[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

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Supporting Information

Introduction

Figure S1 illustrates the genes that make up the Escherichia coli ISC operon and the proteins that they encode.

Experimental Procedures

Sample preparation

Unlabeled and uniformly-[15N]-labeled ([U-15N]) IscU1,2 and unlabeled IscS3 were prepared as described previously. We used a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) to change the codon for residue 328 of IscS from Cys to Ser. We expressed and purified IscS(C328S) with the same protocols used for wild-type IscS.

The DNA fragment encoding Fdx was obtained by PCR from the pUC19isc plasmid containing the E. coli isc operon, which was a generous gift from Dr. Patricia J. Kiley (University of Wisconsin-Madison). We inserted this DNA fragment into the pENTR/SD/D-TOPO vector by employing a pENTR™/SD/D-TOPO Cloning Kit (Invitrogen), and subsequently transferred the Fdx gene to the pDEST24 vector by using a Gateway LR Clonase II enzyme mix (Invitrogen). To express Fdx, the expression plasmid was transformed into Rosetta(DE3)pLysS competent cells (Novagen). The cells were spread onto an LB agar plate supplemented with 100 μg/mL ampicillin. After overnight incubation, a single colony was transferred to 5 mL LB liquid medium containing 100 μg/mL ampicillin, which was incubated for 6–8 h at 37 ºC. Subsequently, 10 μL of the grown medium was transferred to 50 mL of LB liquid medium containing 100 μg/mL ampicillin, which was incubated overnight at 37 ºC. The whole cells harvested by centrifugation were transferred to one of the following media. (1) For the production of unlabeled Fdx, the cells were transferred to 1-L of LB liquid medium supplemented with 10 μM ferric chloride and 100 μg/mL ampicillin. (2) For the production of [U-15N]-Fdx, the cells were transferred to 1-L M9 minimal medium containing 1 g/L [15NH4]Cl, 3 g/L glucose, 10 μM ferric chloride, and 100 μg/mL ampicillin. (3) For the production of [U-13C, U-15N]-Fdx, the cells were transferred to 1-L of M9 minimal medium containing 1 g/L [15NH4]Cl, 2 g/L [U-13C]-glucose, 10 μM ferric chloride, and 100 μg/mL ampicillin. Each inoculated medium was incubated at 37 ºC. When the optical density (OD) at 600 nm reached ~1.0, 0.4 mM IPTG was added to induce overexpression of Fdx. After an additional 6–8 h of incubation, the cells were harvested by centrifugation, and stored at -80ºC until used.
Purification of Fdx was initiated by resuspending the cell pellet with a buffer consisting of 50 mM Tris-HCl, 0.1 mM EDTA, and 1 mM dithiothreitol (DTT) at pH 7.5. After sonication of the cells, 0.2 mM PMSF was added, and the insoluble fraction was separated from the lysate by centrifugation for 40 min at 25,000 g. The soluble fraction was loaded onto a DE52 anion exchange column (GE Healthcare), and the column was subsequently eluted by applying a 0-0.25 M NaCl gradient. The fractions containing Fdx were identified by the brown color of Fdx and by SDS-PAGE analysis. The fractions containing Fdx were combined, concentrated with an Amicon Ultra-15 centrifugal filter (Millipore), and subsequently loaded onto a HiLoad 16/60 Superdex-75 gel filtration column (GE Healthcare). The buffer for gel filtration contained 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, and 150 mM NaCl at pH 7.5. The fractions displaying brown color were analyzed for purity by SDS-PAGE. The final Fdx samples were concentrated, frozen with liquid nitrogen, and stored at -80 °C until used. An aliquot of the sample was analyzed by mass-spectrometry, which gave a consistent result with the expected mass of E. coli Fdx.

Reduced-Fdx was prepared as follows. After introduction into an anaerobic chamber (Coy Laboratory) filled with 90% nitrogen, 10% hydrogen, and less than 5 ppm of oxygen, the Fdx sample was extensively buffer-exchanged with an anaerobic buffer. Subsequently, a 10-fold excess of sodium dithionite was added to the sample, and the sample was incubated for 30 min. The reduction of Fdx was monitored by the color change from brown to light pink, and by taking a uv/vis spectrum. Finally, the Fdx was buffer-exchanged and concentrated to remove excess dithionite.

The CyaY gene from E. coli genomic DNA was cloned into the pE-SUMO-Kan vector (Lifesensors) by a PCR and restriction enzyme based cloning technique. The forward DNA primer for PCR contained a BsaI site, and the reverse primer contained an XhoI site; this enabled directional cloning of the gene into the pE-SUMO-Kan vector. The PCR product was ligated by using T4 DNA ligase to the enzyme-digested vector, and the ligated plasmid was transformed into E. coli 10G competent cells (Lucigen). Positive recombinants containing the target gene were identified by colony PCR.

