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[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

ABSTRACT: *Escherichia coli* [2Fe-2S]-ferredoxin (Fdx) is encoded by the *isc* operon along with other proteins involved in the 'house-keeping' mechanism of iron–sulfur cluster biogenesis. Although it has been proposed that Fdx supplies electrons to reduce sulfane sulfur (S^0) produced by the cysteine desulfurase (IscS) to sulfide (S^{2-}) as required for the assembly of Fe–S clusters on the scaffold protein (IscU), direct experimental evidence for the role of Fdx has been lacking. Here, we show that Fdx (in either oxidation state) interacts directly with IscS. The interaction face on Fdx was found to include residues close to its Fe–S cluster. In addition, C328 of IscS, the residue known to pick up sulfur from the active site of IscS and deliver it to the Cys residues of IscU, formed a disulfide bridge with Fdx in the presence of an oxidizing agent. Electrons from reduced Fdx were transferred to IscS only in the presence of L-cysteine, but not to the C328S variant. We found that Fdx, IscU, and CyaY (the bacterial frataxin) compete for overlapping binding sites on IscS. This mutual exclusion explains the mechanism by which CyaY inhibits Fe–S cluster biogenesis. These results (1) show that reduced Fdx supplies one electron to the IscS complex as S^0 is produced by the enzymatic conversion of Cys to Ala and (2) explain the role of Fdx as a member of the *isc* operon.

Escherichia coli ferredoxin (Fdx) has been one of the most studied iron–sulfur (Fe–S) proteins since its first characterization in 1974.[1] Fdx contains a [2Fe-2S] cluster with a redox potential of approximately −380 mV.[1,2] The crystal structure of oxidized *E. coli* Fdx was found to be highly similar to those of bovine adrenodoxin and *Pseudomonas putida* putidaredoxin, as expected from their close sequence identity and conservation of the cysteine residues (C42, C48, C51, and C87) that coordinate the [2Fe-2S] cluster.[3]

Despite its detailed biophysical characterization, hard evidence for the physiological role of *E. coli* Fdx has been elusive.[4] Because the protein is encoded within the *isc* operon responsible for the production of proteins involved in the ISC Fe–S cluster biogenesis system (Figure S1 in Supporting Information [SI]),[5] it has been proposed that Fdx functions in Fe–S cluster biosynthesis.[6] Fdx has been shown to be critical for Fe–S cluster biosynthesis in *Azotobacter vinelandii*, yeast, and humans.[7−11] Mitochondria contain a homologous ISC system, and defects in this system in humans have been linked to genetic diseases.[12] The essential components of the ISC system in *E. coli* (Figure S1 in SI) include: IscS, the homodimeric pyridoxal phosphate-dependent cysteine desulfurase that generates sulfur by converting Cys to Ala and transfers it to other proteins;[13] IscU, the scaffold protein on which Fe–S clusters are assembled and from which Fe–S clusters are transferred to various apo-proteins;[14] and HscB, the DnaJ-type cochaperone, and HscA, the DnaK-like chaperone, both of which are involved in ATP-dependent cluster transfer.[15] CyaY, the *E. coli* homologue of human frataxin, is not encoded by the *isc* operon, but has been found to inhibit Fe–S cluster assembly.[16] The homodimeric cysteine desulfurase (IscS in *E. coli* and *A. vinelandii*, and Nfs1 in eukaryotes) produces S^0, which needs to be reduced to sulfide (S^{2-}) in order for IscU to assemble the iron–sulfur cluster. Assuming that two electrons are supplied by the oxidation of two ferrous ions,[4] two additional electrons from an external source are required to make one $[2Fe\text{-}2S]^{2+}$ cluster,[17,18] and it has been speculated that Fdx is involved in this reduction.[4]

