Role of the IscU Protein in Iron−Sulfur Cluster Biosynthesis: IscS-mediated Assembly of a $[Fe_2S_2]$ Cluster in IscU

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Iron−sulfur clusters are ubiquitous in nature,[1] but the mechanisms for assembly and insertion of these centers in their cognate proteins are largely unknown. Recent genetic and biochemical studies point to a common mechanism, centered around two proteins termed IscS and IscU in prokaryotes,[2] that has been preserved for general Fe−S cluster biosynthesis in both prokaryotic and eukaryotic cells.[2,3] Functional and primary sequence homologues of IscS and IscU were first identified as the products of the nitrogen fixation *nifS* and *nifU* genes that specifically target the biosynthesis of the nitrogenase Fe−S clusters in *Azotobacter vinelandii*.[4] IscS is closely related to NifS, and both have been shown to be homodimeric, pyridoxyl phosphate-dependent L-cysteine desulfurases, catalyzing the reductive conversion of cysteine to alanine and sulfide via an enzyme-bound persulfide intermediate.[2,5] The importance of IscU for Fe metabolism, in general, and Fe−S cluster biosynthesis, in particular, can be gauged by the fact that it is one of the most conserved sequence motifs in nature.[6] IscU has three conserved cysteine residues (Cys^{37}, Cys^{63}, and Cys^{106} in *A. vinelandii* and *E. coli* IscU)[2] and corresponds to the N-terminal third of NifU,[2,7] which has been overexpressed as a separate domain and termed NifU-1.[7b,8] These similarities, along with the observation that the *nif*-specific iron−sulfur cluster assembly operon is less complicated due to the absence of putative molecular chaperone genes, have led to our using the *nif*-specific pathway as a model for general biological iron−sulfur cluster assembly. Although we were successful in obtaining evidence for NifS-mediated assembly of a transient $[Fe_2S_2]^{2+}$ cluster within NifU,[8] the lability of this cluster and interference from the permanent $[Fe_2S_2]^{2+,+}$ cluster present in the central domain of NifU, impeded unambiguous characterization of this transient cluster in full-length NifU.[8] The involvement of IscU/IscS system in general Fe−S cluster biosynthesis and the lack of a permanent $[Fe_2S_2]$ cluster in IscU, clearly make this a more attractive system for investigating cluster assembly in IscU. Using purified *A. vinelandii* IscU and IscS, we report here the IscS-mediated assembly of a reductively labile $[Fe_2S_2]^{2+}$ cluster in IscU and evidence for the formation of a $\alpha_2\beta_2$ heterotetrameric complex between the homodimeric IscU and IscS proteins. The results support a general mechanism for biological iron−sulfur cluster assembly in which IscU (or NifU) provides a scaffold for IscS (or NifS)-directed assembly of a reductively labile $[Fe_2S_2]$ cluster.

The initial evidence for the assembly of a $[Fe_2S_2]^{2+}$ cluster in *A. vinelandii* IscU was provided by UV−visible absorption spectroscopy, see Figure 1. Samples of IscU do not exhibit a visible chromophore as purified but turn red over a period of 60 min on anaerobic treatment with catalytic amounts of IscS in the presence of excess L-cysteine and a stoichiometric amount of ferric ammonium citrate (based on the concentration of IscU monomer).[9] The resulting spectrum has bands centered at 320, 410, and 456 nm and a pronounced shoulder at 510 nm and is characteristic of a $[Fe_2S_2]^{2+}$ cluster.[10] Control experiments in the absence of IscU confirm that this chromophore is associated with the IscU protein (see inset in Figure 1) and the absorption intensity of this chromophore only increased for ferric concentrations up to 1 equiv per IscU monomer (based on experiments with 0.5, 1.0, 2.0, and 8.0 equiv of ferric ammonium citrate, data not shown). The maximal visible extinction coefficients, $\epsilon_{456} = 4.6\ mM^{-1}\ cm^{-1}$ and $\epsilon_{410} = 4.7\ mM^{-1}\ cm^{-1}$ are indicative of approximately one $[Fe_2S_2]^{2+}$ cluster per IscU dimer based on the published values for well-characterized 2Fe ferredoxins, $\epsilon_{460} = 6-10\ mM^{-1}\ cm^{-1}$ and $\epsilon_{420} = 8-11\ mM^{-1}cm^{-1}$.[10] In addition, the stoichiometry of one $[Fe_2S_2]^{2+}$ cluster per IscU dimer is supported by iron analyses and the A_{456}/A_{280} ratio. In contrast to NifU-1,[8] the $[Fe_2S_2]^{2+}$ cluster assembled in IscU stayed intact during brief exposure to excess EDTA (10-fold excess with respect to iron for <5 min) followed by anaerobic gel filtration and anion-exchange chromatography to remove excess reagents. Samples of $[Fe_2S_2]$-containing IscU prepared using the above procedure with an 8-fold stoichiometric excess of ferric ion per IscU monomer for 3 h and subjected to EDTA treatment followed by chromatographic repurification prior to analysis, yielded 1.0 ± 0.1 Fe per IscU monomer (average of three determinations).[11] The resulting sample exhibited visible absorption characteristics identical to that shown in Figure 1, with $A_{456}/A_{280} = 0.20$. This ratio is approximately half the value observed for 2Fe ferredoxins with similar numbers of UV-absorbing aromatic residues per monomeric unit and therefore indicative of one $[Fe_2S_2]^{2+}$ cluster per IscU dimer.[12]