Expression of the CyaY-SUMO fusion was initiated by transforming the expression plasmid to Rosetta2(DE3) E. coli competent cells (Novagen). The transformed cells were plated on YT plates supplemented with 1% glucose, 50 µg/mL kanamycin, and 35 µg/mL chloramphenicol, and grown overnight at 37 °C. A single colony from the plate was transferred to 1-ml YT liquid medium supplemented with 1% glucose, 50 µg/mL kanamycin, and 35 µg/mL chloramphenicol, and the medium was incubated for 1–3 h at 37 °C in a bench-top shaker (250–300 rpm). A small aliquot from the cultured medium was transferred to 50–100 ml of MDAG medium1 containing 50 µg/mL kanamycin and 35 µg/mL chloramphenicol, and the cells were grown overnight at 25 °C in a bench-top shaker. Subsequently, 10–12 ml of the grown culture was used to inoculate 500 ml of TB-g autoinduction medium4 supplemented with 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. The cells were grown overnight, harvested by a centrifugation, and stored at -80°C until used.

CyaY was isolated and purified by immobilized metal affinity chromatography. The cell pellets were first resuspended in buffer A (20 mM Tris-HCl pH7.5, 300 mM NaCl, and 5–10% glycerol) containing 5 mM imidazole, 1 mM PMSF, 500 ku of rLysozyme™ (Novagen), 500–2500 units of Benzonase® nuclease (Novagen), and 0.1% NP-40 (Fluka). After sonication, the cell lysate was clarified by centrifugation at 25,000 g for 30 min. 0.1% polyethylene imine was added to the supernatant in order to remove nucleic acids, and the lysate was centrifuged again at 25,000g for 30 min. Proteins in the lysate were precipitated by adding 70% ammonium sulfate to the solution in the presence of 2 mM β-mercaptoethanol, and the protein pellet was obtained by centrifugation at 25,000 g for 15–30 min. The pellet was re-dissolved in buffer A containing 35 mM imidazole, and subsequently applied to a Ni-Sepharose column (GE Healthcare). The bound protein was eluted with buffer A containing 500 mM imidazole. The fractions containing the target protein were combined, dialyzed against buffer A containing 5 mM imidazole, and treated with 0.5 mg of SUMO protease in the presence of 1–2 mM DTT. The protease cleavage reaction was run overnight at 4°C, and the reaction mixture was re-applied to a Ni-Sepharose column to capture the His-tagged SUMO protein. The collected flow-through fractions containing CyaY were analyzed by SDS-PAGE, and only the pure fractions were pooled. When necessary, the combined sample was further polished with a HiLoad 16/60 Superdex-
200 gel filtration column (GE Healthcare). The final sample was frozen with liquid nitrogen, and stored at -80°C until used.

NMR spectroscopy
All NMR spectra were obtained at 25°C with a 600 MHz Varian NMRS spectrometer equipped with a z-gradient cryogenic probe. All NMR samples contained 7% D2O, 0.7 mM 2,2-dimethyl-2-silapentane-5-sulfonate, and 0.02% sodium azide. NMRPipe was used for processing raw NMR data, and Sparky was employed to analyze processed data and assign NMR signals.

The NMR experiments involving [U-15N]-Fdx were initiated by taking the 2D 1H-15N HSQC spectra of [U-15N]-Fdx. The samples contained 0.3 mM Fdx with the buffer of 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM DTT, and 150 mM NaCl at pH 7.5. Note that the sample of reduced-Fdx was prepared in the anaerobic chamber as described above, and an air-tight NMR tube (Wilmad) was employed to maintain a reduced environment. After acquiring an initial 2D 1H-15N HSQC spectrum, 0.5- to 1.5 equivalent (subunit) unlabeled IscS was added to the [U-15N]-Fdx sample, and 2D 1H-15N HSQC spectra were collected. When called for, 1 equivalent of unlabeled CyaY was added to the mixture, and 2D 1H-15N HSQC spectra were collected.

The NMR experiments involving [U-15N]-IscU were conducted similarly. Briefly, initial 2D 1H-15N HSQC spectra were taken with a sample containing [U-15N]-IscU alone. The protein concentration was 0.2 mM, and the buffer contained 50 mM Tris-HCl, 0.5 mM EDTA, and 1 mM DTT at pH 7.5. To obtain spectra of IscU complexed with IscS, 1.0 to 1.5 equivalent (subunit) of unlabeled IscS was added to the IscU sample. Subsequently, 1.0 equivalent of unlabeled-Fdx or 1.0 equivalent of unlabeled-CyaY was added to the mixture in order to assess the effects of these proteins on the IscS-IscU complex.

