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Facilitated Transfer of IscU−[2Fe2S] Clusters by Chaperone-Mediated Ligand Exchange

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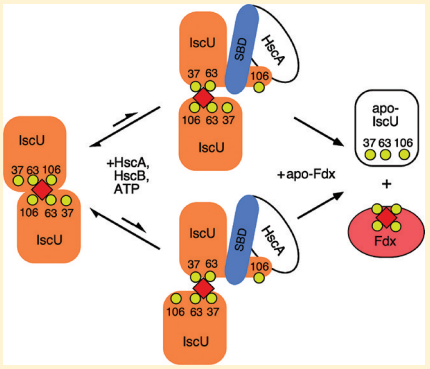
Supporting Information

ABSTRACT: The scaffold protein IscU and molecular chaperones HscA and HscB play central roles in biological assembly of iron−sulfur clusters and maturation of iron−sulfur proteins. However, the structure of IscU−FeS complexes and the molecular mechanism whereby the chaperones facilitate cluster transfer to acceptor proteins are not well understood. We have prepared amino acid substitution mutants of *Escherichia coli* IscU in which potential ligands to the FeS cluster (Cys-37, Cys-63, His-105, and Cys-106) were individually replaced with alanine. The properties of the IscU−FeS complexes formed were investigated by measuring both their ability to transfer preformed FeS clusters to apo-ferredoxin and the activity of the IscU proteins in catalyzing cluster assembly on apo-ferredoxin using inorganic iron with inorganic sulfide or with IscS and cysteine as a sulfur source. The ability of the HscA/HscB chaperone system to accelerate ATP-dependent cluster transfer from each IscU substitution mutant to apo-ferredoxin was also determined. All of the mutants formed FeS complexes with a stoichiometry similar to the wild-type holo-protein, i.e., $IscU_2$[2Fe2S], raising the possibility that different cluster ligation states may occur during iron−sulfur protein maturation. Spectroscopic properties of the mutants and the kinetics of transfer of performed IscU−FeS clusters to apo-ferredoxin indicate that the most stable form of holo-IscU involves iron coordination by Cys-63 and Cys-106. Results of studies on the ability of mutants to catalyze formation of holo-ferredoxin using iron and different sulfur sources were consistent with proposed roles for Cys-63 and Cys-106 in FeS cluster binding and also indicated an essential role for Cys-106 in sulfide transfer to IscU from IscS. Measurements of the ability of the chaperones HscA and HscB to facilitate cluster transfer from holo-IscU to apo-ferredoxin showed that only IscU(H105A) behaved similarly to wild-type IscU in exhibiting ATP-dependent stimulation of cluster transfer. IscU(C63A) and IscU(C106A) displayed elevated rates of cluster transfer in the ±ATP whereas IscU(C37A) exhibited low rates of cluster transfer ±ATP. In interpreting these findings, we propose that $IscU_2$[2Fe2S] is able undergo structural isomerization to yield conformers having different cysteine residues bound to the cluster. On the basis of the crystal structure of HscA complexed with an IscU-derived peptide, we propose that the chaperone binds and stabilizes an isomer of $IscU_2$[2Fe2S] in which the cluster is bound by cysteine residues 37 and 63 and that the [2Fe2S] cluster, being held less tightly than that coordinated by Cys-63 and Cys-106 in free $IscU_2$[2Fe2S], is more readily transferred to acceptor proteins such as apo-ferredoxin.



The biogenesis of iron−sulfur proteins involves a number of specialized proteins that mediate iron−sulfur cluster formation and delivery to acceptor proteins.[1−5] The most widely distributed and conserved system employs a scaffold-like protein for initial assembly of FeS complexes and a Hsp70-type molecular chaperone system to facilitate cluster delivery. In bacteria the IscU protein serves as a general FeS scaffold for preassembly of iron−sulfur clusters,[6] and the specialized HscA/HscB chaperone/cochaperone system functions to regulate cluster release from IscU and transfer to acceptor proteins.[7]

The properties of IscU−FeS complexes have been investigated extensively, but structural characterization of the complexes formed has been limited and complicated by differences in experimental conditions. In vitro studies of IscU from *Azotobacter vinelandii* established formation of forms of the scaffold containing one [2Fe2S], two [2Fe2S], or one [4Fe4S] cluster per dimer, and these may function in maturation of FeS proteins having different nuclearity.[8−10] The amino acid side chains that serve as iron ligands in the different FeS clusters have not been identified, but three conserved cysteine residues and a conserved histidine have been implicated in cluster binding. In vivo studies using *Saccharomyces cerevisiae* showed that mutant alleles in which cysteine residues were individually replaced with alanine were no longer able to complement strains lacking the wild-type scaffold protein.[11] In vivo studies with *Azotobacter vinelandii* demonstrated the importance of homologous cysteine residues as well as a conserved histidine, and growth differences between

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the mutants further established a functional inequivalence of the conserved cysteine residues.[12] In vitro studies using recombinant forms of human IscU also showed the importance of conserved cysteine and histidine residues for cluster assembly when expressed in *Escherichia coli*, but a mutant protein in which the conserved histidine was replaced with alanine was able to reconstitute a native-like FeS cluster in vitro, suggesting a possible role for this histidine in cluster assembly as opposed to an essential role in cluster coordination or stabilization.[13,14]

Structural studies of IscU have revealed several different metal–ligand coordination patterns, suggesting that multiple residues may be involved in metal and FeS cluster binding. Solution NMR structures of a zinc complex of IscU from *Hemohilus influenzae*[15] showed that the metal was bound to conserved cysteine and histidine residues homologous to those identified in mutagenesis studies. The positions of theses residues (Cys-37, Cys-63, His-105, and Cys-106) in the solution structures of the zinc complex of *H. influenzae* IscU and the metal-free form of *Escherichia coli* IscU[a] are shown in Figure 1. Their location in conformationally dynamic loops at

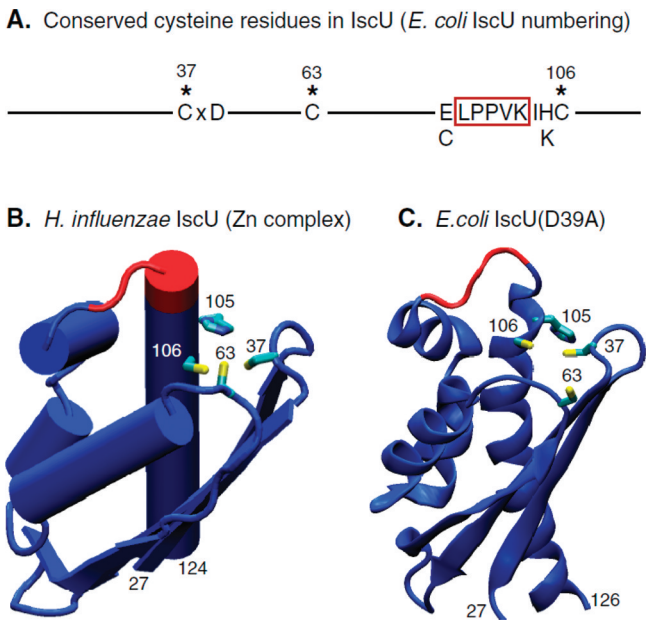

Figure 1. (A) Location of conserved cysteine residues in IscU. The sequence and numbering shown corresponds to that of *E. coli* IscU. The positions of an additional cysteine residue found in some eukaryotes and a lysine residue found in place of His-105 are also indicated. The boxed sequence ^{99}LPPVK^{103} is the sequence recognized by HscA.[23,24] (B) Solution NMR structure of the zinc complex of *H. influenzae* IscU.[15] Side chains of cysteine and histidine residues bound to zinc are shown; the zinc atom is omitted for clarity. The HscA recognition sequence is highlighted in red. (C) Solution NMR structure of the metal-free form of the *E. coli* IscU (D39A) mutant[a] showing side chains of conserved cysteine and histidine residues. The HscA recognition sequence is highlighted in red. Structural drawings were generated using VMD.[41]

