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Multiple Turnover Transfer of [2Fe2S] Clusters by the Iron-Sulfur Cluster Assembly Scaffold Proteins IscU and IscA*[S]

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IscU/Isu and IscA/Isa (and related NifU and SufA proteins) have been proposed to serve as molecular scaffolds for preassembly of [FeS] clusters to be used in the biogenesis of iron-sulfur proteins. *In vitro* studies demonstrating transfer of preformed scaffold-[FeS] complexes to apoprotein acceptors have provided experimental support for this hypothesis, but investigations to date have yielded only single-cluster transfer events. We describe an *in vitro* assay system that allows for real-time monitoring of [FeS] cluster formation using circular dichroism spectroscopy and use this to investigate *de novo* [FeS] cluster formation and transfer from *Escherichia coli* IscU and IscA to apo-ferredoxin. Both IscU and IscA were found to be capable of multiple cycles of [2Fe2S] cluster formation and transfer suggesting that these scaffold proteins are capable of acting "catalytically." Kinetic studies further showed that cluster transfer exhibits Michaelis-Menten behavior indicative of complex formation of holo-IscU and holo-IscA with apo-ferredoxin and consistent with a direct [FeS] cluster transfer mechanism. Analysis of the dependence of the rate of cluster transfer, however, revealed enhanced efficiency at low ratios of scaffold to acceptor protein suggesting participation of a transient, labile scaffold-[FeS] species in the transfer process.

The biosynthesis of iron-sulfur proteins is a multistep process involving a number of specialized proteins that mediate [FeS] cluster formation and delivery to acceptor proteins (reviewed in Refs. 1–3). One of the key features of the current view of the pathway is the proposed participation of protein scaffolds that function as the initial sites for formation of transient [FeS] clusters; these molecular scaffolds may serve to guide cluster assembly, protect nascent clusters in the cellular environment, and/or assist in cluster transfer to acceptor proteins. The concept of scaffold-mediated metallocluster formation emerged from studies on the nitrogenase MoFe protein (4–6), and initial formation of [FeS] clusters on scaffold proteins was first proposed for NifU, a protein required for the biogenesis of [FeS] clusters in the nitrogenase Fe-protein (7). Subsequent studies of [FeS] cluster formation on other proteins implicated in iron-sulfur protein biogenesis, the NifU-related protein IscU/Isu (8, 9), IscA/Isa (10, 11), and SufA (12), led to the suggestion that these proteins may also function as specific scaffolds for preassembly of [FeS] clusters.

In vitro studies demonstrating transfer of preformed scaffold-[FeS] complexes from NifU (13), IscU/Isu (9, 14, 15), IscA/Isa (16–18), and SufA (12, 19) to several apoprotein acceptors have provided experimental support for their proposed roles as transient scaffolds. The mechanism of cluster transfer, however, is poorly understood. The structures of the scaffold-[FeS] complexes are not known, and the exact forms of [FeS] cluster(s) transferred have not been established. The solution NMR (20) and crystal structures (Protein Data Bank code 1SU0) of apo-IscU and the crystal structures of apo-IscA (21, 22) show that cysteine residues presumed to be involved in binding to iron atoms of the clusters are located at the surface of the apo-forms of the scaffold proteins. The scaffold-bound [FeS] clusters are therefore likely to be at least partially exposed to solvent and accessible for transfer to acceptor proteins. Little is known about how the clusters are released, but there is some evidence for a direct transfer involving scaffold-acceptor complexes. Studies employing a modified form of human Isu containing a ^{57}Fe-labeled cluster showed that the label did not exchange with ferrous sulfate present in solution during cluster transfer to apo-ferredoxin (15), and studies with *Escherichia coli* IscA and SufA showed that the iron chelator bathophenanthroline sulfonate does not interfere with cluster transfer to apo-BioB (12). These findings are consistent with a mechanism in which transfer occurs without a significant degree of cluster disassembly and reassembly and suggest that clusters may be captured immediately upon release from the scaffold protein or may be transferred directly in a scaffold-acceptor protein complex. Evidence that complex formation can occur has been obtained for IscA-ferredoxin and SufA-BioB pairs using affinity chromatography (16, 19) and for Isu- and Isa-ferredoxin pairs using chemical cross-linking (9, 11). However, the nature of the scaffold-acceptor interactions has not been characterized, and it has not been firmly established that complex formation is required for cluster transfer.