Here, we demonstrate that Fdx interacts directly with IscS. We observed that most of the $^1H–^{15}N$ NMR signals in the heteronuclear single quantum correlation (HSQC) spectrum of [U-^{15}N]-Fdx, in either its oxidized (Figure 1a) or reduced (Figure 1c) state, broadened beyond detection upon the addition of 1.5 equiv (subunit) of IscS (Figure 1b and 1d, respectively). See SI for detailed experimental procedures. We attribute the line broadening of signals from Fdx (12 kDa) to its association with the much larger homodimeric IscS (~90 kDa). We confirmed the formation of the Fdx–IscS complex by inducing a disulfide bond between the two proteins (Figure 1e and 1f), in analogy to the disulfide-bonded complex of IscU and IscS obtained by exposing the IscU–IscS complex to an oxidizing agent.[19] The covalent Fdx–IscS complex induced by an oxidizing agent failed to form when the Cys residue (C328) of IscS, which serves to transfer sulfur from the catalytic site of IscS to IscU, was substituted by Ser (Figure 1f). These results are consistent with the idea that Fdx interacts with IscS to provide one of the electrons needed to reduce S^0 to S^{2-}. The interaction between Fdx and IscS was also confirmed by a chemical cross-linking experiment (Figure S2 in SI).

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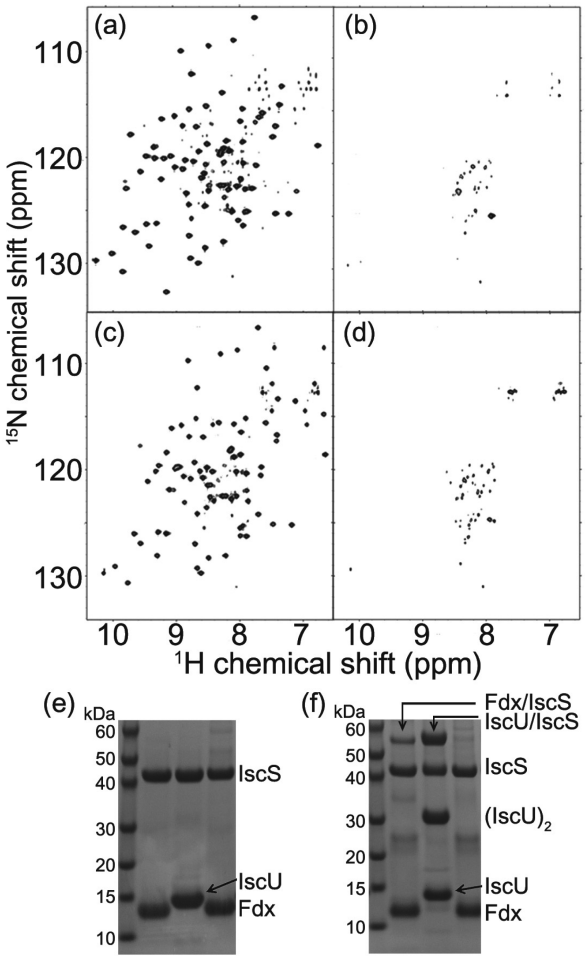

Figure 1. Evidence for direct interaction between ferredoxin (Fdx) and cysteine desulfurase (IscS). 2D ^{1}H−^{15}N HSQC spectra of (a) oxidized [U-^{15}N]-Fdx and (c) reduced [U-^{15}N]-Fdx showing that most signals broaden beyond detection in the presence of 1.5 equiv of IscS: (b) for oxidized [U-^{15}N]-Fdx and (d) for reduced [U-^{15}N]-Fdx. The broadening is attributed to rapid relaxation in the large complex. Formation of the Fdx−IscS complex was verified by inducing a disulfide bond between the two proteins in the presence of (e) excess dithiothreitol or (f) ferric cyanide. The protein product was precipitated with TCA, redissolved in a nonreducing SDS buffer, and analyzed by SDS-PAGE in the absence of a reducing agent. The first lane in each panel contains molecular weight markers. The second lane in each panel shows the product of the reaction between Fdx and IscS. The third lane in each panel shows the product of the reaction between IscU and IscS (positive control). The fourth lane in each panel shows the product of the reaction between Fdx and IscS(C328S), which failed to show a band from a disulfide-bonded complex. Note that the second and fourth lanes of panel (f) show minor bands corresponding to oligomeric forms of Fdx, which presumably result from random encounters between the exposed cysteine residues of Fdx.