one end of the molecule suggests that small structural rearrangements might accommodate binding of FeS clusters. The NMR structure the zinc complex of IscU from *Bacillus subtilis*[a] revealed that whereas the general fold of the protein was similar to that of *H. influenzae* IscU, the zinc atom was bound to three cysteine residues homologous to *H. influenzae* Cys-37, Cys-63, and Cys-106 and a nearby aspartic acid residue homologous to Asp-39. *B. subtilis* IscU has a lysine at the position corresponding to His-105 in *H. influenzae* IscU, and the resulting structural rearrangement allowing aspartate to bind the metal suggests significant flexibility in the regions near these residues. The NMR structure of IscU from *Mus musculus*[a] shows zinc bound to cysteine residues equivalent to Cys-37 and Cys-63 and nearby aspartate and histidine residues equivalent to Asp-39 and His-105 of *H. influenzae* IscU, further underscoring the conformational flexibility of IscU in metal binding.

X-ray crystallographic studies have also provided varied results for IscU metal coordination. Studies of zinc complexes of crystals of IscU from *Streptococcus pyogenes*[16] and *Thermus thermophilus*[a] revealed that cysteine and aspartic acid residues homologous to *H. influenzae* Cys-37, Asp-39, Cys-63, and Cys-106 were involved in metal binding as observed in the solution NMR structures of zinc-bound IscU from *B. subtilis* and *M. musculus*. While aspartate acts as a zinc ligand in these proteins, its role as a FeS cluster ligand is called into question by the finding that mutant IscU proteins in which the homologous aspartic acid is replaced with alanine form FeS complexes with increased stability compared to the wild-type protein.[13,17−20] The only structure determined in which IscU contains a bound FeS cluster was obtained by X-ray crystallographic analysis of a mutant form of IscU from the hyperthermophilic bacterium *Aquifex aeolicus*.[21] The mutant employed contained an alanine substitution for the aspartic acid corresponding to *H. influenzae* Asp-39, a replacement known to stabilize and trap FeS clusters in other IscU proteins.[13,17−20] While the *A. aeolicus* apo-IscU protein exits predominantly as monomeric and dimeric species in solution,[22] the form crystallized was found to be an asymmetric trimer with one [2Fe2S] cluster sequestered and bound entirely within a single protomer. The iron atoms of the cluster were coordinated to cysteine and histidine residues homologous to *H. influenzae* IscU positions Cys-37, Cys-63, His-105, and Cys-106. The involvement of conserved cysteine residues is consistent with IscU mutagenesis studies,[11,12] but the aspartate to alanine substitution in the mutant crystallized may have affected the type of FeS complex formed. In addition, the involvement of histidine contrasts with the finding that this residue is not essential for cluster binding.[14] The functional properties of the *A. aeolicus* $IscU_3$[2Fe2S] complex such as its ability to transfer the [2Fe2S] cluster to acceptor proteins have not been described, and it is therefore difficult to assess the physiological relevance of the structure. Thus, while mutagenesis studies and structural findings point toward possible roles of several conserved residues in FeS cluster binding, the detailed structures of different IscU–FeS complexes and their exact roles in iron–sulfur protein maturation in vivo remain to be determined.

The mechanism by which molecular chaperones facilitate cluster transfer from holo-IscU to apoprotein acceptors have also remained elusive. *E. coli* HscA has been found to bind specifically to a conserved IscU sequence motif, ^{99}LPPVK^{103} located near Cys-106 (see Figure 1), a putative ligand of the IscU–FeS cluster.[23,24] This finding raises the possibility that interaction of IscU with the chaperone could affect how FeS clusters are bound to the scaffold protein. The crystal structure of the substrate binding domain (SBD) of HscA complexed with an IscU-derived peptide containing this recognition sequence has been determined and revealed that the peptide is held in an extended conformation with the N- and C-

terminal ends located on opposite faces of the chaperone SBD.[25] In the case of full-length IscU this mode of binding would position N-terminal residues Cys-37 and Cys-63 on opposite sides of the SBD from C-terminal residues His-105 and Cys-106. This physical separation of potential cluster ligands would preclude simultaneous coordination of N- and C-terminal residues from the same polypeptide chain to the FeS cluster when IscU is bound to HscA. Oligomeric forms of IscU could allow N- and C-terminal residues from *different monomers* to bind to the FeS cluster, but the lack of structural information on how FeS clusters are bound to IscU makes it difficult to predict how this mode of binding to the chaperone might affect the FeS cluster. HscA, in concert with the cochaperone HscB, has been found to enhance the rate of cluster transfer from $IscU_2$[2Fe2S] to apo-ferredoxin in an ATP-dependent manner and to alter the spectroscopic properties of the FeS cluster.[26,27] This suggests that the scaffold−chaperone interaction could have effects on the structure of the FeS cluster that decrease its stability and thereby facilitate its release, but the exact nature of the molecular changes involved is not known.

In the present study we employed substitution mutants of *E. coli* IscU in which the potential cluster ligands Cys-37, Cys-63, His-105, and Cys-106 were individually replaced with alanine. We studied the ability of the mutants to bind FeS-clusters, their activity in FeS cluster transfer and maturation using apo-ferredoxin, and the effects of the HscA/HscB chaperone system on FeS- cluster transfer from the mutants to apo-ferredoxin. The results suggest specific roles for individual cysteine residues in cluster assembly and transfer, and interpretation of the findings in terms the crystal structure of a HscA−IscU peptide complexes allows us to propose a model in which binding of IscU to the chaperone is coupled with exchange of FeS cluster ligands that favors release and transfer to acceptor apoproteins.