Similar NMR studies were carried out with [U-15N]-CyaY. A 2D 1H-15N HSQC spectrum was acquired with 0.2 mM [U-15N]-CyaY in a buffer consisting of 50 mM Tris HCl, 0.5 mM EDTA, and 1 mM DTT at pH 7.5. Next, a 2-fold excess of unlabeled IscS was added, and a 2D 1H-15N HSQC spectrum was acquired. Finally, a 2-fold excess of unlabeled-IscU was added and a 2D 1H-15N HSQC spectrum was acquired.

The backbone signals of [U-13C, U-15N]-Fdx in its oxidized and reduced states were assigned by collecting and analyzing the following NMR spectra: 2D 1H-15N HSQC, 3D HNCO, 3D HN(CA)CO, 3D HNCACB, and 3D CBCA(CO)NH. Reduced-[U-13C, U-15N]-Fdx was prepared and transferred to air-tight NMR tube in the anaerobic chamber as described above. To maintain the reduced state long enough to collect 3D NMR spectra, it was found necessary to add 2 mM sodium dithionite to the sample. As expected, the backbone signals from residues close to the Fe-S cluster were not observed. In addition to the proline residues, H40-H52, S86-Q88, and R102-H107 of oxidized-Fdx and Q10, L28, D29, H40-C53, L73, L85-A89, R102, N106, and H107 of reduced-Fdx failed to exhibit 1H-15N signals (colored red in Figure 2). The assignment results for oxidized-Fdx and reduced-Fdx were deposited to Biological Magnetic Resonance data Bank with accession numbers i8991 and i8992, respectively.

Induction of intermolecular disulfide bonds
An intermolecular disulfide bond between native sulfhydryl groups of Fdx and IscS was induced by following the protocol adapted from the previous study of IscU and IscS. Three pairs of proteins, Fdx and IscS, IscU and IscS, and Fdx and IscS(C328S), were mixed with excess DTT. The same three pairs of proteins were mixed in a separate experiment with excess ferric cyanide. The concentration of each protein was 5 mg/mL, and the buffer contained 50 mM Tris-HCl at pH 7.5. After a ~30-min incubation at ambient temperature, the protein mixtures were precipitated by the addition of 10% TCA. The samples were subsequently centrifuged for 10 min at 25,000 g to obtain the protein pellets. After discarding the supernatant, the pellets were washed three times with ddH2O, dissolved in 1x NuPAGE LDS Sample Buffer (Invitrogen), and analyzed with SDS-PAGE. The positive result from the mixture of IscU and IscS served as a positive control that our experimental setup was valid for observing a disulfide-bonded complex, while the experiment with Fdx and IscS(C328S) served as a negative control (Figure 1e and 1f).
**Chemical cross-linking experiment**

SM(PEG)\(_2\) (Thermo Scientific Pierce) a reagent containing heterobifunctional groups with spacer arm length of 17.6 Å was employed in the chemical cross-linking experiment. One end of this reagent reacts with a primary amine group, while the other end reacts with a sulfhydryl group. Fdx was first treated with excess DTT. After removing DTT from the Fdx solution by use of a Zeba Spin Desalting column (Thermo Scientific), 20-fold SM(PEG)\(_2\) was added. The protein concentration was 1 mg/mL, and the reaction buffer consisted of 50 mM HEPES-NaOH and 0.5 mM EDTA at pH 7.5. After 30 min incubation at ambient temperature, unreacted cross-linking chemicals were removed by desalting, and the solution containing reacted-Fdx was divided in two: one was analyzed by SDS-PAGE without further treatment, and the other was mixed with 1 mg/mL IscS. The IscS sample had been treated with DTT and put through a desalting column to remove DTT immediately before adding to the reaction. The mixture of labeled-Fdx and IscS was incubated for 30 min, and the cross-linking between two proteins was analyzed by SDS-PAGE (Figure S2). Similar experimental procedures were followed in attempting to cross link CyaY, IscU, and IscS. CyaY was first labeled with SM(PEG)\(_2\), and excess crosslinking reagent was removed by a desalting column. Then labeled-CyaY was mixed with IscU and IscS in the absence of free cross-linking agent; and the product was analyzed by PAGE (results not shown).