The addition of a substoichiometric quantity of IscS (0.5 equivalent subunit) to [U-^{15}N]-Fdx led to broadening of subset of the ^{1}H−^{15}N HSQC cross peaks suggesting that these peaks correspond to residues in the interaction site (Figure S3 in SI). We used conventional methods to assign the NMR signals of [U-^{13}C, U-^{15}N]-Fdx in both its oxidized and reduced states (deposited in the Biological Magnetic Resonance data Bank, BMRB, under accession numbers 18991 and 18992, respectively, for oxidized and reduced Fdx). These assignments allowed us to identify the residues corresponding to the NMR signals that broadened preferentially upon the addition of IscS. For oxidized Fdx, these corresponded to the backbone signals from I27, L28, E39, C53, N68−G78, E80, E82−L85, and A89 (Figure 2a); for reduced Fdx, these corresponded to I8, E39,

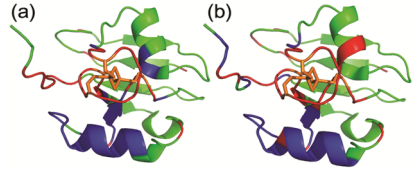

Figure 2. X-ray structure of oxidized *E. coli* ferredoxin (Fdx) (PDB 1I7H) colored to show residues from (a) oxidized [U-^{15}N]-Fdx and (b) reduced [U-^{15}N]-Fdx whose NMR signals were perturbed preferentially upon the addition of cysteine desulfurase (IscS) (see Figure S3 in SI). Residues of [U-^{15}N]-Fdx whose ^{1}H−^{15}N NMR signals were broadened by a substoichiometric amount (0.5 equiv) of IscS are colored blue; the [2Fe-2S] cluster and its cysteine ligands are colored orange; and residues whose NMR signals were unassigned are colored red. The preferentially perturbed residues of Fdx are adjacent to the Fe−S cluster, which is consistent with a model in which the Fdx−IscS complex serves to transfer an electron from reduced Fdx to the active site of IscS.

E67−M72, D74−E80, E82−R84, I100, Y103, T104, A108, and R109 (Figure 2b). In both oxidation states, these regions are adjacent to the [2Fe-2S] cluster.

To investigate the physiological relevance of the interaction between Fdx and IscS we took advantage of the large difference in the optical spectra of oxidized and reduced Fdx (Figure S4 in SI) to determine whether an electron from reduced-Fdx is transferred to IscS. We observed that the spectrum of Fdx alone (black trace in Figure 3a) and the spectrum of Fdx mixed with one equivalent (subunit) of IscS (red trace in Figure 3a) did not change over the period of 0.5 h. However, the addition of 5 equiv of L-cysteine to the mixture of reduced-Fdx and IscS led to rapid oxidation of Fdx (blue trace in Figure 3a). This result indicates that electrons from reduced-Fdx are transferred to the product of IscS and Cys. Intriguingly, we observed that reduced Fdx is more stable against air oxidation when complexed with IscS than when alone. Samples containing reduced-Fdx alone were found to oxidize over a period of 2 h, whereas reduced Fdx in a 1:1 mixture with IscS (subunit) failed to oxidize even after 6 h (data not shown). This result is consistent with the proposed binding interface, which shields the Fe−S cluster of Fdx from solvent.