MATERIALS AND METHODS

Proteins. Recombinant forms of *E. coli* IscU,[28] ferredoxin,[29] HscA, and HscB[30] were prepared according to previously published procedures, and protein concentrations were determined as described therein. Alanine substitution mutants of IscU were prepared by site-specific mutagenesis (QuikChange, Stratagene) using the pTrcIscU plasmid,[28] and mutations were confirmed by DNA sequencing (Laragen Inc.). Mutant IscU proteins were expressed in an iscU strain of *E. coli* M6 1655, in which the IscU gene was disrupted by the *cat* gene (designated strain OD110, James Imlay, University of Illinois, Champagne−Urbana), and cells were grown under selection with chloramphenicol and ampicillin during expression. IscU mutants were isolated as for wild-type IscU and behaved similarly during purification indicating no significant structural effects were caused by the alanine replacements. Concentrations of the holo-forms of IscU and ferredoxin are reported in terms of the [2Fe2S] complex of each protein. Under the conditions employed IscU behaves as a dimer, and concentrations are therefore reported in terms of the $(IscU)_2$[2Fe2S] complex;[8−10,28] ferredoxin is monomeric and contains a single [2Fe2S] cluster.[29] IscS was purified as previously described[31] and exhibited a specific activity of ∼150 μmol of sulfide/(min mg).

Sample Handling and Cluster Reconstitution. Sample manipulations and reagent preparation were carried out anaerobically under argon in septum-capped vials or cuvettes, and stainless steel needles and cannulae were used for sample transfers as described previously.[31] The buffer used was 0.1 M Tris-HCl, pH 8.0, containing 5 mM dithiothreitol (TD buffer). Where indicated, in experiments involving HscA and HscB, 10 mM $MgCl_2$ and 150 mM KCl were added to TD buffer (referred to as TDMK buffer). Unless otherwise specified, the temperature was maintained at 23 °C.

$IscU_2$[2Fe2S] was prepared by three sequential additions at 5 min intervals of 0.32 equiv of ferric ammonium citrate and lithium sulfide to 0.5 mM apo-IscU in TD buffer on ice. The final solution thus contained slightly substoichiometric concentrations of iron and sulfide required for formation of holo-IscU. The same pairwise progressive addition of aliquots of iron salts and sulfide was used for titration experiments aimed at assessing the stoichiometry of cluster formation on IscU. Formation of the [2Fe2S] cluster was monitored by visible region CD spectroscopy. At concentrations in the 0.5 mM range reconstituted $IscU_2$[2Fe2S] and $IscU(H105A)_2$[2Fe2S] were found to be stable for at least 18 h under anaerobic conditions in TD buffer at 0 °C. Single cysteine mutants of $IscU_2$[2Fe2S] were stable for at least 3 h under similar conditions.

Native ferredoxin was isolated as the [2Fe2S] complex.[29] Apo-ferredoxin was prepared by precipitating the holo-protein in 10% trichloroacetic acid containing 5 mM DTT for 10 min at 0 °C. The apo-ferredoxin pellet was collected by centrifugation, washed once with 1% trichloroacetic acid and twice in cold water under anaerobic conditions, and dissolved anaerobically in TD buffer to give solutions in the 0.3−0.5 mM range.

Cluster Transfer Studies. Unless otherwise specified, reactions were initiated by addition of apo-ferredoxin (final concentration 40−45 μM) to a solution containing 0.90−0.95 equiv of $IscU_2$[2Fe2S] in TD or TDMK buffer at 23 °C. Where indicated, HscA and/or HscB were added to the buffer prior to other proteins, and ATP was added to a final concentration of 2 mM from a 20 mM stock solution immediately after the addition of apo-ferredoxin. Mixtures were prepared directly in septum-capped 1 mL anaerobic cuvettes. Cluster transfer rates were calculated from the increase in ellipticity at 435 nm.[27,32]

Catalysis of Cluster Assembly. When lithium sulfide was used as the source of cluster sulfur, reactions were started by the addition of this reagent to a final concentration of 0.1 mM to a solution containing 40−45 μM apo-ferredoxin, 0.1 mM ferric ammonium citrate, in the absence and in the presence of a largely substoichiometric concentration of IscU (typically, 10 μM as the monomer) in TD buffer at 23 °C. When IscS and cysteine were used as the source of cluster sulfur, reactions were started by the addition of IscS to a final concentration of 2 μM to a solution containing 0.25 mM cysteine in addition to the components listed above. In both cases, cluster assembly was measured by monitoring the increase in ellipticity at 435 nm.[27,32]

Analytical Methods. Analyses for iron and sulfide were carried out as described previously.[32] Circular dichroism measurements were recorded at 23 °C in 1 cm path anaerobic cuvettes using a Jasco J-810 spectropolarimeter and analyzed using Jasco software.

RESULTS

Properties of Mutant IscU FeS Complexes. Recombinant forms of *E. coli* IscU having alanine substitution for Cys-37, Cys-63, His-105, or Cys-106 were expressed in an $iscU^-$ strain of *E. coli*. Each of the mutants behaved similarly to recombinant wild-type IscU during purification, and each

exhibited full activity in stimulating HscA ATPase activity ± HscB,[28] indicating that these amino acid replacements do not cause major structural alterations in the protein. In addition, as observed with wild-type IscU, each mutant was isolated in the apo-form, indicating that none of the replacements acted to stabilize bound FeS clusters as is observed with the IscU-(D39A) mutant.[b] Visible region absorption and CD spectra of wild-type IscU and the mutant proteins following reconstitution of FeS clusters using ferric iron and sulfide are presented in Figure 2. While the spectra differ from one another, each of the

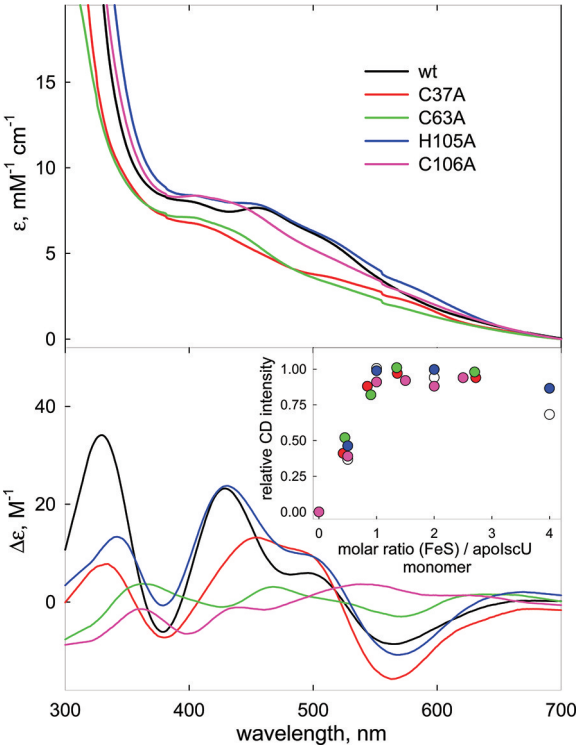

Figure 2. Absorbance (upper panel) and CD spectra (lower panel) of FeS complexes formed with wild-type and mutant forms of IscU. Complexes were prepared by sequential additions of ferric ammonium citrate and lithium sulfide to IscU in TD buffer. The final ratio of iron and sulfide to IscU monomer was 2:1, and spectra were recorded 1 h following the last addition of sulfide. None of the proteins gave appreciable absorption or CD signals in the absence of iron and sulfide. Inset: titrations of wild-type and mutant forms of IscU with iron and sulfide. IscU samples in TD buffer were treated with aliquots of ferric ammonium citrate followed by lithium sulfide after 5 min, and CD spectra were recorded following 20−30 min equilibration. CD peak-to-trough signal amplitudes were measured at the following wavelengths: 430−570 nm (wild-type and H105A); 450−570 nm (C37A); 470−580 nm (C63A); 430−540 nm (C106A).

mutant proteins appears capable of hosting an FeS center. The position of maxima in the visible region absorbance spectra may be taken as an indication that 4Fe4S clusters may form with some of the protein, as reported for wt IscU at high protein concentration.[9,10,33] However, spectral changes monitored by CD during titrations with iron and sulfide indicate that the wt protein and each of the mutants formed a complex having $IscU_2$[2Fe2S] stoichiometry (Figure 2B, inset). These findings suggest that IscU has sufficient conformational flexibility to form FeS complexes with different combinations of amino acid side chains serving as ligands to the cluster.