Although these *in vitro* studies provide support for scaffold proteins in [FeS] cluster assembly and delivery, the cluster transfers described to date have been relatively inefficient. In some cases the overall yield of cluster transfer has approached 100%, but the transfer rates observed have generally been slow ($k_{obs} < 0.1\ min^{-1}$) and in some cases much slower than the cell division time of the source organism. This raises the question of whether the observed transfers reflect the physiological mechanism of cluster formation and transfer or whether they are simply a thermodynamic consequence of the greater stability of

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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the acceptor protein-[FeS] complexes compared with that of the scaffold-[FeS] complexes. In addition, none of the studies reported has demonstrated that the scaffold protein is able to function in the manner expected for a carrier-type catalyst, *i.e.* to mediate multiple cluster transfer reactions. In each study a stoichiometric or excess amount of preformed scaffold-[FeS] complex was used to deliver cluster to the acceptor protein, and turnover or recycling of the scaffold proteins has not been demonstrated. This raises the question of whether [FeS] complexes of scaffold proteins can function sufficiently rapidly to support the iron-sulfur protein requirements of the cell.

In the studies described herein we have developed an *in vitro* assay system that allows for real-time monitoring of [FeS] cluster formation in solutions containing both scaffold and acceptor proteins. We have used this system to investigate *de novo* [FeS] cluster formation and transfer from the *E. coli* proteins IscU and IscA to *E. coli* apo-ferredoxin. This system has allowed us to observe multiple [FeS] cluster transfer cycles and to investigate the dependence of transfer rate on scaffold and acceptor protein concentrations.

MATERIALS AND METHODS

Protein—Recombinant forms of *E. coli* apo-IscU (23), apo-IscA (22), and ferredoxin (24) were prepared according to previously published procedures, and protein concentrations were determined as described therein. Concentrations of the holo-forms of IscU, IscA, and ferredoxin are reported in terms of the [2Fe2S] complex of each protein. IscU behaves as a dimer in both the apo- and holo-forms (8, 23), and concentrations are therefore reported in terms of the $(IscU)_2$-[2Fe2S] complex. The oligomeric state of IscA is less well defined (11, 16, 18), but the crystal structure of apo-IscA suggests that two [2Fe2S] clusters can be bound to a tetramer (22), and concentrations are therefore reported in terms of an $(IscA)_2$-[2Fe2S] complex. Holo-ferredoxin contains a single [2Fe2S] cluster (24, 25).

Sample Handling and Cluster Reconstitution—Sample manipulations, including reagent preparation and chromatography, were carried out anaerobically under argon in septum-capped vials, cuvettes, or columns, and stainless steel needles, tubing, and cannulae were used for sample transfers. Unless otherwise specified the buffer used was 0.1 M Tris-HCl, pH 8.0, containing 5 mM dithiothreitol (TD buffer), and the temperature was maintained at 25 °C.

Holo-IscU was prepared by three sequential additions at 5-min intervals of 0.5 eq of ferric ammonium citrate and lithium sulfide to apo-IscU; the final solution thus contained 1.5-fold greater concentrations of iron and sulfide than required for formation of the $(IscU)_2$-[2Fe2S] complex. Holo-IscA was prepared similarly using ferrous ammonium sulfate in place of the ferric salt. For both proteins formation of [2Fe2S] clusters was monitored by visible region CD spectroscopy (*cf.* Fig. 1). In some cases $(IscU)_2$-[2Fe2S] and $(IscA)_2$-[2Fe2S] were purified by sequential gel permeation and ion exchange anaerobic chromatography on small G25 and DE52 columns; both holo-forms were found to be stable for at least 24 h under anaerobic conditions at 0 °C. Elemental analyses of holo-IscU and holo-IscA for iron and sulfide were consistent with the presence of one [2Fe2S] cluster per dimer.

Ferredoxin was isolated as the [2Fe2S] complex (24). Apo-ferredoxin was prepared by precipitating the holo-form in 10% trichloroacetic acid containing 10 mM dithiothreitol for 10 min at 0 °C. The sample was collected by centrifugation, washed twice in water under anaerobic conditions, and dissolved anaerobically in TD buffer.

Cluster Transfer Studies—Unless otherwise specified, apo-IscU (or apo-IscA) and apo-ferredoxin in TD buffer were mixed at 25 °C in a 1-ml cuvette with 0.2 mM ferric (or ferrous) salt, typically equivalent to five times the amount of apo-ferredoxin (*i.e.* 2.5-fold that required for formation of the [2Fe2S]-ferredoxin). Lithium sulfide was used as the source of sulfide rather than the physiological donor cysteine and IscS (8) to ensure that sulfide availability was not rate-limiting. Reactions were initiated by the addition of five equivalents of lithium sulfide (0.2 mM final concentration), and the kinetics of cluster assembly and transfer were monitored by CD spectroscopy. Control experiments, in the absence of scaffold protein, were also carried out to determine the background rates of ferredoxin-[2Fe2S] formation.