We next examined whether reduced Fdx donates its electron for IscU-mediated Fe−S cluster reconstitution. In the presence of 5 equiv of ferrous ammonium sulfate and 5 equiv of L-cysteine, the UV/vis spectrum of reduced Fdx (black trace in Figure 3b) and the UV/vis spectrum of equimolar reduced Fdx and IscU (subunit) (scarlet trace in Figure 3b) remained unperturbed for over 1 h, indicating that Fdx remained reduced. By contrast, the addition of a catalytic amount (0.02 equivalent subunit) of IscS to the solution of equimolar reduced Fdx and IscU in the presence of 5 equiv of ferrous ammonium sulfate and 5 equiv of L-cysteine led to the rapid oxidation of Fdx (green traces in Figure 3b). The subtraction of the UV/vis spectrum of oxidized Fdx, from the final UV/vis spectrum (top green trace in Figure 3b) yielded a UV/vis spectrum (Figure S5 in SI) similar to that of [2Fe-2S]IscU indicating cluster formation. These results indicate that donation of an electron

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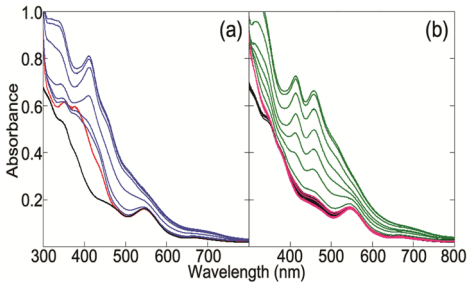

Figure 3. Evidence from UV/vis spectra for the transfer of an electron from reduced-ferredoxin (Fdx) to cysteine desulfurase (IscS) in the presence of L-cysteine (Cys). (a) (Black) UV/vis spectrum of reduced Fdx. (Red) UV/vis spectrum of equimolar reduced Fdx and IscS (subunit). (Blue) UV/vis spectra of equimolar reduced-Fdx and IscS taken every 5 min for 30 min following the addition of 5-fold Cys. The spectra show that reduced Fdx is oxidized only in the presence of both IscS and Cys. The small increase in absorbance at 400 nm in the presence of IscS is attributed to its pyridoxal 5′-phosphate cofactor (Figure S4 in SI). (b) (Black) UV/vis spectrum of reduced Fdx in the presence of 5 equiv of Cys and 5 equiv of ferrous ammonium sulfate. (Scarlet) UV/vis spectrum of equimolar reduced Fdx and scaffold protein (IscU) in the presence of 5-fold Cys and 5-fold ferrous ammonium sulfate. (Green) UV/vis spectra taken every 10 min for 1 h of equimolar reduced Fdx and IscU in the presence of 5-fold Cys and 5-fold ferrous ammonium sulfate following the addition of a catalytic quantity (0.02 equivalent subunit) of IscS. Reduced Fdx was oxidized only when all the components of the Fe–S cluster assembly system were present, indicating that electrons from reduced-Fdx are donated for cluster assembly.

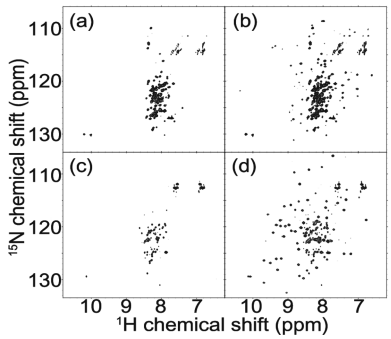

Figure 4. Binding of bacterial frataxin (CyaY) to cysteine desulfurase (IscS) displaces the scaffold protein (IscU) from the IscS–IscU complex; similarly, binding of CyaY to IscS displaces ferredoxin (Fdx) from the Fdx–IscS complex. (a) The 2D ^{1}H–^{15}N HSQC spectrum of [U-^{15}N]-IscU mixed with 1.5-fold (subunit) unlabeled IscS showed peaks from the D (disordered) state of IscU characteristic of the IscU–IscS complex.[25] (b) The addition of one equivalent of unlabeled CyaY to the solution shown in (a) yielded a mixture of peaks from the S (structured) and D states characteristic of free IscU.[25] (c) The 2D ^{1}H–^{15}N HSQC spectrum of oxidized [U-^{15}N]-Fdx mixed with equimolar unlabeled IscS showed only a few peaks from mobile groups characteristic of the Fdx–IscS complex. (d) Addition of equimolar unlabeled CyaY to the sample shown in (c) led to the appearance peaks from free Fdx.