The absorption and CD properties of the IscU-$(H105A)_2$[2Fe2S] complex are similar to those of wild-type $IscU_2$[2Fe2S], indicating that His-105 is not likely to serve as an iron ligand in the holo-protein. On the other hand, the spectra of the cysteine mutants differ more significantly from wild-type IscU. Because of the sensitivity of CD to cluster coordination and geometry,[32] we have focused on the CD properties of the mutants (signal intensities and positions of wavelength maxima and minima) in comparing them to wild-type IscU. Among the cysteine mutants only IscU(C37A) exhibits a CD spectrum that has features that resemble those of wild-type IscU (i.e., wavelength extrema and approximate intensities). We interpret this similarity to indicate that Cys-37 does not serve as a ligand in the predominant form of the wild-type holo-protein present in solution. In contrast, FeS complexes of the IscU(C63A) and IscU(C106A) mutants display relatively weak CD signals and have wavelength minima and maxima that differ significantly from those of wild-type protein and of the His-105 and Cys-37 mutants. We interpret these results to indicate that Cys-63 and Cys-106 serve as the cluster ligands in the predominant form of wild-type $IscU_2$[2Fe2S] and that when either Cys-63 or Cys-106 is replaced with alanine, structural rearrangements allow other amino acid side chains to bind and stabilize the cluster.

Transfer of Preformed $IscU_2$[2Fe2S] Clusters. Wild-type IscU2[2Fe2S] is able to transfer its [2Fe2S] cluster quantitatively to apo-ferredoxin.[32] The kinetics observed suggest that the [2Fe2S] cluster is transferred directly from holo-Iscu to apo-ferredoxin, a finding consistent with reports that transfer does not involve cluster disassembly and reassembly.[19,34] The ability of each of the IscU mutants to transfer preformed [2Fe2S] clusters was assessed by comparing the kinetics of cluster transfer to apo-ferredoxin with that observed for wild-type IscU. As shown in Figure 3, all mutants

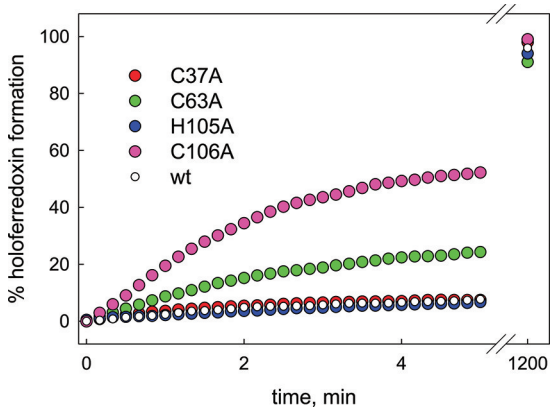

Figure 3. Rates of [2Fe2S] cluster transfer from wild-type and mutant forms of holo-IscU to apo-ferredoxin. Cluster transfer was followed by monitoring CD ellipticity increases at 435 nm following the addition of 48 μM apo-ferredoxin to 50 μM $IscU_2$[2Fe2S] complexes in TD buffer. All $IscU_2$[2Fe2S] were prepared by three sequential additions at 5 min intervals of 0.32 equiv of ferric ammonium citrate and lithium sulfide to the appropriate apo-IscU in TD buffer on ice and used 60 min after the last addition of reagents. Cluster formation followed a hyperbolic time course for all samples, and visible region CD spectra of samples at 20 h are shown in Figure S1.

were able to provide [2Fe2S] complexes; the final yields of holo-ferredoxin were similar to that obtained using wild-type IscU (Figure 1S). However, the initial rate of cluster transfer

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differed significantly among the mutants. The rates observed using IscU(C37A)$_2$[2Fe2S] and IscU(H105A)$_2$[2Fe2S] were similar to that using wild-type IscU$_2$[2Fe2S], suggesting that the [2Fe2S] cluster is bound similarly to that of wild-type IscU. In contrast, IscU(C63A)$_2$[2Fe2S] and IscU(C106A)$_2$[2Fe2S] exhibited faster initial rates of holo-ferredoxin formation, indicating that the [2Fe2S] cluster present in the cysteine mutants is more readily released than the cluster bound in wild-type IscU. These findings suggest that Cys-63 and in particular Cys-106 are most critical for [2Fe2S] cluster binding stability, consistent with the spectral findings described above, suggesting that Cys-63 and Cys-106 serve as cluster ligands in the predominant form of IscU$_2$[2Fe2S].

IscU Catalysis of Cluster Assembly. Previous work has established that IscU can function catalytically in mediating [2Fe2S] cluster formation on apo-ferredoxin and suggested that labile intermediate IscU−FeS species may be involved in the cluster maturation process.[32] We assessed the ability of the IscU mutants to mediate catalysis of cluster assembly on apo-ferredoxin using iron together with one of two sources of sulfur: either inorganic sulfide or IscS and cysteine. Initial rates of holo-ferredoxin formation using iron and sulfide in the absence of IscU or in the presence of wild-type IscU or the IscU mutants are shown in Figure 4. Similar final yields of

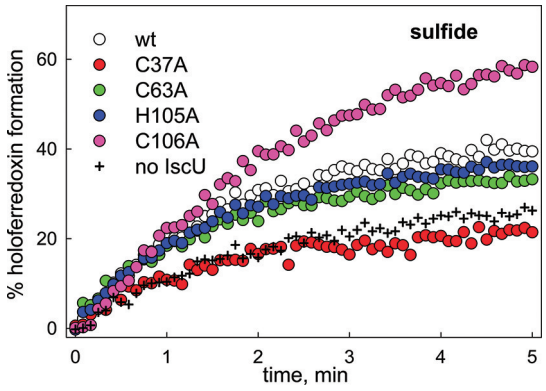

Figure 4. Catalysis of [2Fe2S]-ferredoxin maturation by wild-type and mutant forms of IscU with inorganic sulfide as the sulfur source. Samples contained 45 μM apo-ferredoxin, 100 μM ferric ammonium citrate, and 10 μM of individual forms of apo-IscU (as monomer). Reactions were carried out in TD buffer and initiated by addition of lithium sulfide to a final concentration of 100 μM. Rates of formation of holo-ferredoxin were followed by monitoring the increase in CD ellipticity at 435 nm. Visible region CD spectra of samples at 20 h are shown in Figure S2.