Analytical Method—Analyses for iron and sulfide were carried out as described previously (26). CD measurements were recorded at 25 °C in 1-cm path anaerobic cuvettes using a Jasco J-810 spectropolarimeter.

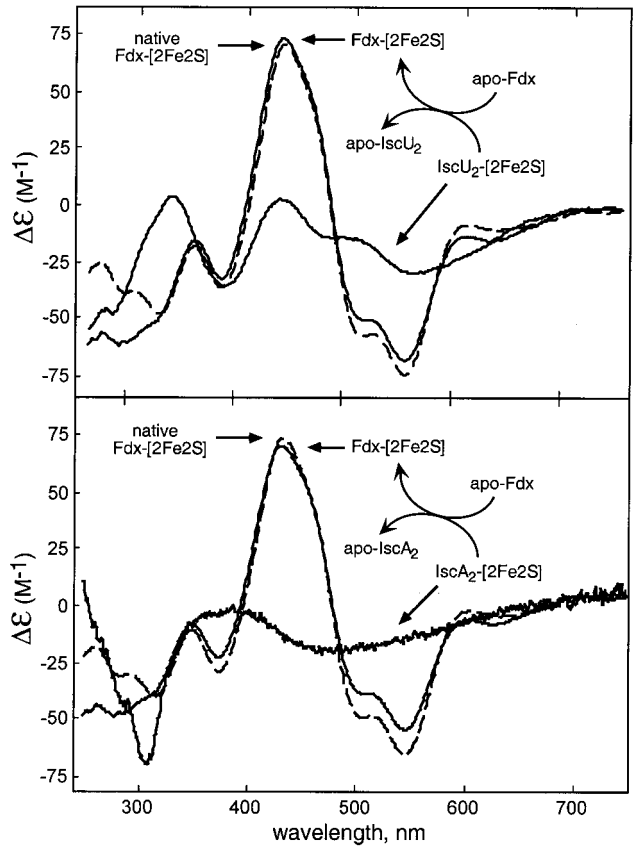

FIG. 1. **Circular dichroism spectra of [2Fe2S] complexes of ferredoxin (*Fdx*), IscU, and IscA.** *Upper panel*, CD spectra of a sample containing purified $IscU_2$-[2Fe2S] were recorded prior to and 22 h following addition of 1.6 equivalents of apo-ferredoxin. *Lower panel*, CD spectra of a sample containing purified $IscA_2$-[2Fe2S] were recorded prior to and 18 h following addition of 1.7 equivalents of apo-ferredoxin. The CD spectra of purified holo-ferredoxin (*native Fdx-[2Fe2S]*) is shown in each panel as a *dashed line* for comparison with the spectra of the scaffold-reconstituted samples.

RESULTS AND DISCUSSION

Circular Dichroism of [FeS] Complexes—Previous [FeS] cluster transfer studies utilized UV-visible absorption spectroscopy, analytical gel electrophoresis, or biochemical assays to determine the amount of acceptor complex formed. The electrophoretic and biochemical methods employed require interrupting the transfer reaction and/or separation of scaffold and acceptor proteins and do not allow for real-time characterization of reaction progress (9, 13–15, 17, 18). UV-visible spectroscopy has been used to directly monitor cluster transfer with IscA and SufA (12, 16, 19), but the general similarity and the absence of protein-specific signature features of the absorption spectra of [FeS] complexes makes it difficult to determine whether intermediate species having different coordination and/or geometry might occur during the transfer reaction.

CD spectra of metal complexes are generally more complex than absorption spectra and provide increased sensitivity for detecting differences in cluster properties. The near UV-visible region CD of the [2Fe2S] complex of *E. coli* IscU (39) is qualitatively similar to that of *E. coli* ferredoxin-[2Fe2S] (24), consistent with similar cluster coordination, but the CD intensity of $IscU_2$-[2Fe2S] is weaker suggesting different cluster environments in the two proteins. The CD of IscA has not been reported, but models of [2Fe2S] complexes of IscA based on the crystal structure (22) suggest that the cluster environment is likely to be much different from that of ferredoxin (25). We sought to determine whether CD could provide a more sensitive means of mon-

itoring cluster transfer from IscU and/or IscA to ferredoxin.

