *E. coli* cells grown at 15°C first and then transferred to 37°C exhibited two obvious [2Fe-2S] cluster binding peaks at 415 nm and 459 nm (Fig. 5A). Thus, the iron-bound Fdx can be converted into iron-sulfur cluster binding forms in *E. coli* cells. However, it remained unclear if Fdx could assemble a cluster by itself during the shift from cold to warmer temperatures, because the other ISC components were also present under these conditions and may compensate for iron-sulfur cluster assembly under increasing temperatures.

To further analyze whether iron-bound Fdx could transform into the iron-sulfur cluster binding form *in vitro*, the iron binding form of Fdx was first obtained by Mono Q separation (Fig. 4D). The iron-bound Fdx was incubated with cysteine desulfurase IscS and L-cysteine in the presence of dithiothreitol at 37°C for 28 min. Following incubation, two [2Fe-2S] cluster binding peaks from Fdx at 415 nm and 459 nm also appeared, indicating the formation of [2Fe-2S] clusters in Fdx (Fig. 5B). Furthermore, adding additional free Fe^{2+} into the reaction buffer under the same conditions led to quicker assembly of the [2Fe-2S] cluster in Fdx (see Fig. S1 in the supplemental

FIG 4 Separation of the iron-binding form of Fdx from a purified recombinant protein expressed in *E. coli* cells. The recombinant Fdx was purified from *E. coli* cells grown in M9 minimal medium supplemented with 1 μ M ferric citrate at 15°C. The purified Fdx was loaded onto a Mono Q column preequilibrated with buffer containing 20 mM Tris (pH 8.0). Different forms of Fdx were isolated, as described in Materials and Methods. (A) The elution profile of Fdx. Inset, SDS-PAGE gel analysis of the eluted fractions. (B) UV-visible absorption spectra of Fdx before Mono Q isolation. The UV-visible absorption spectra of fractions 23, 25, 30, and 35 from panel A are shown in panels C, D, E, and F, respectively.

material), indicating that Fdx can use its bound iron or free Fe^{2+} to self-assemble its iron-sulfur center.

These results demonstrated that the intracellular assembly efficiency of the clusters in Fdx increases during upshifts in environmental temperatures and that the iron-binding form of Fdx could be converted to the iron-sulfur cluster-bound form in *E. coli* cells and *in vitro*.

The function of Fdx depends on its bound iron-sulfur cluster. The bound iron in Fdx can be used to assemble iron-sulfur clusters; thus, the binding sites of iron and iron-sulfur clusters may be the same so that Fdx can improve the assembly efficiency of iron-sulfur clusters. To test this hypothesis, an Fdx mutant (Fdx-4M) was generated in which the four cysteine residues responsible for binding the [2Fe-2S] cluster (Cys 42,

FIG 5 Iron-binding form of Fdx could be converted to the iron-sulfur cluster-bound form in *E. coli* cells and *in vitro*. (A) UV-visible absorption spectra of recombinant Fdx purified from *E. coli* cells grown aerobically in LB medium at 15°C first (spectrum 1) and then transferred to fresh LB medium added with 34 μ g/ml chloramphenicol and cultured at 37°C for continued growth (spectrum 2). (B) Reconstitution of the iron-sulfur cluster in the iron-binding form of Fdx isolated from the experiments described in Fig. 4 (fraction 25).

FIG 6 Iron and [2Fe-2S] clusters bound in Fdx were required for the biogenesis of the iron-sulfur cluster in *E. coli* cells. UV-visible absorption spectra of recombinant Fdx (spectrum 1) and Fdx-4M (spectrum 2) purified from *E. coli* cells grown at 37°C (A) and 15°C (B). The insets show photographs of SDS-PAGE gels of the purified protein. (C) Growth curve of wild-type *E. coli* MC4100-introduced pBAD empty vector (closed circles), *E. coli fdx* knockout mutant-introduced pBAD empty vector (closed squares), wild-type Fdx expression vector (hollow squares), and Fdx variant expression vector (Fdx-4M; four cysteine residues of bound [2Fe-2S] cluster were replaced by serine residues) (closed triangles) in LB medium containing 0.002% arabinose and 100 μ g/ml ampicillin under aerobic growth conditions. The results are representative of three experiments. (D) NADH dehydrogenase I activity of cells in panel C after being harvested and washed twice with buffer.

Cys 48, Cys 51, and Cys 87) were replaced with serine residues. Wild-type Fdx and Fdx-4M were then expressed in *E. coli* cells grown at 37°C and 15°C, followed by purification of each protein. The purified Fdx from *E. coli* cells grown at 37°C and 15°C could bind the [2Fe-2S] cluster (Fig. 6A) and iron (Fig. 6B). In contrast, the Fdx mutant (Fdx-4M) failed to bind any [2Fe-2S] cluster or iron in the *E. coli* cells grown at both temperatures. These results indicated that the [2Fe-2S] cluster and iron share the same binding site in the protein. Importantly, the mutation of each single conserved cysteine residue of Fdx resulted in a failure to bind [2Fe-2S] clusters but did not affect the iron binding activity. Nevertheless, mutating both Cys 48 and Cys 51 into serine residues significantly decreased the iron binding capacity of Fdx (Fig. S2). Thus, the iron-binding behavior is different from that of the iron-sulfur clusters. For iron-only binding, two or three of these cysteine residues are enough.

The *fdx* gene was then deleted from wild-type *E. coli* strain MC4100, followed by an analysis of mutant cell growth and iron-sulfur cluster biogenesis. The Fdx defect led to slow growth (Fig. 6C) and low activity of NADH dehydrogenase I (Fig. 6D) phenotypes in *E. coli* cells. Concomitantly, the [4Fe-4S] cluster assembly in endonuclease III (Nth) and [2Fe-2S] cluster assembly in SoxR were inhibited in the *fdx* mutant cells (data not shown). This indicated that Fdx was generally required for iron-sulfur cluster biosynthesis and cell growth in *E. coli* cells.