[2Fe2S]-ferredoxin were obtained in all cases (Figure S2), but the initial rates of catalysis varied among the mutants. IscU(C106A) was the only mutant that exhibited enhanced catalysis of cluster assembly relative to wild-type IscU, suggesting that the cluster complex formed in this mutant was less stable and more readily transferred than the complexes bound to other forms of IscU. IscU(H105A) exhibited catalytic activity close to that of wild-type IscU, again suggesting that His-105 is not essential for cluster formation or transfer. IscU(C63A) exhibited slightly decreased activity compared to wild-type IscU. Because IscU(C63A)[2Fe2S] is more effective in cluster transfer (cf. Figure 3), the decreased activity observed in this experiment suggests that the kinetics of cluster formation on IscU(C63A) are somewhat impaired compared to wild-type IscU. A striking effect was observed with IscU(C37A) which displayed a rate similar to the sample lacking scaffold protein and thus appeared to be unable to catalyze cluster maturation. Because IscU(C37A) is able to form a [2Fe2S] complex and to transfer the cluster at rate similar to wild-type IscU (see Figures 2 and 3), the failure to facilitate cluster formation on apo-ferredoxin suggests that Cys-37 may play a role in initial cluster assembly or may be necessary for formation of an intermediate species involved in the cluster transfer process.

We also measured rates of holo-ferredoxin formation using iron together with IscS and cysteine for each of the mutants. In contrast to the findings using sulfide a brief lag ~30 s in the rate of cluster formation was observed for all samples. The basis for this delay is not clear but could reflect events involved in cluster formation on apo-IscU or the requirement for dissociation of holo-IscU from IscS prior to cluster transfer to apo-ferredoxin. High final yields of [2Fe2S]-ferredoxin were observed in all cases (Figure S3), but initial rates of holo-ferredoxin differed among the mutants (Figure 5). IscU(H105A) gave an initial

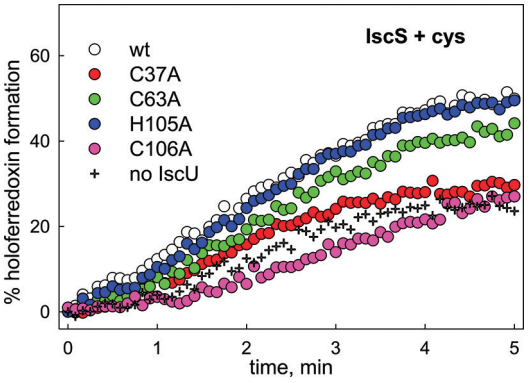

Figure 5. Catalysis of [2Fe2S]-ferredoxin maturation by wild-type and mutant forms of IscU with IscS and cysteine as the sulfur source. Samples contained 45 μM apo-ferredoxin, 100 μM ferric ammonium citrate, 0.25 mM cysteine, and 10 μM of individual forms of apoIscU (as monomer). Reactions were carried out in TD buffer and initiated by addition of IscS to a final concentration of 2 μM. Rates of formation of holo-ferredoxin were followed by monitoring the increase in CD ellipticity at 435 nm. Visible region CD spectra of samples at 20 h are shown in Figure S3.

rate indistinguishable from wild-type IscU consistent with a lack of involvement of His-105 in sulfide delivery from IscS and in cluster binding and transfer. IscU(C63A) again exhibited slightly lower activity compared to wild-type IscU, suggesting a somewhat slower rate of cluster formation. Also as before, IscU(C37A) displayed low activity, only marginally greater than the sample lacking IscU and consistent with a role in cluster assembly and/or transfer. The most dramatic change in activity using different sulfur sources was observed with IscU(C106A). Whereas this mutant was more effective than wild-type IscU when inorganic sulfide was used (Figure 4), IscU(C106A) was completely inactive inhibitory when IscS and cysteine was used. Because IscU(C106A)$_2$[2Fe2S] is also more effective in transferring preformed clusters (Figure 3), the lower rate using IscS and cysteine suggests that Cys-106 plays a critical role in sulfur delivery from IscS to IscU.

Chaperone Effects on IscU Cluster Transfer. Previous studies using purified components from *A. vinelandii*[26] and *E. coli*[27] revealed that HscA together with HscB enhance the rate of cluster transfer from IscU$_2$[2Fe2S] to apo-ferredoxin in an ATP-dependent manner. We measured the effect of HscA and

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HscB on the rate of cluster transfer from the IscU mutants to apo-ferredoxin to determine whether the [2Fe2S] complexes present in the different mutants also exhibited a similar rate enhancement. Figure 6 shows the initial rates of [2Fe2S]-ferredoxin formation for wild-type and mutant forms of IscU in the absence and presence of ATP (open and filled symbols, respectively), and Figure S4 shows the final yields of holo-ferredoxin in the presence of ATP for each form. In agreement with previous studies, the chaperones had no effect on wild-type IscU in the absence of ATP but enhanced the initial rate of holo-ferredoxin formation 5−10-fold in the presence of ATP. IscU(H105A) behaved similarly to wild-type IscU, indicating that His-105 is not critical for chaperone effects on cluster transfer.

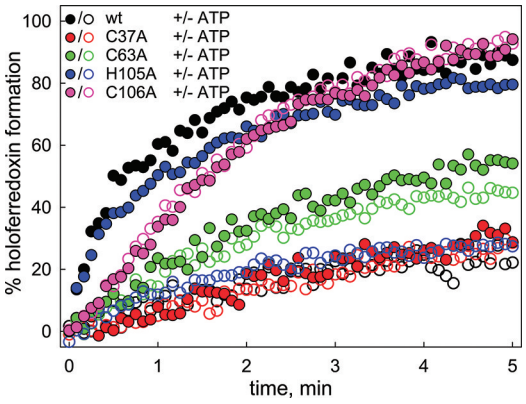

Figure 6. Effects of HscA, HscB, and ATP on the rate of [2Fe2S] cluster transfer from wild-type and mutant forms of holo-IscU to apo-ferredoxin. Samples contained 40 μM $IscU_2$[2Fe2S] complexes, 40 μM HscA, and 40 μM HscB in TDMK buffer. All $IscU_2$[2Fe2S] were prepared by three sequential additions at 5 min intervals of 0.32 equiv of ferric ammonium citrate and lithium sulfide to the appropriate apo-IscU in TD buffer on ice and were used 60 min after the last addition of reagents. Reactions were initiated by addition of apo-ferredoxin to a final concentration of 45 μM. Where indicated, ATP was added immediately after apo-ferredoxin to a final concentration of 2 mM. Rates of formation of [2Fe2S]-ferredoxin were followed by monitoring the increase in CD ellipticity at 435 nm. Visible region CD spectra of samples at 20 h are shown in Figure S4.