Fig. 1 shows near UV-visible region CD spectra of purified [2Fe2S] forms of IscU, IscA, native ferredoxin, and ferredoxin reconstituted using the scaffold proteins. Native ferredoxin-[2Fe2S] was isolated as described previously (24), and $IscU_2$-[2Fe2S] and $IscA_2$-[2Fe2S] were prepared and purified as described under "Materials and Methods." Apo-ferredoxin was reconstituted by equilibration for 18–22 h under anaerobic conditions with the purified $IscU_2$-[2Fe2S] or $IscA_2$-[2Fe2S] complexes. An excess of apo-ferredoxin (1.6 eq) was used to favor complete cluster transfer from the scaffold proteins. For both IscU and IscA the CD spectrum of the reconstituted ferredoxin is qualitatively indistinguishable from the native ferredoxin-[2Fe2S] complex, consistent with a lack of effect of the source of the [FeS] cluster on the properties of the ferredoxin product. In addition, UV-visible absorption spectra and iron and sulfide analysis of the isolated reconstituted ferredoxins confirmed that the products were the expected [2Fe2S] complexes. With $IscU_2$-[2Fe2S] as the donor the yield of ferredoxin-[2Fe2S] was essentially quantitative (≅98%) with respect to the amount of cluster available, whereas the yield was somewhat lower with $IscA_2$-[2Fe2S] (≅90%). In both cases the large difference in CD intensity of ferredoxin-[2Fe2S] compared with that of the scaffold complexes and the spectral detail over this wavelength range provide a sensitive means to monitor [FeS] cluster transfer.

Catalysis of Cluster Transfer by IscU—The results in Fig. 1 establish transfer of *preformed* [FeS] clusters from $IscU_2$-[2Fe2S] and $IscA_2$-[2Fe2S] to apo-ferredoxin. To determine whether IscU and IscA are able to mediate *multiple cluster transfer cycles* requires: 1) use of substoichiometric amounts of the scaffold protein relative to apo-ferredoxin, 2) a system for continuous regeneration of the scaffold-[2Fe2S] complex, and 3) that the rate of cluster assembly on the scaffold is greater than the rate of unassisted ferredoxin cluster assembly. As described under "Materials and Methods" we found that $IscU_2$-[2Fe2S] was formed in good yield using ferric ammonium citrate and lithium sulfide in TD buffer, and we monitored the rate of cluster formation at 25 °C by single-wavelength CD measurements at 540 nm. Supplemental Fig. 1 shows that using 100 μM apo-IscU, formation of $IscU_2$-[2Fe2S] is >90% complete within 5 min with $k_{obs} \cong 0.3$ min^{-1}. Spontaneous formation of ferredoxin-[2Fe2S] under similar conditions, in contrast, is much slower and requires several hours to reach completion (see below). Therefore under these conditions formation of holo-IscU is not expected to be rate-limiting, and the rate of holo-ferredoxin formation in mixtures of apo-IscU and apo-ferredoxin will reflect the rate of cluster transfer from holo-IscU to apo-ferredoxin and/or ferredoxin cluster maturation.

Initial studies to test for a catalytic role of IscU in mediating multiple cluster transfers were carried out using apo-$IscU_2$ and apo-ferredoxin in a molar ratio of 0.1 to 1. In the experiment shown in Fig. 2 apo-ferredoxin alone or apo-ferredoxin and apo-IscU were pre-equilibrated in the presence of ferric ammonium citrate, and cluster formation was initiated by addition of lithium sulfide. IscU was found to significantly enhance cluster assembly on ferredoxin. The initial rate of ferredoxin-[2Fe2S] formation is ≅9-fold greater in the presence of IscU than in its absence suggesting that scaffold-mediated preassembly and transfer is more efficient than direct assembly on the ferredoxin. The final yield of holo-ferredoxin is also greater (≅100 *versus* ≅75%) suggesting that the scaffold-mediated process may prevent unproductive side reactions from occurring. The CD spectra at different stages of completion of the reaction are qualitatively similar and exhibit distinct isodichroic points at