from reduced Fdx is essential to cluster assembly on IscU. Notably, when the inactive IscS mutant, IscS(C328S), was substituted in place of wild-type IscS in the above experiments, electron transfer failed to occur even in the presence of L-cysteine (Figure S6 in SI).

CyaY has been reported to negatively regulate in vitro Fe–S cluster reconstitution[16] and to form a ternary CyaY–IscU–IscS complex.[20] Titration of [U-^{15}N]-CyaY with unlabeled IscS (Figure S7b in SI) confirmed that CyaY forms a complex with IscS. Subsequent addition of excess unlabeled IscU failed to displace the bound [U-^{15}N]-CyaY (Figure S7c in SI). However, the addition of CyaY was found to displace [U-^{15}N]-IscU from the [U-^{15}N]-IscU–IscS complex (Figure 4ab). These results argue against the formation of a reported ternary CyaY–IscS–IscU complex.[20] The addition of CyaY also was found to displace [U-^{15}N]-Fdx from the [U-^{15}N]-Fdx–IscS complex (Figure 4 cd). Taken together, these results indicate that CyaY binds more tightly to IscS than either IscU or Fdx and that either interaction could account for the reported inhibition of cluster assembly. A recent study[21] reported that IscU reduces the desulfurase activity of IscS, yet the addition of CyaY partially restored the desulfurase activity. The displacement of IscU by CyaY may account for this observation. Although our NMR results appear to rule out the formation of a stable CyaY–IscS–IscU ternary complex, we did capture a ternary complex containing CyaY, IscU, and IscS by chemical cross-linking (data not shown), in which CyaY was first labeled with cross-linking agents, and IscU and IscS were subsequently added in the absence of additional cross-linking reagents. It is known that chemical cross-linking can capture weak or transient protein–protein interactions.[22]

In eukaryotes, frataxin plays an opposite role.[23] In forming a ternary complex with human cysteine desulfurase (Nfs1) and the human scaffold protein (ISCU), human frataxin, the eukaryotic orthologue of CyaY, accelerates the rate of Fe–S cluster biogenesis.[23]

Finally, we found that Fdx and IscU compete for overlapping binding sites on IscS. The addition of unlabeled Fdx to the [U-^{15}N]-IscU–IscS complex resulted in the displacement of [U-^{15}N]-IscU (Figure S8 in SI). This result suggests that Fdx binds more tightly to IscS than IscU. Together with the data for CyaY binding, it appears that the binding sites on IscS for CyaY, Fdx, and IscU are overlapping and that the order of affinity for IscS is CyaY > Fdx > IscU.

Our results suggest that one of the two electrons needed for reduction of S^0 is transferred from Fdx bound to IscS prior to the transfer of the sulfur to IscU. Where this electron is bound remains to be determined, although it could be as a persulfide radical anion.[24] It has been proposed that the second electron required to generate S^{2-} is donated by the oxidation of Fe(II) to Fe(III).[4] Whether this occurs before or after IscU displaces Fdx is currently under investigation. A second round of this reaction cycle would be required to deliver the second sulfur and iron required for [2Fe-2S] cluster assembly. Our earlier studies[25,26] have suggested that the formation of a cluster or nascent cluster involving the Cys residues of IscU will perturb the IscU conformational equilibrium from the partially disordered state (D) to the structured state (S) state observed in the X-ray structure of [2Fe-2S]IscU.[27]

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, the result of a chemical cross-linking experiment, additional NMR spectra, and additional UV/vis spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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