**Electron transfer experiment**

The samples used in electron transfer experiments were prepared in an anaerobic chamber (Coy Laboratory). Samples used for uv/vis spectroscopy were transferred to 1-cm path-length cuvettes, sealed with rubber septa, and analyzed with a UV-1700 spectrophotometer (Shimadzu). The buffer contained 50 mM Tris-HCl at pH 7.5. The raw uv/vis spectral data were processed with UVProbe 2.21 software (Shimadzu).

The electron transfer from reduced-Fdx to IscS was monitored as follows. Three 1-mL samples were prepared. The first sample contained 50 μM reduced-Fdx alone. The second sample contained 50 μM reduced-Fdx and 50 μM IscS (subunit). The IscS sample was buffer-exchanged extensively with an anaerobic buffer before being mixed with Fdx. The third sample contained 50 μM of both proteins. Immediately after adding 250 μM L-cysteine to each sample, uv/vis spectra were taken at every 5 min for 30 min in total (Figure 3a).

The donation of electrons from reduced-Fdx to the IscU-mediated Fe-S cluster reconstitution was monitored similarly. Three 1-mL samples were prepared in the anaerobic chamber. All samples contained 250 μM ferrous ammonium sulfate and 250 μM L-cysteine. The first sample contained 50 μM reduced-Fdx only, the second sample contained 50 μM reduced-Fdx with 50 μM IscU, and the third sample contained 50 μM reduced-Fdx with 50 μM IscU and 1 μM IscS (subunit). The IscU sample was pre-treated with excess DTT, incubated for ~30 min, and buffer-exchanged before being mixed with the other proteins. Shortly after the sample preparation, the uv/vis spectrum of each sample was taken at every 10 min for 1 h.

Finally, the above procedures were repeated with IscS(C328S) to test whether electron transfer takes place with inactive IscS. The experiment involving wild-type IscS was conducted as a positive control. Each experiment was repeated at least twice to check reproducibility.

**Results**

The addition of a sub-stoichiometric amount of IscS to [U-\(^{15}\)N]-Fdx led to selective broadening of peaks (Figure 5a) serving to identify the interface of Fdx that contacts IscS. The oxidation state of Fdx was monitored by uv-vis spectroscopy (Figure S4). The product of the cluster assembly reaction consisting of IscS, IscU, reduced Fdx, L-cysteine, and Fe(II) was analyzed by uv/vis spectroscopy; subtraction of the spectrum of Fdx led to a spectrum (Figure 5b) resembling that of holo-IscU. Uv/vis spectroscopy was used to monitor the oxidation state of reduced Fdx in the presence of IscS(C328S) with added L-cysteine; Fdx remained oxidized (Figure 6a), in contrast to the mixture containing wild-type IscS which led to oxidation of Fdx (Figure 3). The in vitro cluster assembly reaction mixture in which IscS was replaced by IscS(C328S)
failed to assemble clusters (Figure S6b). Broadening of signals from [U-\(^{15}\)N]-CyaY upon the addition of excess unlabeled IscS (Figure S7b) confirmed that the two proteins form a stable complex. The addition of excess unlabeled IscU to the [U-\(^{15}\)N]-CyaY-IscS complex failed to displace [U-\(^{15}\)N]-CyaY (Figure S7c). Because the addition of CyaY was found to displace [U-\(^{15}\)N]-IscU from the [U-\(^{15}\)N]-IscU-IscS complex (Figure 4), we conclude that CyaY binds much more tightly to IscS than does IscU. Similar titration studies showed that [U-\(^{15}\)N]-IscU complexed to IscS (Figure S8a) was displaced by the addition of excess unlabeled Fdx (Figure S8b).

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Supplemental Figures

**Figure S1.** The *isc* operon of *Escherichia coli* and its products that are involved in the ISC Fe-S cluster biogenesis mechanism.\(^9\) IscR is a homodimeric repressor that regulates the expression of the *isc* operon in response to the endogenous Fe-S cluster level.\(^9\) IscS is the homodimeric pyridoxal phosphate-dependent cysteine desulfurase that mediates removal and transfer of sulfane sulfur from L-cysteine to IscU.\(^9\) IscU is the scaffold protein on which Fe-S clusters are assembled and from which Fe-S clusters are transferred to various apo-proteins.\(^9\) IscA is a potential iron donor protein.\(^1,1\) HscB and HscA are the DnaJ-type co-chaperone and the DnaK-like chaperone, respectively. HscB binds to holo-IscU and targets it to HscA, where, in an ATP-dependent reaction, the cluster bound to IscU is transferred to an apo acceptor protein.\(^1,1\) Fdx is the [2Fe-2S]-ferredoxin, which has been proposed as the source of electrons required for Fe-S cluster assembly.\(^1,1\) IscX is a small acidic protein, whose function is yet to be elucidated.\(^1,1\)