To further analyze the physiological role of iron and iron-sulfur clusters within Fdx with respect to cell growth and iron-sulfur cluster assembly in *E. coli*, the Fdx-4M construct from mutants that had lost the ability to bind iron and [2Fe-2S] clusters (Fig. 6A and B) was transformed into Fdx-deleted *E. coli* cells. Unlike wild-type Fdx, Fdx-4M failed to restore cell growth (Fig. 6C) and NADH dehydrogenase I activity (Fig. 6D) of Fdx-deleted cells. The activity of NADH dehydrogenase I is dependent on multiple

FIG 7 Kinetics of iron-sulfur cluster reassembly in Fdx and FumB in *E. coli* cells grown during the temperature transition from 15°C to 37°C. UV-visible absorption spectra of recombinant Fdx (A) and FumB (B) purified from *E. coli* cells grown aerobically in LB medium at 15°C first and then transferred to fresh LB medium supplemented with 34 μ g/ml chloramphenicol at 37°C for 0, 1, 2, 4, and 8 h. The purified recombinant proteins expressed at 37°C were used as a control sample (CTL; red line). (C) Comparison of the repair rates of the iron-sulfur cluster in Fdx (circles) and FumB (squares) during the temperature transition from 15°C to 37°C.

iron-sulfur clusters within the complex; thus, the activity of NADH dehydrogenase I indirectly reflects the level of iron-sulfur cluster biogenesis in *E. coli* cells. These results indicated that the function of Fdx was dependent on its bound [2Fe-2S] clusters. Given that iron and [2Fe-2S] clusters share the same binding site in Fdx and the bound iron could be reconstituted into the [2Fe-2S] cluster, the iron binding activity of Fdx is also required for the Fdx functions crucial for the iron-sulfur cluster biogenesis and cell growth in *E. coli*.

Fdx prioritized the restoration of its iron-sulfur cluster in *E. coli* cells after increased temperatures. Given that Fdx, including the iron and [2Fe-2S] cluster bound in the protein, is required for the biogenesis of iron-sulfur clusters in *E. coli* cells, we speculated that reassembly of the [2Fe-2S] cluster in Fdx was prioritized. To test this hypothesis, cold-stressed *E. coli* cells that expressed recombinant iron-sulfur proteins at 15°C were transferred to fresh LB medium supplemented with the protein synthesis inhibitor chloramphenicol and allowed to incubate at either 37°C (Fig. 7) or 25°C (Fig. S3). Obvious [2Fe-2S] cluster binding in preproduced Fdx in *E. coli* cells was not observed when grown at 15°C (Fig. 7A). However, the [2Fe-2S] cluster of Fdx was rapidly restored when the growth temperature for cells was shifted upwards to 37°C, during which the [2Fe-2S] cluster content accumulated linearly within 0 to 4 h, plateauing after 4 h (Fig. 7C). The maximum amount of [2Fe-2S] cluster binding in Fdx was slightly higher than that of the control sample that was purified from *E. coli* cells grown at 37°C. In contrast, while the [4Fe-4S] cluster was assembled correctly in FumB purified from cells grown at 37°C, FumB failed to assemble any iron-sulfur clusters when cells were grown at 15°C (Fig. 7B). The [4Fe-4S] cluster assembly in FumB and subsequent

fumarase activity could not be recovered even if the cells were transferred from 15°C to 37°C and incubated for an additional 8 h (Fig. 7B and C) in fresh LB medium supplemented with chloramphenicol. These results indicated that the [2Fe-2S] cluster of Fdx was reassembled prior to the [4Fe-4S] cluster in FumB.

To further explore the iron-sulfur cluster reassembly of other [2Fe-2S] and [4Fe-4S] proteins, two examples of each were investigated, including the redox transcription factor SoxR, which requires an intact [2Fe-2S] cluster to mediate redox regulation activity, and the endonuclease III (Nth), which requires a stable [4Fe-4S] cluster to catalyze DNA base excision repair activity. The [2Fe-2S] cluster in SoxR and the [4Fe-4S] cluster in Nth failed to be assembled when the *E. coli* cells were grown at 15°C (Fig. S3), and they were difficult to restore when the *E. coli* cells were transferred from 15°C to 25°C with incubation for an additional 4 h in the presence of chloramphenicol in fresh LB. In contrast, Fdx failed to assemble the [2Fe-2S] cluster when expressed at 15°C, but the cluster was quickly restored after *E. coli* cells were transferred from 15°C to 25°C. This result indicated that the [2Fe-2S] cluster in Fdx was reassembled prior to the [4Fe-4S] cluster in Nth and the [2Fe-2S] cluster in SoxR. Thus, a distinct and unique iron-sulfur cluster reassembly pathway of Fdx may exist in *E. coli*. Furthermore, the iron binding in Fdx may contribute to the maintenance of protein stability and the rapid assembly of the iron-sulfur center upon temperature rises.