In contrast to wild-type IscU and IscU(H105A), the cysteine mutants showed little or no ATP-dependent chaperone effects. The rate of cluster transfer from $IscU(C37A)_2$[2Fe2S] remained low ± ATP. The lack of a chaperone effect could reflect an inability of $IscU(C37A)_2$[2Fe2S] to bind to HscA and/or HscB or a requirement for Cys-37 for efficient cluster transfer to apo-ferredoxin. Apo-IscU(C37A) interacts normally with both HscA and HscB as evidenced by its activity in stimulating HscA ATPase activity and synergism with HscB, but binding of the [2Fe2S]-cluster to IscU(C37A) could affect the interactions as discussed below. For IscU(C63A) the rate of cluster transfer was slightly elevated −ATP as was observed in the absence of the chaperones (Figure 3) and is consistent with a somewhat less stable cluster in this mutant. The rate of cluster transfer from $IscU(C63A)_2$[2Fe2S] increased only modestly +ATP, suggesting poor binding to the chaperone(s) or a requirement for Cys-63 for efficient cluster transfer. For IscU(C106A) the initial rate of cluster transfer was rapid in the absence of ATP and showed no further increase in the presence of ATP. In both cases the rate approached that observed for wild-type IscU +ATP. The rapid transfer ±ATP and the similarity to that seen with wild-type IscU in the presence of ATP raises the possibility that the IscU-$(C106A)_2$[2Fe2S] complex may have structural features in common with that of the chaperone-activated wild-type $IscU_2$[2Fe2S] complex. In the following section we interpret these findings in terms of a model in which the interaction of IscU with HscA, HscB, and ATP facilitates cluster release and transfer by favoring a form of $IscU_2$[2Fe2S] with altered cluster coordination.

DISCUSSION

Iron−sulfur clusters are thermodynamically stable structures that form readily from free iron and sulfide, and their assembly can occur even in the absence of protein binding sites.[35] However, in cellular systems spontaneous assembly of FeS centers is precluded by the limited solubility of iron and sulfur and by their sequestration to prevent toxicity. For these reasons specialized systems have evolved that employ molecular scaffolds to preassemble FeS clusters and to regulate cluster delivery and maturation of iron−sulfur proteins. To function effectively as hosts of FeS centers scaffold proteins must balance the stability of the FeS−scaffold complex with the ability to release the cluster to acceptor proteins. In the case of IscU flexibility of the protein scaffold may play an important role in determining the stability and release of bound FeS clusters by allowing formation of multiple conformational states having different FeS cluster binding affinities. Several lines of evidence suggest that the IscU polypeptide is highly flexible. Biophysical studies have established that IscU can accommodate FeS complexes having different nuclearity,[8−10] indicating that structural rearrangements can occur in regions near the cluster binding site. Structural studies have also shown that IscU can form zinc complexes and employ different metal binding ligands (refs 15 and 16 and footnote [a]), establishing that several potential metal binding residues are located in flexible regions of the protein. In addition, the crystal structure of a mutant form of IscU revealed distinct conformations for each IscU monomer in the trimeric form that was crystallized.[21] Solution NMR studies of apo-IscU also suggest that the protein backbone is inherently flexible. *H. influenzae* apo-IscU showed significant disorder when zinc was removed,[15] and studies of *E. coli* apo-IscU indicated the existence of two or more distinct conformers, of which only one was highly ordered.[36] The results described herein provide additional evidence that the IscU protein is conformationally flexible and suggest that this allows for formation of FeS complexes having different coordination patterns and different binding stabilities as evidenced by differences in their ability to transfer FeS clusters to apo-ferredoxin.

Our studies focused on replacement of conserved IscU residues previously implicated as ligands to Fe clusters, viz., Cys-37, Cys-63, His-105, and Cys-106. Among the four alanine substitution mutants investigated only IscU(H105A) behaved similarly to wild-type IscU in all studies. These results argue against His-105 as a ligand to the [2Fe2S] cluster as well as against His-105 having a critical role in the [2Fe2S] cluster transfer process. In contrast, IscU(C37A), IscU(C63A), and IscU(C106A) each exhibited changes in the properties of their respective $IscU_2$[2Fe2S] complexes compared to wild-type IscU, indicating essential roles for Cys-37, Cys-63, and Cys-106 in cluster assembly and/or transfer. Given the close proximity of these three conserved cysteine residues in the folded

structure of IscU (Figure 1), the conformational flexibility of the IscU protein (discussed above), and the inherent stability of the FeS clusters and their avidity for cysteine coordination,[35] we propose that the remaining cysteine pairs serve as the [2Fe2S] cluster ligands in these IscU mutants. Thus, the clusters present in the C37A, C63A, and C106A mutants will be bound by Cys-63 and Cys-106, Cys37 and Cys-106, or Cys-37 and Cys-63, respectively, of each monomer in the $IscU_2$[2Fe2S] complex. Additional physicochemical characterization is needed to more extensively characterize the mutant complexes, but we have used these proposed structures to interpret our findings on the ability of each mutant to transfer preformed [2Fe2S] complexes and to catalyze [2Fe2S] cluster maturation.

Properties of IscU Cysteine-to-Alanine Mutants. The most striking effects were observed with the IscU(C106A) mutant. The altered spectral properties of $IscU(C106A)_2$[2Fe2S] are consistent with Cys-106 acting as an iron ligand in wild-type $IscU_2$[2Fe2S], and the rapid release of the [2Fe2S] cluster to apo-ferredoxin suggests that Cys-106 is essential for complex stability. We propose that the [2Fe2S] cluster in the IscU(C106A) mutant is bound by Cys-37 and Cys-63 to yield a complex that is less stable than that formed with wild-type IscU. IscU(C106A) also enhanced catalysis of holo-ferredoxin formation using iron and sulfide. We interpret this as resulting from more facile release of the [2Fe2S] cluster bound to Cys-37 and Cys-63 in the mutant compared to cluster bound to Cys-63 and Cys-106 in wild-type IscU. Surprisingly, the IscU(C106A) mutant was found to be ineffective in catalysis of holo-ferredoxin formation when iron was used together with IscS and cysteine instead of with free sulfide. This result suggests that Cys-106 plays an essential role in the delivery of sulfur from IscS to the nascent FeS complex on IscU. The mechanism of sulfur transfer from IscS to IscU is not known, but Cys-106 could play a role in binding of IscU to IscS or could be involved directly (e.g., in persulfide exchange with IscS) or indirectly (e.g., in iron binding prior to capture of free sulfide). Studies conducted with *E. coli* IscU and IscS under nonreducing conditions have identified a covalent heterodisulfide complex between IscS and IscU Cys-63,[37] and this finding was interpreted to indicate involvement of IscU Cys-63 in the transfer process. However, because cysteine was not required for adduct formation the observed complex may reflect an artificial oxidation event rather than a reaction intermediate. In addition, our finding that IscU(C63A) is active in catalysis of holo-ferredoxin formation using IscS and cysteine (see below) also argues against an essential role for Cys-63 in sulfur transfer. Studies using mutants of *A. vinelandii* IscU and IscS showed that each of three single site cysteine mutants of IscU were able to accept sulfur from IscS, implying that the sulfur transfer mechanism may be nonselective.[38] However, in this study the kinetics of sulfur transfer from IscS to the cysteine mutants were not examined, and the relative efficiency of individual cysteine residues is not known. A crystal structure has been determined for a complex formed between *E. coli* IscU and IscS,[39] but the structure does not provide insight into the mechanism of sulfur transfer or the roles of individual residues. The crystal structure of the *E. coli* IscS−IscU complex reveals a single molecule of IscU bound to each active site of IscS,[39] and evidence for a similar stoichiometry was observed in SAXS experiments.[40] In the complex crystallized all three cysteine residues of IscU were observed to be >12 Å from the active site of IscS, suggesting that significant conformational rearrangements of the individual proteins or of the complex itself may be required for sulfur transfer to occur. These findings suggest that oligomerization to form the IscU2[2Fe2S] complex that we observe may occur during cluster assembly and subsequent to sulfur transfer. There is current consensus on the fact that IscU dimerization is necessary for formation of the IscU2[4Fe4S] complex in later stages of the assembly process or upon reductive coupling of 2Fe2S clusters.