**Figure S2.** Results from a chemical cross-linking experiment providing evidence for direct interaction between ferredoxin (Fdx) and cysteine desulfurase (IscS). In this experiment, Fdx was first labeled with the cross-linking reagent SM(PEG)\(_2\) (Thermo Scientific). After removal of excess cross-linking agent by desalting gel exclusion chromatography, an aliquot of the labeled-Fdx (Fdx*) was removed for analysis and the remainder was mixed with IscS. The first lane of the SDS-PAGE contained molecular weight markers; the second lane contained Fdx* prior to mixing with IscS; and the third lane contained Fdx* after mixing with IscS. The results show evidence for the formation of a covalently linked Fdx-IscS complex at the expected molecular weight of ~57 kDa.
Figure S3. The binding interface of ferredoxin (Fdx) in its complex with cysteine desulfurase (IscS) was identified by following the 2D $^{1}$H-$^{15}$N HSQC spectrum of [U-$^{15}$N]-Fdx as function of added IscS. (Red) The spectrum of oxidized [U-$^{15}$N]-Fdx alone. (Green) The spectrum of oxidized [U-$^{15}$N]-Fdx after the addition of 0.5 equivalent of IscS. The red peaks missing in green spectrum (see the overlaid spectra in the right panel) are presumed to correspond to residues in the contact region between the two proteins affected by exchange broadening. Similar results were obtained with reduced [U-$^{15}$N]-Fdx (data not shown).

Figure S4. The uv/vis spectra of ferredoxin (Fdx) and IscS under various conditions: (red) oxidized Fdx; (black) reduced Fdx; (green) IscS; and (blue) reduced Fdx in the presence of equimolar IscS subunit. The difference between the black and blue spectra is attributed to the green spectrum of the pyridoxal 5'-phosphate cofactor of IscS. The samples contained 50 µM protein in 50 mM Tris-HCl buffer at pH 7.5.
Figure S5. Uv/vis difference spectrum obtained by subtracting the spectrum of oxidized Fdx (Figure S4, orange trace) from the spectrum of the final product (after one hour) of the cluster reconstitution experiment (Figure 3b, top green trace). This difference spectrum is very similar to that of $[2\text{Fe}-2\text{S}]$-IscU.$^{18}$

Figure S6. Ferredoxin failed to transfer electrons to IscS(C328S), the inactive IscS mutant. (a) Reduced-Fdx was mixed with equimolar (subunit) IscS(C328S). After addition of 5 equivalents of L-cysteine, uv/vis spectra were collected at every 5 min for 30 min. The unchanged spectra revealed that Fdx remained reduced. (b) Reduced-Fdx was mixed with equimolar IscU and a catalytic amount (0.02 equivalent by subunit) of IscS(C328S) in the presence of 5 equivalents of ferrous ammonium sulfate. After the addition of 5 equivalents of L-cysteine, uv/vis spectra of the mixture were taken every 10 min for 1 h. The unchanged spectra indicate that Fdx remained reduced and that no Fe-S clusters were formed.
**Figure S7.** CyaY forms a complex with IscS, and added IscU does not displace CyaY from the CyaY-IscS complex. (a) 2D $^1$H-$^{15}$N HSQC spectrum of [U-$^{15}$N]-CyaY. (b) $^1$H-$^{15}$N HSQC spectrum of [U-$^{15}$N]-CyaY following the addition of a 2-fold excess of unlabeled IscS. Most of the signals are broadened out because of the high molecular mass of the complex. (c) $^1$H-$^{15}$N HSQC spectrum of the [U-$^{15}$N]-CyaY-IscS complex following the addition of a 2-fold excess of unlabeled IscU. The lack of signals resembling those in (a) indicates that IscU fails to displace [U-$^{15}$N]-CyaY from the IscS complex.

**Figure S8.** The addition of Fdx displaces IscU from the IscU-IscS complex. (a) 2D $^1$H-$^{15}$N HSQC spectrum of [U-$^{15}$N]-IscU in the presence of equimolar (subunit) unlabeled IscS exhibiting the set of poorly-dispersed peaks representative of the [U-$^{15}$N]-IscU-IscS complex. (b) The addition of one equivalent of unlabeled-Fdx to the solution in (a) resulted in the appearance of the mixture of well-dispersed (S-state) and poorly dispersed (D-state) peaks characteristic of free IscU. These results indicate that Fdx binding to IscS displaces IscU.