The biogenesis of the iron-sulfur cluster in Fdx is unique in *E. coli* cells. Given that the [2Fe-2S] cluster of Fdx was assembled prior to the iron-sulfur cluster in other proteins, we speculated that the assembly mechanism of the [2Fe-2S] cluster in Fdx differs from that of other iron-sulfur proteins in *E. coli* cells. In *E. coli* cells, the proteins encoded by *iscSUA-hscBA-fdx* are the major source of iron-sulfur cluster biogenesis activity, with cysteine desulfurase IscS, the scaffold protein IscU, and iron chaperone IscA being three core iron-sulfur cluster assembly proteins. To determine whether the three proteins are required for [2Fe-2S] cluster assembly in Fdx, the *iscSUA* gene cluster that encodes the three proteins was first knocked out, followed by analysis of [2Fe-2S] cluster assembly in Fdx within the *iscSUA* mutant cells. Fdx purified from the wild-type and *iscSUA* mutant cells exhibited similar UV-visible absorption spectra, indicating the presence of the [2Fe-2S] cluster (Fig. 8A). The [2Fe-2S] cluster binding peaks at 415 nm and 459 nm were very similar for both samples. In contrast, SoxR purified from *iscSUA* mutant cells had smaller [2Fe-2S] cluster binding peaks at 414 nm, 448 nm, and 548 nm than SoxR purified from wild-type cells (Fig. 8B), along with weaker EPR signals of the reduced [2Fe-2S] cluster of SoxR (Fig. 8C). In addition, FumB purified from *iscSUA* mutant cells almost lost the [4Fe-4S] cluster binding peak at 420 nm (Fig. 8D), in addition to fumarase activity (Fig. 8E), compared with FumB purified from wild-type cells. These results indicated that the [2Fe-2S] cluster assembly in Fdx is independent of the *iscSUA* core system in *E. coli* cells.

Additional experiments were also conducted with mutant *E. coli* cells, including the complete knocking out of *isc* or *suf* systems. Fdx purified from MC4100 and *isc* or *suf* mutant cells had almost exactly the same UV-visible absorption spectra, showing the [2Fe-2S] cluster binding peaks (Fig. S4A), and the proteins contained the same iron and sulfide contents (data not shown). While the NADH dehydrogenase I activity of *suf* mutant cells was almost the same as that of wild-type MC4100, the NADH dehydrogenase I activity of the *isc* mutant was less than 20% that of wild-type MC4100 (Fig. S4B). These results indicated that the biogenesis of the [2Fe-2S] cluster in Fdx was independent of the entire *isc* or *suf* iron-sulfur assembly system in *E. coli* cells. Furthermore, the restoration of the iron-sulfur cluster in Fdx expressed in *iscSUA* mutants was also observed after increased temperatures from 15°C to 37°C. An alternative possibility is that either the Isc or Suf pathway transfers the iron-sulfur cluster to Fdx. Nevertheless, the assembly of the iron-sulfur cluster in Fdx is unique within *E. coli* cells compared to other iron-sulfur proteins.

FIG 8 Biogenesis of the iron-sulfur cluster in Fdx was independent of the *iscSUA* core system in *E. coli*. (A) UV-visible absorption spectra of Fdx purified from wild-type *E. coli* of MC4100 (spectrum 1, black line) and the *iscSUA* deletion mutant (spectrum 2, green line) grown in LB medium under aerobic growth conditions. (B) UV-visible absorption spectra of SoxR purified from wild-type *E. coli* MC4100 (spectrum 1, black line) and the *iscSUA* deletion mutant (spectrum 2, green line) grown in LB medium under aerobic growth conditions. (C) EPR spectra of 60 μ M purified SoxR proteins from panel B reduced with 1 mM sodium dithionite. (D) UV-visible absorption spectra of fumarase B (FumB) purified from wild-type *E. coli* MC4100 (spectrum 1, black line) and the *iscSUA* deletion mutant (spectrum 2, green line) grown in LB medium under aerobic growth conditions. (E) The relative fumarase activity of purified FumB samples from panel D.

DISCUSSION

The function of *E. coli* Fdx has yet to be fully elucidated, although the small size of the protein, its stability, and the highly negative reduction potential (-300 mV or less) of its [2Fe-2S] center suggest a role as a cellular electron transfer protein (30, 31). Several studies have evaluated the physiological electron donor and acceptor partners for Fdx and its homologous proteins, which have contributed to an established model of a biological redox system involved in the biogenesis of iron-sulfur clusters (16, 31–33). Interestingly, however, holo-Fdx is a [2Fe-2S] cluster-containing protein in addition to an iron-sulfur cluster assembly protein. This unique dual identity raises the question of how its [2Fe-2S] cluster is assembled. Here, we observed that the [2Fe-2S] cluster assembly in Fdx was normal in *isc* or *suf* mutant *E. coli* cells but was inhibited by cold stress. We also confirmed that recombinant Fdx purified from cold-stressed cells has iron binding activity, with an absorption peak at 320 nm shown by UV-visible spectroscopy. The bound iron contributes to stabilizing Fdx and can also be assembled into an iron-sulfur cluster through a chemical reaction initiated by IscS and L-cysteine in the presence of DTT under aerobic conditions. However, there are only substoichiometric amounts of iron in the iron-only form of Fdx, and cellularly available iron ions are required during *in vivo* conversion to the [2Fe-2S] cluster from the iron-only species. Thus, we propose a model of [2Fe-2S] cluster biogenesis in Fdx in *E. coli* cells (shown in Fig. 9). First, apo-Fdx initially recruits intracellular free iron and forms iron-loaded Fdx (Fe-Fdx). Fe-Fdx can then transform to [2Fe-2S] cluster-loaded Fdx ([2Fe-

FIG 9 Proposed mechanism of [2Fe-2S] cluster biogenesis in Fdx in *E. coli*. *E. coli* apo-Fdx initially recruits intracellular free iron, forms iron-loaded Fdx (Fe-Fdx), and then converts to [2Fe-2S] cluster-loaded Fdx ([2Fe-2S]-Fdx) in the presence of a currently uncertain sulfur source. This conversion was controlled by growth temperature *in vivo*. Fpr, ferredoxin-NADP⁺ reductase.