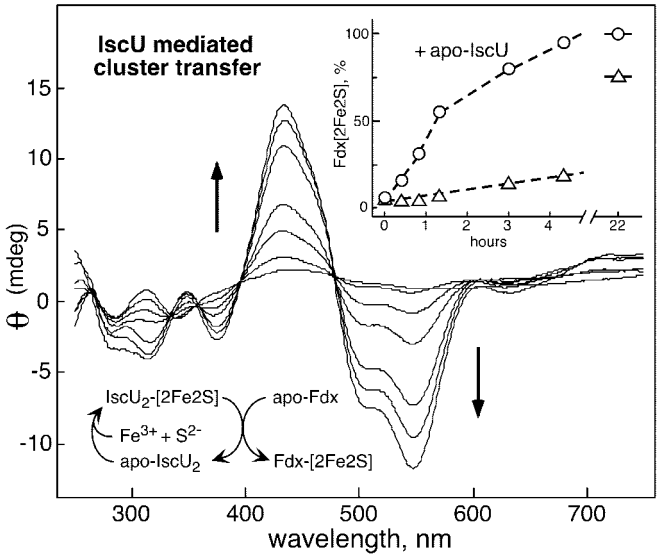

FIG. 2. **Time course of holo-ferredoxin (*Fdx*) formation during chemical reconstitution in the presence of catalytic amounts of apo-$IscU_2$.** CD spectra of a solution containing 50 μM apo-ferredoxin, 0.2 mM ferric ammonium citrate, and 5.5 μM apo-$IscU_2$ were taken immediately before and 25, 50, 80, 180, 260, and 1320 min after the addition of lithium sulfide to a final concentration of 0.2 mM. *Inset*, comparison of holo-ferredoxin formation in the presence (*circles*) and in the absence (*triangles*) of apo-IscU. Holo-ferredoxin formation was calculated using the change in ellipticity at 540 nm and compared with that of the native ferredoxin-[2Fe2S] complex.

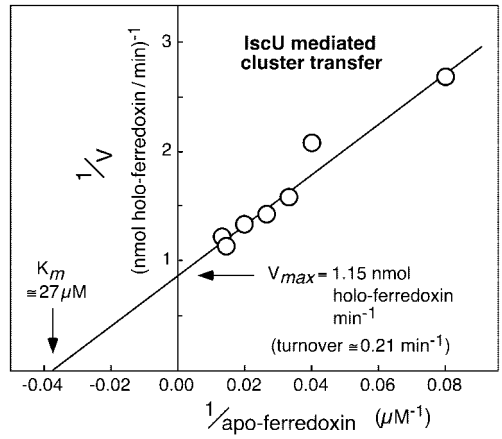

FIG. 3. **Lineweaver-Burk analysis of the dependence of the rate of apo-$IscU_2$-catalyzed holo-ferredoxin formation on apo-ferredoxin concentration.** Initial rates of holo-ferredoxin formation were monitored by CD spectroscopy at 540 nm in mixtures containing 5.5 μM apo-$IscU_2$, 0.2 mM ferric ammonium citrate, and the indicated concentration of apo-ferredoxin. Reactions were initiated by the addition of lithium sulfide to a final concentration of 0.2 mM. Rates were corrected for the rate of background chemical reconstitution at each apo-ferredoxin concentration in the absence of apo-IscU.

333, 357, 394, and 478 nm. The occurrence of isodichroic wavelengths during the course of the reaction indicates that the $IscU_2$-[2Fe2S] and ferredoxin-[2Fe2S] complexes are the only spectroscopically detectable species and suggests that if intermediate [FeS] species are formed during cluster transfer, they do not accumulate to a significant degree.

The reaction kinetics of the IscU-mediated cluster transfer process were further investigated by varying the concentration of apo-ferredoxin. Experiments were carried out using 10–75 μM apo-ferredoxin in the absence and presence of 5.5 μM IscU, and rates of ferredoxin-[2Fe2S] formation in the presence of IscU were corrected for spontaneous formation in the absence of IscU. The results are presented in Fig. 3 in the form of a

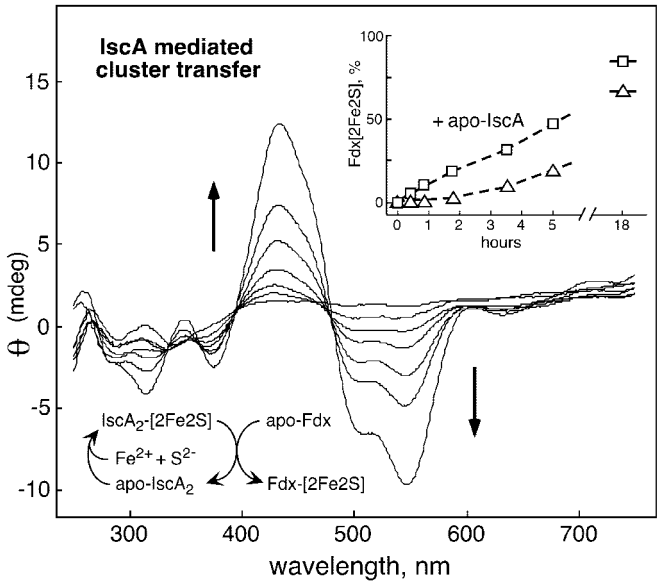

FIG. 4. **Time course of holo-ferredoxin (*Fdx*) formation during chemical reconstitution in the presence of catalytic amounts of apo-IscA$_2$.** CD spectra of a mixture containing 50 μM apo-ferredoxin, 0.2 mM ferrous ammonium sulfate, and 5.7 μM apo-IscA$_2$ were taken immediately before and 25, 50, 105, 210, 300, and 1440 min after the addition of lithium sulfide to a final concentration of 0.2 mM. *Inset*, comparison of holo-ferredoxin formation in the presence (*squares*) and in the absence (*triangles*) of apo-IscA. Holo-ferredoxin formation was calculated using the change in ellipticity at 540 nm and compared with that of the native ferredoxin-[2Fe2S] complex.