The $IscU(C63A)_2$[2Fe2S] mutant also exhibited altered spectral properties and released its [2Fe2S] cluster to apo-ferredoxin more readily than wild-type $IscU_2$[2Fe2S]. These results are consistent with Cys-63 serving as a second iron ligand in wild-type $IscU_2$[2Fe2S] and contributing to the stability of the complex. On the basis of these results and those for IscU(C106A), we propose that Cys-63 and Cys-106 serve as cluster ligands in wild-type $IscU_2$[2Fe2S] and that the cluster is bound by Cys-37 and Cys-106 in the IscU(C63A) mutant. IscU(C63A) was active in catalyzing ferredoxin maturation using iron with sulfide, but catalysis was not enhanced relative to wild-type IscU. Given the decreased stability of the $IscU(C63A)_2$[2Fe2S] complex, we expected that IscU(C63A) would be somewhat more effective than wild-type IscU as was observed with IscU(C106A). The reason for the lack of enhancement is not known but could be due to a slower rate of formation of a [2Fe2S] cluster having Cys-37 and Cys-106 as ligands compared to the rate of formation of a [2Fe2S] cluster having Cys-37 and Cys-63 as ligands. IscU(C63A) showed slightly decreased activity in catalyzing ferredoxin maturation using iron with IscS and cysteine but did not exhibit the complete lack of activity displayed by IscU(C106A) under similar conditions. We interpret this result to indicate the Cys-63, unlike Cys-106, is not required for sulfur transfer from IscS.

In the case of IscU(C37A) the spectral properties of the FeS complex resemble those of wild-type $IscU_2$[2Fe2S] in their general characteristics. This suggests that Cys-37, unlike Cys-63 and Cys-106, is not a cluster ligand under the conditions used. The rate of transfer of preformed FeS-clusters from $IscU(C37A)_2$[2Fe2S] to apo-ferredoxin was similar to that of wild-type IscU consistent with minor effects if any of the cysteine-to-alanine substitution on cluster stability. However, the ability of IscU(C37A) to catalyze cluster maturation on holo-ferredoxin was significantly impaired relative to wild-type IscU using either sulfide or IscS and cysteine as the sulfur source. The decreased activity of IscU(C37A) in catalyzing cluster maturation could reflect a role for Cys-37 in initial cluster assembly on IscU and/or in the formation of an intermediate species involved in cluster transfer from IscU to apo-ferredoxin. The lack of an effect of chaperones on transfer of preformed clusters from IscU(C37A) to apo-ferredoxin described below suggests that Cys-37 plays a role in the formation of a unstable species involved in cluster transfer.

Holo-IscU as an Equilibrium Mixture of Species. The finding that each of the individual cysteine mutants studied is able to form a [2Fe2S] complex suggests that wild-type $IscU_2$[2Fe2S] may exist as an equilibrium mixture of structural isomers having different pairs of cysteine residues as ligands to the [2Fe2S] cluster. Relatively minor conformational changes in the region of the cluster binding site are required to switch cysteine side chains that bind to the [2Fe2S] cluster, and the flexibility of the IscU polypeptide could allow such structural rearrangements to occur with minor energetic costs. In addition, the inherent stability of the [2Fe2S] cluster would be expected to allow the cluster to remain intact during a ligand

exchange process. For a symmetrical $IscU_2$[2Fe2S] complex three cysteine-ligated conformational isomers are possible with the cluster bound to Cys-37 and Cys-63, Cys-37 and Cys-106, or Cys-63 and Cys-106 (designated as C37/C63, C37/C106, and C63/C106, respectively). These species would be expected to differ in their relative stability depending upon solution conditions. Our results indicate that the form of wild-type IscU having Cys-63 and Cys-106 as cluster ligands (C63/C106) is the most stable species and is thus the predominant form present under the conditions used. Small amounts of IscU having Cys-37 and Cys-63 (C37/C63) or Cys-37 and Cys-106 (C37/C106) as ligands may also be present, but the exact amount of each isomer and the rates of interconversion between the different species are not known and are difficult to measure. Our finding that the cysteine substitution mutants exhibit different rates of transfer of preformed [2Fe2S] clusters to apo-ferredoxin raises the possibility that interconversion between species having different ligation could be important in determining the rate of cluster release from $IscU_2$[2Fe2S] and delivery to acceptor proteins.

Effects of Chaperones on [2Fe2S] Cluster Release. HscA and HscB enhanced the rate of [2Fe2S] cluster transfer from holo-IscU to apo-ferredoxin in an ATP-dependent manner for wild-type $IscU_2$[2Fe2S] and for IscU-$(H105A)_2$[2Fe2S]. In contrast, none of the cysteine substitution mutants showed an ATP-dependent chaperone enhancement, and each displayed [2Fe2S] cluster transfer rates similar to those observed in the absence of the chaperone system. In the following we interpret these findings in terms of the structures proposed for the mutant $IscU_2$[2Fe2S] complexes discussed above and the crystal structure of HscA containing a bound peptide fragment derived from IscU.[24] Figure 7 presents a model of a hypothetical complex formed between IscU and the substrate binding domain of HscA. In the crystal structure of the HscA(SBD)-IscU peptide complex the ^{98}ELPPVKIHC^{106} peptide is held in an extended conformation with the N- and C-termini positioned on opposite sides of the SBD. To create a model with full-length IscU, the N- and C-terminal regions of IscU were taken separately from the solution NMR structure of the IscU−Zn complex[15] and joined to the HscA-bound IscU peptide. The model illustrates the physical separation of Cys-106 from both Cys-37 and Cys-63 that occurs when IscU is bound to the chaperone. The HscA(SBD) is ~20 Å across and creates a barrier that blocks Cys-106 from being near Cys-37 and/or Cys-63 and prevents coordination of Cys-106 to a [2Fe2S] cluster bound to either Cys-37 or Cys-63 in the IscU−chaperone complex. Based on this model the most stable form of wild-type $IscU_2$[2Fe2S] in which the cluster is coordinated to Cys-63 and Cys-106 would not bind to HscA. Instead, prior to binding to the chaperone, IscU must undergo a conformational rearrangement and exchange ligands to form an $IscU_2$[2Fe2S] complex with Cys-37 and Cys-63 coordinating the cluster, i.e., the C37/C63 conformational isomer. On the basis of our findings that the IscU(C106A) mutant forms a less stable [2Fe2S] complex, we would expect the HscA-bound C37/C63 isomer of wild-type IscU to release its [2Fe2S] cluster more readily than free wild-type IscU in which the cluster is held by Cys-63 and Cys-106. This is indeed the case and suggests that the chaperone acts to facilitate cluster transfer by binding and favoring an $IscU_2$[2Fe2S] conformational isomer having decreased [2Fe2S] cluster affinity.