2S]-Fdx) in the presence of a currently uncertain sulfur source. The conversion from the iron-only bound form of Fdx to holo-Fdx was controlled by the growth temperature in *E. coli* cells.

pCold I was used as a recombinant plasmid in these experiments to express proteins in *E. coli* MC4100 cells. The pCold vector is one of the most effective *E. coli* heterologous protein expression systems and allows improved expression levels and stability of the proteins of interest in the soluble fraction. Consequently, the vector is suitable for investigating iron-sulfur cluster assembly mechanisms under cold stress conditions using recombinant iron-sulfur proteins as the reporter. Iron-sulfur cluster assembly in FumB, SoxR, and Nth was inhibited when cells were grown at 15°C (Fig. 7; see also Fig. S3 in the supplemental material). This phenomenon raises the question as to how iron-sulfur cluster formation is regulated under cold stress. Under cold stress conditions, the growth rate of *E. coli* cells sharply decreases. In addition, harvested *E. coli* cells grown at 15°C looked paler than those that were grown at 37°C, which may be the result of reduced iron-sulfur protein expression. Gene expression analysis in *Trichomonas vaginalis* previously revealed that its iron-sulfur cluster assembly machinery was induced under cold stress, although numerous iron-sulfur proteins, enzymes involved in energy metabolism, showed significant downregulation during cold stress (34). In this study, we report that iron-sulfur cluster biogenesis is affected by cold stress. Our results suggested that Fdx acts as an essential factor to regulate Isc system-mediated iron-sulfur biogenesis when environmental temperatures fluctuate by assembling its own [2Fe-2S] clusters or by binding iron atoms only. This study also has important implications for the heterologous production of iron-sulfur proteins in *E. coli* at low temperatures, because the cluster synthesis or insertion is inhibited and this group of proteins can be stably expressed even without the clusters.

Cold stress can also restrict sulfide delivery during iron-sulfur cluster biogenesis. L-Cysteine desulfurase IscS purified from *E. coli* cells grown at 15°C had lower absorption peaks at 390 nm than did that from *E. coli* cells grown at 37°C (Fig. S5), indicating decreasing binding of the cofactor pyridoxal phosphate (PLP) in IscS under cold stress. Indeed, the desulfurase activity of IscS purified from stressed cells was consistently lowered. However, the levels of protein expression in *E. coli* cells at 37°C and 15°C were the same. These results suggested that cold stress impairs IscS activity by inhibiting PLP binding activity or the biosynthesis of PLP in *E. coli* cells, resulting in diminished sulfide delivery during iron-sulfur cluster biogenesis.

Isc system components contribute to promoting Fdx expression in *E. coli* cells (35). Previous studies suggested that IscA/SufA paralogs are required for [4Fe-4S] cluster assembly but are dispensable for [2Fe-2S] cluster assembly in *E. coli* cells under normal growth conditions, suggesting that the biogenesis of the [4Fe-4S] and the [2Fe-2S] clusters result from very distinct cellular pathways (28). Most [2Fe-2S] proteins have four cysteine/histidine residues for coordinating to the iron-sulfur center. Indeed, the richness of cysteine/histidine residues that form a pocket-like spatial structure is suited for binding metals. This study provides a model example of [2Fe-2S] proteins like Fdx directly binding to iron and assembling or receiving iron-sulfur clusters, such that the iron donors IscA and/or SufA are not needed for [2Fe-2S] protein maturation. However, Ollagnier-de-Choudens et al. provided evidence supported by *in vitro* experiments that IscA is involved in Fdx cluster assembly and activation (36). This apparent discrepancy suggests that *in vitro* experiments allow limited conclusions to be drawn regarding intracellular cluster assembly mechanisms, consequently necessitating more molecular and *in vivo* experiments. A previous study showed that zinc accumulation in *E. coli* cells strongly inhibits iron-sulfur biogenesis, with Fdx binding zinc ions instead of iron-sulfur clusters (37). Thus, Fdx is likely a multimetal binding protein.

Similar to IscA and hIscA, whose roles are proposed as iron donors in iron-sulfur assembly pathways (38, 39), Fdx may also be involved in iron delivery for physiological processes. Minimal iron-bound Fdx was isolated by the Mono Q column from Fdx purified from *E. coli* cells grown at 37°C, consistent with previous results (40). Iron binding in Fdx is likely instantaneous, thereby forming an intermediate state wherein the bound iron could be quickly utilized in response to metabolic demands.

Our results demonstrated that the assembly of iron-sulfur clusters in Fdx is not completely dependent on the cysteine desulfurase IscS. The subsequent questions are whether Fdx can bind iron in cells and initiate building of its own [2Fe-2S] clusters and where the sulfur atoms are delivered from. In *E. coli* cells, there are two other cysteine desulfurases that share similarity to IscS, including SufS and CsdA. IscS, from the Isc system, appears to mediate a general sulfur delivery for cluster assembly, whereas SufS, together with other products encoded by the *suf* operon, plays a role in cluster assembly under stressful conditions (41, 42). The physiological conditions under which the third cysteine desulfurase, CsdA, is involved in iron-sulfur cluster biogenesis remain unclear (43). A knockout of *csdA* in *E. coli* cells did not significantly influence cell growth and iron-sulfur cluster assembly under aerobic conditions (data not shown). It is possible that the similar functionalities among the three cysteine desulfurases lead to compensation by one of them if another is inactivated, which may allow continuous sulfur provisioning functions for [2Fe-2S] cluster assembly in Fdx. Tanaka et al. constructed a series of gene disruption mutants for the *isc* operon in the *suf* mutant background and observed that inactivating each gene that encoded IscS, IscU, HscB, or HscA in *suf* mutant *E. coli* cells abolished the production of holo-Fdx (44). We speculate that Fdx can effectively accept clusters from multiple sources dependent on physiological changes, but when temperatures increase, Fdx can quickly obtain [2Fe-2S] clusters via a self-assembly mechanism.