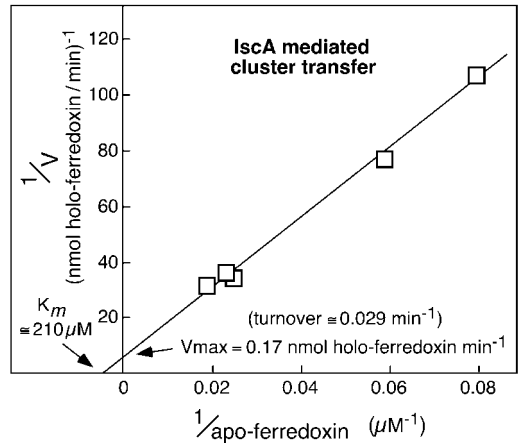

FIG. 5. **Lineweaver-Burk analysis of the dependence of the rate of apo-IscA$_2$-catalyzed holo-ferredoxin formation on apo-ferredoxin concentration.** Initial rates of holo-ferredoxin formation were monitored by CD spectroscopy at 540 nm in mixtures containing 5.7 μM apo-IscA$_2$, 0.2 mM ferrous ammonium sulfate, and the indicated concentration of apo-ferredoxin. Reactions were initiated by the addition of lithium sulfide to a final concentration of 0.2 mM, and rates were corrected for the rate of background chemical reconstitution at each apo-ferredoxin concentration in the absence of apo-IscA.

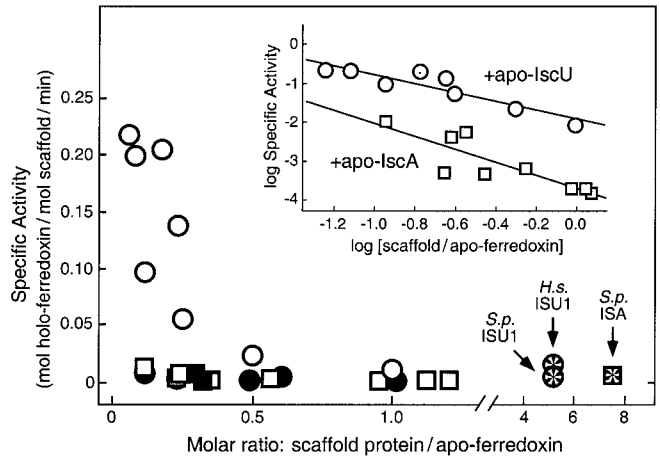

FIG. 6. **Effect of scaffold:acceptor ratio on specific activity of IscU- and IscA-mediated holo-ferredoxin assembly.** The initial rates of holo-ferredoxin formation in experiments employing apo-ferredoxin concentrations from 45 to 72 mM are compared for different concentrations of IscU$_2$ and IscA$_2$. Rates in experiments employing apo-IscU$_2$ (*open circles*) or apo-IscA$_2$ (*open squares*) were corrected for those observed in the absence of the scaffold proteins. *Filled symbols* correspond to rates determined in experiments employing preformed holo-IscU$_2$-[2Fe2S] (*solid circles*) or holo-IscA$_2$-[2Fe2S] (*solid squares*) complexes. Specific activities for cluster transfer from preformed human holo-Isu$_2$ (*H.s. ISU1*) to human apo-ferredoxin (15), from preformed *Schizosaccharomyces pombe* holo-Isu$_2$ (*S.p. ISU1*) to *S. pombe* apo-ferredoxin (15), and from preformed *S. pombe* holo-Isa$_2$ (*S.p. ISA*) to *S. pombe* apo-ferredoxin (17) are from Table I. Daisy wheel symbols (*circles* for ISU1 and *square* for ISA1) were used to distinguish these data (Refs. 15 and 17) from our data (this study). *Inset*, double logarithmic plot for the dependence of the rate of holo-ferredoxin formation on the molar ratio between the apo-IscU$_2$ or apo-IscA$_2$ and apo-ferredoxin. *Lines* shown represent fits of each set of data to a linear regression function.

double reciprocal plot. Ferredoxin-[2Fe2S] formation in the presence of IscU exhibits Michaelis-Menten behavior suggesting formation of a complex between IscU$_2$-[2Fe2S] and apo-ferredoxin with rapid association/dissociation kinetics followed by slower transfer of the [FeS] cluster. The apparent K_m for the reaction is ≅27 μM suggesting a relatively weak interaction between the two proteins. The maximal turnover number extrapolated to saturating levels of apo-ferredoxin is ≅0.21 mol holo-ferredoxin/mol IscU$_2$ min^{-1} indicating that cluster transfer in this system is a relatively slow process.