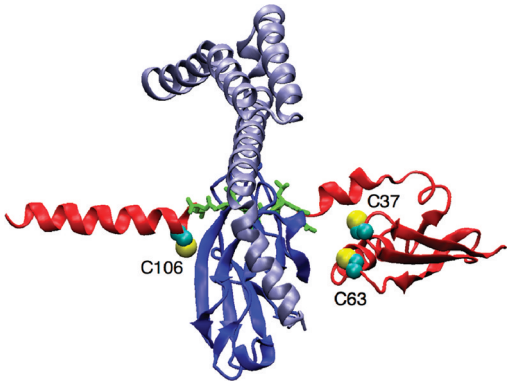

Figure 7. Hypothetical model showing the positions of the N- and C-terminal regions of IscU when bound to the substrate binding domain of HscA. The backbone structure of the HscA(SBD) is from ref 26; the β-subdomain is shown in blue, and the α-subdomain is shown in gray. The backbone structure of IscU N-terminal residues 28−97 and C-terminal residues 105−128 were taken separately from ref 15 and are shown in red. Side chains of cysteine residues 37, 63, and 106 are shown as space-filling models (carbon, cyan; sulfur, yellow). The structure of IscU residues 98−104 bound to the HscA(SBD) is from ref 25 and is shown as a green stick figure.

The lack of an effect of the chaperone system on the cysteine mutants is also consistent with this model. The [2Fe2S] cluster bound to IscU(C106A) is proposed to by coordinated by Cys-37 and Cys-63 and thus mimics the C37/C63 conformational isomer of chaperone-bound wild-type $IscU_2$[2Fe2S]. The $IscU(C106A)_2$[2Fe2S] mutant complex exhibits a high "basal" rate of cluster transfer that is not further enhanced by the chaperone system as expected if the [2Fe2S] cluster in the mutant is bound similarly to that of wild-type $IscU_2$[2Fe2S] bound to HscA. In contrast, the IscU(C37A) and IscU(C63A) mutants, having the [2Fe2S] cluster bound to Cys-106 and either Cyst-63 or Cys-37, respectively, are unable to form a [2Fe2S] complex that mimics the C37/C63 conformational isomer required for binding to HscA. Thus, neither IscU-$(C37A)_2$[2Fe2S] nor $IscU(C63A)_2$[2Fe2S] would be expected to exhibit a significant enhancement of cluster transfer using the chaperone system, and this is what is observed. For $IscU(C63A)_2$[2Fe2S] the rate of cluster transfer is slightly elevated in the presence and absence of the chaperone system compared to wild-type IscU in the absence of chaperones, suggesting that cluster bound to Cys-37 and Cys-106 is held somewhat less tightly than cluster bound to Cys-63 and Cys-106. For $IscU(C37A)_2$[2Fe2S] the rate of cluster transfer is similar to that of wild-type IscU in the absence of chaperones. This is expected if the cluster is bound to Cys-63 and Cys-106 because this mutant will resemble the stable C63/C106 isomer of free wild-type $IscU_2$[2Fe2S].

Mechanism of Chaperone-Catalyzed [2Fe2S] Cluster Transfer. A schematic model depicting steps involved in the interaction of HscA with $IscU_2$[2Fe2S] is presented in Figure 8. The C63/C106 conformer of $IscU_2$[2Fe2S] is proposed to be the most stable and predominant form in solution but is unable to bind to HscA because the ^{99}LPPVK^{103} recognition sequence is structurally constrained by the coordination of Cys-106 to the [2Fe2S] cluster. However, isomerization to form the C37/C63 conformer frees the C-terminal region from the cluster and allows interaction of the LPPVK residues with the chaperone. Our previous work showed that HscA was able to maximally stimulate cluster transfer at a stoichiometry of one HscA per

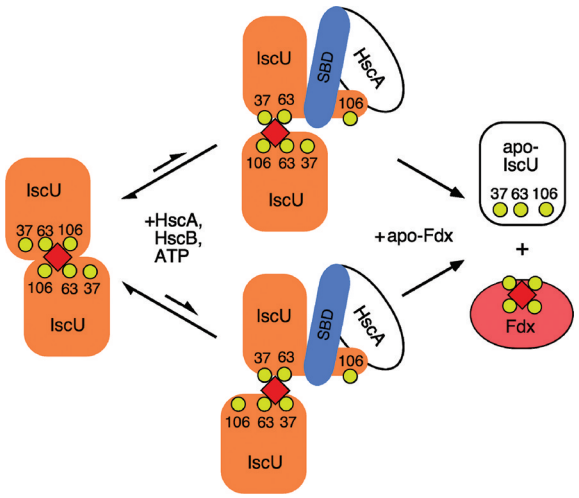

Figure 8. Proposed mechanistic scheme for chaperone-facilitated FeS cluster transfer from $IscU_2$[2Fe2S] to apo-acceptor proteins. See text for details.

IscU dimer,[27] but we do not know whether binding of a single HscA molecule is sufficient to stabilize a symmetric C37/C63 conformer (upper path) or whether the chaperone might interact with a "mixed C37/C63 + C63/C106 isomer" like that shown in the lower pathway. In either case, the chaperone-bound IscU species having altered cluster ligation is proposed to bind the [2Fe2S] cluster less tightly than free $IscU_2$[2Fe2S] to facilitate cluster transfer. The existence of mixed ligation isomers is not known, but our results with the cysteine-to-alanine mutants indicate that the symmetric C37/C63 isomer can form and that the [2Fe2S] cluster of this form is more readily released to acceptor proteins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental figures showing CD spectra at equilibrium of holoferredoxin formed under the various conditions used: (1) transfer from $IscU_2$[2Fe2S]; (2) catalysis, sulfide; (3) catalysis, IscS and cysteine; (4) transfer from $IscU_2$[2Fe2S] in the presence of HscA, HscB, and ATP. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CD, circular dichroism; TD buffer, 0.1 M Tris-HCl, pH 8.0, 5 mM dithiothreitol; TDMK buffer, 0.1 M Tris-HCl, pH 8.0, 5 mM dithiothreitol, 10 mM $MgCl_2$, 150 mM KCl.

■ ADDITIONAL NOTES

[a]Unpublished results from the Protein Data Bank. Organism and PDB identifier code: *B. subtilis* (1XJS); *E. coli* (2KQK); *M. musculus* (1WFZ); *T. thermophilus* (2QQ4).
[b]Ta, D. T., and Vickery, L. E., unpublished results.

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