In conclusion, (i) this study identifies the iron binding activity of Fdx (Fe-Fdx), which can be converted into the [2Fe-2S] cluster-bound form ([2Fe-2S]-Fdx) by a putative self-assembly mechanism, thereby providing novel insights into ISC component functions. (ii) Cold stress inhibits iron-sulfur cluster assembly by impairing the functions of Fdx and IscS. (iii) Fdx expressed in *E. coli* cells is sensitive to cold stress, and the iron binding form of Fdx may be an intermediate state or a way to store iron so that it can quickly restore its [2Fe-2S] cluster to fulfill related functions in response to changing environmental or cellular conditions.

MATERIALS AND METHODS

Protein expression and purification under cold stress conditions. The DNA fragments encoding Fdx were amplified from the wild-type *E. coli* genomic DNA by PCR. The sequences of the primers are the following: Fdx-1, 5'-GTAGGCATATGCCAAAGATTGTTATTTGCCT-3'; Fdx-2, 5'-CTGAATTCTTAATGCTCAGCGCATGGTTGAT-3'.

The PCR products were ligated into the cold shock expression plasmid pCold I, digested by NdeI and EcoRI using a one-step cloning kit (Vazyme Biotech Co., Ltd.). The cloned genes were confirmed by direct sequencing. The same method was also used to construct the recombinant expression plasmids FumB (fumarase B)-pCold and SoxR-pCold. Each recombinant plasmid was introduced into *E. coli* MC4100. The pBAD plasmids expressing Fdx, endonuclease III (Nth), SoxR, and FumB were prepared previously. The overnight *E. coli* culture was diluted (1:50) into fresh LB medium at 37°C with aeration (250 rpm). The growth medium was placed on ice for 30 min to cool after *E. coli* cells grew to an optical density (OD) at 600 nm of 0.6. Protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to the medium for an additional 12 h at a cold temperature of 15°C with aeration (250 rpm). Proteins were purified according to the procedures described in Yang et al. (45). The purity of the purified protein was determined by SDS-PAGE followed by Coomassie brilliant blue staining. The concentration of purified protein (apo-form) was determined from the absorption peak at 280 nm using the published extinction coefficient. Before determination, those purified protein samples were transiently incubated with 10 mM hydrochloric acid (HCl) to abolish the impacts of Fe binding or iron-sulfur cluster binding in proteins on the 280-nm peak. The concentration of apo-Fdx was calculated based on a molar absorption coefficient at 280 nm of $7.81 \text{ mM}^{-1} \text{ cm}^{-1}$. The protein concentration was also determined with the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) using bovine serum albumin as a standard. The results from both methods were almost the same.

Gene knockout in *E. coli* cells and site-directed mutagenesis. Fdx, a key molecule involved in the biogenesis of iron-sulfur clusters, was deleted from *E. coli* MC4100 according to the procedures described in reference 46. The constructed *E. coli* fdx mutant cells grow more slowly in LB medium than wild-type cells. The gene deletion was confirmed by PCR (Vazyme Biotech). Genes encoding IscS, IscU, and IscA were also deleted from wild-type *E. coli* cells (MC4100). The Fdx-4M mutant, in which the four conserved cysteine residues (C42, C48, C51, and C87) were replaced with serine residues, was constructed by site-directed mutagenesis. These specific mutations were confirmed by direct sequencing.

Iron and sulfide content analyses. The iron content of the protein samples was measured using the iron indicator ferroZine according to the procedures described in reference 47. The protein samples were heated to 85°C for 30 min with 500 μM ferroZine and 2 mM L-cysteine. After incubation, the protein samples were centrifuged for 5 min at 12,000 rpm to remove the precipitate. The concentration of the iron-ferroZine complex was measured at 564 nm using an extinction coefficient of $27.9 \text{ mM}^{-1} \text{ cm}^{-1}$. The iron content of protein samples was also analyzed by inductively coupled plasma mass spectrometry (ICP-MS; 8800; Agilent) for confirmation. The results from both methods were almost the same. The total sulfide content of protein samples was measured using the method reported by Siegel (48). Protein samples were incubated at 37°C for 20 min with 2 mM *N,N*-dimethyl-*p*-phenylene-diamine sulfate (DPD) and 3 mM FeCl_3 . After the incubation time, all samples were centrifuged at room temperature for 10 min to remove any precipitates. The amount of sulfide was then determined from the absorption peak at 669 nm. *E. coli* Nth was used as a standard. The iron content in *E. coli* Nth was calculated from the iron-ferroZine determination as previously described, because *E. coli* Nth contains a stable [4Fe-4S] cluster and the protein should have equal amounts of iron and sulfur.

Iron-binding analysis in Fdx. Recombinant Fdx was expressed at 15°C in *E. coli* cells grown in M9 minimal medium containing glycerol (0.2%), thiamine (5 $\mu\text{g}/\text{ml}$), and 20 amino acids (100 $\mu\text{g}/\text{ml}$). Different concentrations of ferric citrate (0 to 2 μM) were added to the M9 medium before protein expression was induced by IPTG. For *in vitro* experiments, apo-Fdx (25 μM) was incubated with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0 to 25 μM) in the presence of 10 mM DTT in open-air microtubes at 25°C for 8 h. The Fdx samples were then repurified by passing them through a HiTrap desalting column. The amplitudes of the absorption peaks of purified Fdx at 320 nm were used to estimate the iron binding in Fdx.

Mono Q analysis. Fdx, purified from *E. coli* cells grown in M9 minimal medium supplemented with 1 μM ferric citrate at 15°C, was dissolved into buffer A containing 20 mM Tris-HCl (pH 8.0) before passing through a Mono Q column (1 ml) to separate protein molecules with different anions in solution. After washing the column with 10 column volumes of buffer A, each protein solution (5 ml) was loaded onto the Mono Q column and eluted with a linear gradient of NaCl (0 to 0.5 M) in buffer B containing 20 mM Tris (pH 8.0) at a flow rate of 1 ml/min. Based on the elution profile, each eluted fraction (0.5 ml) was immediately collected and analyzed by UV-visible absorption spectroscopy. The eluted fractions were then subjected to SDS electrophoresis gel analysis.