Catalysis of Cluster Transfer by IscA—Studies were also carried out to determine whether IscA is able to catalyze multiple cluster transfer cycles. Because cluster assembly on IscA is more efficient with ferrous salts, apo-IscA and apo-ferredoxin were equilibrated with ferrous ammonium sulfate, and the reaction was initiated by addition of lithium sulfide. Fig. 4 shows a time course experiment employing a molar ratio of apo-IscA$_2$:apo-ferredoxin of 0.1 to 1. The stimulation of the initial rate of ferredoxin-[2Fe2S] formation (≅4-fold) is not as great as that observed with IscU, but enhancement continues throughout the reaction consistent with IscA mediating multiple catalytic cycles. The final yield (18 h) of holo-ferredoxin in the presence of IscA is also greater than in its absence (≅80 *versus* ≅65%) suggesting that the scaffold-mediated process is somewhat more effective. As found for IscU, the CD spectra at intermediate stages exhibit distinct isodichroic points (335, 392, and 478 nm) indicating that IscA$_2$-[2Fe2S] and ferredoxin-[2Fe2S] are the only detectable chromophoric species.

The kinetics of IscA-mediated cluster transfer were further investigated using 10–75 μM apo-ferredoxin in the absence and presence of 5.5 μM IscA, and rates were corrected for spontaneous ferredoxin-[2Fe2S] formation. Fig. 5 shows that IscA-mediated ferredoxin-[2Fe2S] formation exhibits Michaelis-Menten behavior indicative of formation of an IscA$_2$-[2Fe2S]·apo-ferredoxin complex with fast kinetics followed by slower cluster transfer. The apparent K_m observed (≅210 μM) suggests a weaker interaction between IscA-[2Fe2S] and apo-ferredoxin than that for IscU$_2$-[2Fe2S] (K_m ≅ 27 μM). In addition, the maximal extrapolated turnover number (≅0.03 min^{-1}) is ~7-fold slower than that observed with IscU$_2$-[2Fe2S] (≅0.2 min^{-1}) indicating that IscA is a less effective catalyst for cluster transfer to apo-ferredoxin. The lower affinity and poorer catalysis are consistent with the finding that the final yield obtained with IscA (≅75%, Fig. 4) is less than that obtained with IscU (≅100%, Fig. 2).

TABLE I
Rates of [FeS] cluster transfer from scaffold proteins to apoprotein acceptors

Scaffold	Acceptor	Scaffold:acceptor ratio[a]	Specific activity[b]	Reference
			mol/mol × min^{-1}	
IscU (*E. coli*)	Fdx (*E. coli*)	0.05:1	0.2	This work[c]
IscU (*E. coli*)	Fdx (*E. coli*)	1:1	0.01	This work[c]
Isu (*human*)	Fdx (*human*)	5.2:1	0.017	15[d]
Isu (*S. pombe*)	Fdx (*S. pombe*)	5.2:1	0.006	15[d]
IscA (*E. coli*)	Fdx (*E. coli*)	0.1:1	0.011	This work[c]
IscA (*E. coli*)	Fdx (*E. coli*)	1:1	0.0003	This work[c]
Isa (*S. pombe*)	Fdx (*S. pombe*)	7.6:1	0.005	17[d]

[a] The scaffold:acceptor ratio is calculated assuming that scaffold proteins function as dimeric complexes containing a single [2Fe2S] cluster.
[b] k_{obs}/scaffold:acceptor ratio.
[c] Apo-$IscU_2$ or apo-$IscA_2$ were used to mediate cluster assembly.
[d] Preformed Isu-[FeS] or Isa-[FeS] complexes were used as cluster donors. The specific activity for cluster transfer using preformed human holo-Isu_2 and human apo-ferredoxin (Fdx) and using preformed *S. pombe* holo-Isu_2 and *S. pombe* apo-ferredoxin were calculated from Table 1 of Ref. 15. The specific activity for cluster transfer using preformed *S. pombe* holo-Isa_2 and *S. pombe* apo-ferredoxin was calculated from Table 1 of Ref. 17.