Iron-sulfur cluster assembly in Fdx. Iron-loaded Fdx was prepared as previously described. In typical iron-sulfur cluster assembly reaction mixtures, 200 μM iron-bound Fdx was incubated with 1 μM cysteine desulfurase IscS in buffer containing 2 mM DTT, 500 mM NaCl, and 20 mM Tris-HCl at 37°C for 4 min, and 1 mM L-cysteine was then added to initiate iron-sulfur cluster assembly. The iron-sulfur cluster assembly in Fdx was monitored by a UV-visible spectrometer equipped with a temperature controller.

EPR measurements. Iron-loaded Fdx (400 μM) was prepared by incubating apo-Fdx with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ at a 1:1 molar ratio in the presence of dithiothreitol, followed by repurification by chromatography first onto a nickel-nitrilotriacetic acid (Ni-NTA) affinity column and then onto a HiTrap desalting column. For the reduced samples, freshly prepared sodium dithionite was added (at a final concentration of 4 mM) before the samples were transferred to EPR tubes and frozen immediately in liquid nitrogen. The X-band EPR spectra were recorded using a Bruker EMX plus 10/20 spectrometer equipped with an Oxford ESR910 liquid helium cryostat. Routine EPR conditions were the following: microwave frequency, 9.39 GHz; microwave power, 2.0 mW; modulation frequency, 100 kHz; modulation amplitude, 2 G; temperature, 10 K; receiver gain, 1×10^4 .

Enzyme activity assays for fumarase B, NADH dehydrogenase I, and IscS. The activity of purified fumarase B was measured by monitoring the reaction product (fumaric acid) in a reaction mixture

containing 50 mM sodium phosphate (pH 7.4) and 50 mM substrate (malate) at 250 nm using an extinction coefficient of $1.48 \text{ cm}^{-1} \text{ mM}^{-1}$. Notably, the rapid detection of fumarase B activity after purification is necessary. NADH dehydrogenase I activity was measured using deamino-NADH as a specific substrate. In the assay, the inverted membrane vesicles of *E. coli* cells were prepared by passing the cells through a low-temperature ultrahigh-pressure continuous flow cell disrupter (JN-3000 PLUS) once. Inverted membrane vesicles were added to the reaction solution containing Tris (20 mM, pH 8.0), NaCl (200 mM), deamino-NADH (50 μM), sodium azide (400 mM), and plumbagin (400 μM). The NADH dehydrogenase I activity was determined by measuring the oxidation of deamino-NADH at 340 nm at room temperature. The cysteine desulfurase activity of purified *E. coli* IscS was measured using the sulfide detection method. Briefly, purified cysteine desulfurase (1 μM) was incubated with L-cysteine (1 mM) in buffer containing Tris (20 mM [pH 8.0]), NaCl (200 mM), and dithiothreitol (2 mM) at 37°C for 10 min. Reactions were terminated by the addition of DPD (20 mM) (in 7.2N HCl) and FeCl_3 (0.3 M) (in 1.2N HCl). The produced sulfide was detected using the aforementioned method.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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We declare that we have no competing interests.

REFERENCES

- Rouault TA. 2015. Mammalian iron-sulphur proteins: novel insights into biogenesis and function. *Nat Rev Mol Cell Biol* 16:45–55. <https://doi.org/10.1038/nrm3909>.
- Stehling O, Wilbrecht C, Lill R. 2014. Mitochondrial iron-sulfur protein biogenesis and human disease. *Biochimie* 100:61–77. <https://doi.org/10.1016/j.biochi.2014.01.010>.
- Zheng L, Cash VL, Flint DH, Dean DR. 1998. Assembly of iron-sulfur clusters. Identification of an *iscSUA-hscBA-fdx* gene cluster from *Azotobacter vinelandii*. *J Biol Chem* 273:13264–13272. <https://doi.org/10.1074/jbc.273.21.13264>.
- Hoff KG, Silberg JJ, Vickery LE. 2000. Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of *Escherichia coli*. *Proc Natl Acad Sci U S A* 97:7790–7795. <https://doi.org/10.1073/pnas.130201997>.
- Agar JN, Krebs C, Frazzoni B, Huynh BH, Dean DR, Johnson MK. 2000. IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry* 39:7856–7862. <https://doi.org/10.1021/bi000931n>.
- Kakuta Y, Horio T, Takahashi Y, Fukuyama K. 2001. Crystal structure of *Escherichia coli* Fdx, an adrenodoxin-type ferredoxin involved in the assembly of iron-sulfur clusters. *Biochemistry* 40:11007–11012. <https://doi.org/10.1021/bi010544t>.
- Kim JH, Bothe JR, Frederick RO, Holder JC, Markley JL. 2014. Role of IscX in iron-sulfur cluster biogenesis in *Escherichia coli*. *J Am Chem Soc* 136:7933–7942. <https://doi.org/10.1021/ja501260h>.
- Schwartz CJ, Giel JL, Patschkowski T, Luther C, Ruzicka FJ, Beinert H, Kiley PJ. 2001. IscR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. *Proc Natl Acad Sci U S A* 98:14895–14900. <https://doi.org/10.1073/pnas.251550898>.
- Ding H, Clark RJ, Ding B. 2004. IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible free iron conditions. *J Biol Chem* 279:37499–37504. <https://doi.org/10.1074/jbc.M404533200>.
- Beilschmidt LK, Puccio HM. 2014. Mammalian Fe-S cluster biogenesis and its implication in disease. *Biochimie* 100:48–60. <https://doi.org/10.1016/j.biochi.2014.01.009>.
- Kim JH, Fuzery AK, Tonelli M, Ta DT, Westler WM, Vickery LE, Markley JL. 2009. Structure and dynamics of the iron-sulfur cluster assembly scaffold protein IscU and its interaction with the cochaperone HscB. *Biochemistry* 48:6062–6071. <https://doi.org/10.1021/bi9002277>.
- Chandramouli K, Johnson MK. 2006. HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. *Biochemistry* 45:11087–11095. <https://doi.org/10.1021/bi061237w>.
- Shimomura Y, Takahashi Y, Kakuta Y, Fukuyama K. 2005. Crystal structure of *Escherichia coli* YfhJ protein, a member of the ISC machinery involved in assembly of iron-sulfur clusters. *Proteins* 60:566–569. <https://doi.org/10.1002/prot.20481>.
- Pastore C, Adinolfi S, Huynen MA, Rybin V, Martin S, Mayer M, Bukau B, Pastore A. 2006. YfhJ, a molecular adaptor in iron-sulfur cluster formation or a frataxin-like protein? *Structure* 14:857–867. <https://doi.org/10.1016/j.str.2006.02.010>.
- Tokumoto U, Takahashi Y. 2001. Genetic analysis of the *isc* operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. *J Biochem* 130:63–71. <https://doi.org/10.1093/oxfordjournals.jbchem.a002963>.
- Yan R, Adinolfi S, Pastore A. 2015. Ferredoxin, in conjunction with NADPH and ferredoxin-NADP reductase, transfers electrons to the IscS/IscU complex to promote iron-sulfur cluster assembly. *Biochim Biophys Acta* 1854:1113–1117. <https://doi.org/10.1016/j.bbapap.2015.02.002>.
- Yan R, Konarev PV, Iannuzzi C, Adinolfi S, Roche B, Kelly G, Simon L, Martin SR, Py B, Barras F, Svergun DI, Pastore A. 2013. Ferredoxin competes with bacterial frataxin in binding to the desulfurase IscS. *J Biol Chem* 288:24777–24787. <https://doi.org/10.1074/jbc.M113.480327>.
- Kim JH, Frederick RO, Reinen NM, Troupis AT, Markley JL. 2013. [2Fe-2S]-ferredoxin binds directly to cysteine desulfurase and supplies an electron for iron-sulfur cluster assembly but is displaced by the scaffold protein or bacterial frataxin. *J Am Chem Soc* 135:8117–8120. <https://doi.org/10.1021/ja401950a>.
- Lange H, Kaut A, Kispal G, Lill R. 2000. A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins. *Proc Natl Acad Sci U S A* 97:1050–1055. <https://doi.org/10.1073/pnas.97.3.1050>.

20. Chandramouli K, Unciuleac MC, Naik S, Dean DR, Huynh BH, Johnson MK. 2007. Formation and properties of [4Fe-4S] clusters on the IscU scaffold protein. *Biochemistry* 46:6804–6811. <https://doi.org/10.1021/bi6026659>.
21. Knaff DB, Hirasawa M. 1991. Ferredoxin-dependent chloroplast enzymes. *Biochim Biophys Acta* 1056:93–125. [https://doi.org/10.1016/S0005-2728\(05\)80277-4](https://doi.org/10.1016/S0005-2728(05)80277-4).
22. Lambeth JD, Seybert DW, Lancaster JR, Jr, Salerno JC, Kamin H. 1982. Steroidogenic electron transport in adrenal cortex mitochondria. *Mol Cell Biochem* 45:13–31. <https://doi.org/10.1007/BF01283159>.
23. Webert H, Freibert SA, Gallo A, Heidenreich T, Linne U, Amlacher S, Hurt E, Muhlenhoff U, Banci L, Lill R. 2014. Functional reconstitution of mitochondrial Fe/S cluster synthesis on Isu1 reveals the involvement of ferredoxin. *Nat Commun* 5:5013. <https://doi.org/10.1038/ncomms6013>.
24. Spiegel R, Saada A, Halvardson J, Soiferman D, Shaag A, Edvardson S, Horovitz Y, Khayat M, Shalev SA, Feuk L, Elpeleg O. 2014. Deleterious mutation in FDX1L gene is associated with a novel mitochondrial muscle myopathy. *Eur J Hum Genet* 22:902–906. <https://doi.org/10.1038/ejhg.2013.269>.
25. Mons C, Botzanowski T, Nikolaev A, Hellwig P, Cianferani S, Lescop E, Bouton C, Golinelli-Cohen MP. 2018. The H₂O₂-resistant Fe-S redox switch MitoNEET acts as a pH sensor to repair stress-damaged Fe-S protein. *Biochemistry* 57:5616–5628. <https://doi.org/10.1021/acs.biochem.8b00777>.
26. Gupta V, Sendra M, Naik SG, Chahal HK, Huynh BH, Outten FW, Fontecave M, Ollagnier-de Choudens S. 2009. Native *Escherichia coli* SufA, coexpressed with SufBCDSE, purifies as a [2Fe-2S] protein and acts as an Fe-S transporter to Fe-S target enzymes. *J Am Chem Soc* 131:6149–6153. <https://doi.org/10.1021/ja807551e>.
27. Lipper CH, Karmi O, Sohn YS, Darash-Yahana M, Lammert H, Song L, Liu A, Mittler R, Nechushtai R, Onuchic JN, Jennings PA. 2018. Structure of the human monomeric NEET protein MiNT and its role in regulating iron and reactive oxygen species in cancer cells. *Proc Natl Acad Sci U S A* 115:272–277. <https://doi.org/10.1073/pnas.1715842115>.
28. Tan G, Lu J, Bitoun JP, Huang H, Ding H. 2009. IscA/SufA paralogues are required for the [4Fe-4S] cluster assembly in enzymes of multiple physiological pathways in *Escherichia coli* under aerobic growth conditions. *Biochem J* 420:463–472. <https://doi.org/10.1042/BJ20090206>.
29. Wang W, Huang H, Tan G, Si F, Liu M, Landry AP, Lu J, Ding H. 2010. In vivo evidence for the iron-binding activity of an iron-sulfur cluster assembly protein IscA in *Escherichia coli*. *Biochem J* 432:429–436. <https://doi.org/10.1042/BJ20101507>.
30. Ta DT, Vickery LE. 1992. Cloning, sequencing, and overexpression of a [2Fe-2S] ferredoxin gene from *Escherichia coli*. *J Biol Chem* 267:11120–11125. [https://doi.org/10.1016/S0021-9258\(19\)49883-9](https://doi.org/10.1016/S0021-9258(19)49883-9).
31. Knoell HE, Knappe J. 1974. *Escherichia coli* ferredoxin, an iron-sulfur protein of the adrenodoxin type. *Eur J Biochem* 50:245–252. <https://doi.org/10.1111/j.1432-1033.1974.tb03893.x>.
32. Blaschkowski HP, Neuer G, Ludwig-Festl M, Knappe J. 1982. Routes of flavodoxin and ferredoxin reduction in *Escherichia coli*. CoA-acylating pyruvate: flavodoxin and NADPH: flavodoxin oxidoreductases participating in the activation of pyruvate formate-lyase. *Eur J Biochem* 123:563–569. <https://doi.org/10.1111/j.1432-1033.1982.tb06569.x>.