Additional studies with other apo-protein acceptors will be required to determine whether the reduced effectiveness of IscA compared with IscU reflects a fundamental difference in the mechanism of cluster transfer by IscA and IscU or results from differences in their specific apo-protein acceptor preferences. There is some indication that $IscA_2$-[2Fe2S] may be more stable than $IscU_2$-[2Fe2S], and this could contribute to differences in rates of cluster transfer. *In vitro* studies have shown that clusters on $IscU_2$-[2Fe2S] can be transferred to apo-IscA, whereas $IscA_2$-[2Fe2S] will not transfer its cluster to apo-IscU (19). However, the exact cellular role of IscA has not been established. In addition to forming [FeS] complexes, IscA binds iron and may play a role in iron delivery to IscU for cluster assembly rather than as an alternate [FeS]-scaffold protein (27, 28).

Effect of Scaffold Concentration—Additional cluster transfer experiments were carried out using molar ratios of apo-IscU and apo-IscA to apo-ferredoxin ranging from 0.05:1 to 1.2:1. An analysis of the results in terms of the observed specific activity (mol holo-ferredoxin/mol scaffold/min) *versus* the ratio of $IscU_2$ or $IscA_2$ to apo-ferredoxin is presented in Fig. 6. Surprisingly, the specific activities observed with both IscA and IscU were found to vary with the scaffold:apo-ferredoxin ratio. Higher specific activities were observed at lower ratios, and the specific activity increased >10-fold as the ratio of scaffold:acceptor decreased from about 1:1 to less than 0.1:1. Reliable rate measurements at low $IscA_2$:apo-ferredoxin ratios could not be obtained, but the higher activity of IscU made it possible to determine rates at $IscU_2$:apo-ferredoxin ratios as low as 0.05:1. The increased activity of IscU at low ratios is especially evident at $IscU_2$:apo-ferredoxin ratios lower than 0.3:1. Specific activities increased ~20-fold from ≅0.01 to ≅0.2 mol holo-ferredoxin/mol $IscU_2$/min as the scaffold:acceptor ratio decreased from 1:1 to 0.05:1. The specific activities observed using these low amounts of apo-IscU are also greater than those reported for transfer of preformed clusters in other systems. Table I gives a comparison of the specific activities observed in our multiple turnover assays with specific activities calculated from rates of transfer from preformed Isu and Isa complexes reported by others. The specific activity using a low ratio of apo-IscU is 10–30-fold higher than single-transfer reactions using preformed Isu-[FeS] complexes. IscA is less effective than IscU, and the specific activities we observed in multiple turnover reactions differed only slightly from the single-cluster transfer activities using preformed Isa-[FeS] clusters.

The basis for the observed enhanced cluster transfer at low scaffold-to-acceptor ratios is not known. One explanation is that cluster transfer may involve a labile scaffold-[FeS] species that is transiently formed during initial cluster assembly, and high levels of apo-protein acceptor may favor capture and transfer of this species prior to its maturation to a more stable scaffold-[2Fe2S] complex. A more labile species may also be favored at low scaffold concentrations. The reactive scaffold-[FeS] species may differ from the mature complex in the structure of the [FeS] cluster and/or the structure of the scaffold protein. Transient [FeS] complexes having different nuclearity have been described for both IscU (8) and IscA (10), and intermediate [FeS] species may participate in the transfer process. Solution NMR studies of apo-IscU from *Thermotoga maritima* (29, 30), human,[1] yeast[1] and *E. coli*[2] indicate presence of significant dynamic flexibility in the protein backbone, and a specific $IscU_2$-[2Fe2S] species with a conformation that differs from the major component could be involved in interactions with acceptor proteins and in cluster transfer.

Cellular Mechanism of Cluster Transfer—The maximal rates of scaffold-mediated cluster transfers observed in the *in vitro* experiments reported to date are slow (<1 min^{-1}), and participation of additional cellular components may be required to achieve efficient cluster transfer *in vivo*. Molecular chaperones have been implicated in iron-sulfur protein biosynthesis (*cf.* Refs. 1–3), and these could play a role in generating or stabilizing reactive scaffold-[FeS] species and thereby facilitate cluster transfer. Recently a report appeared describing studies on the effect of the heat shock chaperone DnaK from *T. maritima* on transfer of preformed [FeS] complexes of *T. maritima* IscU or human Isu to human ferredoxin (31). The results from that study suggested that DnaK has only a minor influence on scaffold-[FeS] stability or transfer, and nucleotides and the co-chaperone DnaJ did not elicit effects expected for a specific, chaperone-assisted system (31). Other bacteria and eukaryotes, however, have specialized chaperone/co-chaperone systems that display specific interactions with IscU/Isu homologs (32–38), and these may function to regulate [FeS] cluster transfer *in vivo*.

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Multiple Turnover Transfer of [2Fe2S] Clusters by the Iron-Sulfur Cluster Assembly Scaffold Proteins IscU and IscA

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