33. Vorpal MA, Bruna C, Wandersleben T, Dagnino-Leone J, Lobos-Gonzalez F, Uribe E, Martinez-Oyanedel J, Bunster M. 2017. Molecular and functional characterization of ferredoxin NADP(H) oxidoreductase from *Gracilaria chilensis* and its complex with ferredoxin. *Biol Res* 50:39. <https://doi.org/10.1186/s40659-017-0144-5>.
34. Fang YK, Huang KY, Huang PJ, Lin R, Chao M, Tang P. 2015. Gene-expression analysis of cold-stress response in the sexually transmitted protist *Trichomonas vaginalis*. *J Microbiol Immunol Infect* 48:662–675. <https://doi.org/10.1016/j.jmii.2014.07.013>.
35. Takahashi Y, Nakamura M. 1999. Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in *Escherichia coli*. *J Biochem* 126:917–926. <https://doi.org/10.1093/oxfordjournals.jbchem.a022535>.
36. Ollagnier-de Choudens S, Mattioli T, Takahashi Y, Fontecave M. 2001. Iron-sulfur cluster assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. *J Biol Chem* 276:22604–22607. <https://doi.org/10.1074/jbc.M102902200>.
37. Li J, Ren X, Fan B, Huang Z, Wang W, Zhou H, Lou Z, Ding H, Lyu J, Tan G. 2019. Zinc toxicity and iron-sulfur cluster biogenesis in *Escherichia coli*. *Appl Environ Microbiol* 85:e01967–18. <https://doi.org/10.1128/AEM.01967-18>.
38. Lu J, Bitoun JP, Tan G, Wang W, Min W, Ding H. 2010. Iron-binding activity of human iron-sulfur cluster assembly protein hIscA1. *Biochem J* 428:125–131. <https://doi.org/10.1042/BJ20100122>.
39. Landry AP, Cheng Z, Ding H. 2013. Iron binding activity is essential for the function of IscA in iron-sulphur cluster biogenesis. *Dalton Trans* 42:3100–3106. <https://doi.org/10.1039/c2dt32000b>.
40. Yang W, Rogers PA, Ding H. 2002. Repair of nitric oxide-modified ferredoxin [2Fe-2S] cluster by cysteine desulfurase (IscS). *J Biol Chem* 277:12868–12873. <https://doi.org/10.1074/jbc.M109485200>.
41. Nachin L, El Hassouni M, Loiseau L, Expert D, Barras F. 2001. SoxR-dependent response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SufC, an orphan ABC ATPase. *Mol Microbiol* 39:960–972. <https://doi.org/10.1046/j.1365-2958.2001.02288.x>.
42. Outten FW, Djaman O, Storz G. 2004. A suf operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. *Mol Microbiol* 52:861–872. <https://doi.org/10.1111/j.1365-2958.2004.04025.x>.
43. Loiseau L, Ollagnier-de Choudens S, Lascoux D, Forest E, Fontecave M, Barras F. 2005. Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting Fe-S cluster biogenesis in *Escherichia coli*. *J Biol Chem* 280:26760–26769. <https://doi.org/10.1074/jbc.M504067200>.
44. Tanaka N, Kanazawa M, Tonosaki K, Yokoyama N, Kuzuyama T, Takahashi Y. 2016. Novel features of the ISC machinery revealed by characterization of *Escherichia coli* mutants that survive without iron-sulfur clusters. *Mol Microbiol* 99:835–848. <https://doi.org/10.1111/mmi.13271>.
45. Yang J, Bitoun JP, Ding H. 2006. Interplay of IscA and IscU in biogenesis of iron-sulfur clusters. *J Biol Chem* 281:27956–27963. <https://doi.org/10.1074/jbc.M601356200>.
46. Lu J, Yang J, Tan G, Ding H. 2008. Complementary roles of SufA and IscA in the biogenesis of iron-sulfur clusters in *Escherichia coli*. *Biochem J* 409:535–543. <https://doi.org/10.1042/BJ20071166>.
47. Ding H, Harrison K, Lu J. 2005. Thioredoxin reductase system mediates iron binding in IscA and iron delivery for the iron-sulfur cluster assembly in IscU. *J Biol Chem* 280:30432–30437. <https://doi.org/10.1074/jbc.M504638200>.
48. Siegel LM. 1965. A direct microdetermination for sulfide. *Anal Biochem* 11:126–132. [https://doi.org/10.1016/0003-2697\(65\)90051-5](https://doi.org/10.1016/0003-2697(65)90051-5).