Conclusive evidence that the chromophore assembled in IscU is a $[Fe_2S_2]^{2+}$ cluster was provided by resonance Raman spectroscopy, see Figure 2. The spectrum in the Fe−S stretching region is uniquely indicative of a $[Fe_2S_2]^{2+}$ center,[13] and the individual bands are readily assigned on the basis of the isotope shifts and normal mode calculations reported for 2Fe ferredoxins and model complexes,[14] see Table 1. Although the Fe−S

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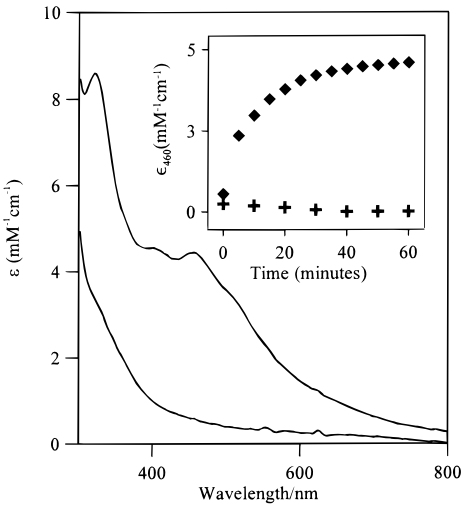

Figure 1. IscS-mediated in vitro assembly of a $[Fe_2S_2]^{2+}$ cluster in IscU. UV−visible absorption spectrum of *A. vinelandii* IscU before (lower spectrum) and after 30 min of IscS-mediated cluster assembly (upper spectrum). Cluster biosynthesis was initiated by addition of 4 mM L-cysteine to 28 μM IscU in the presence of 0.4 μM IscS and 5 mM β-mercaptoethanol under strictly anaerobic conditions. The inset shows the time course monitored at 460 nm for reactions carried out in the presence (◆) and absence (+) of IscU.

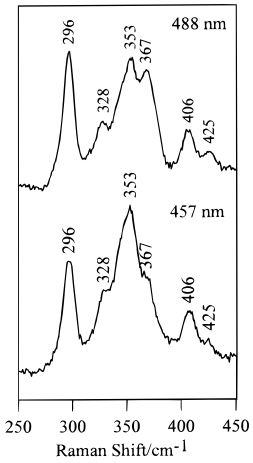

Figure 2. Low-temperature (18 K) resonance Raman spectra of *A. vinelandii* IscU after IscS-mediated cluster assembly. The sample was prepared as described in Figure 1 with the reaction allowed to proceed for 3 h. The spectra were recorded using 488- and 457-nm excitation with 70-mW of laser power at the sample (~2 mM).

stretching modes of the $[Fe_2S_2]^{2+}$ cluster assembled in *A. vinelandii* IscU all occur at higher frequencies, compared to that assembled in *A. vinelandii* NifU-1 and the all-cysteinyl-ligated $[Fe_2S_2]^{2+}$ clusters present in as-purified samples of *A. vinelandii* NifU and human ferrochelatase, the relative intensities of individual modes using 488- and 457-nm excitation are strikingly similar in all four of these proteins.[7a,8,15] This strongly suggests analogous all-cysteine coordination environments for the $[Fe_2S_2]^{2+}$ centers in all four proteins with slightly stronger Fe−S(Cys) and Fe−S(bridging) bonds being responsible for the higher frequencies of the $[Fe_2S_2]^{2+}$ center in IscU.[16] Coordination by four cysteine residues requires the cluster to be bridging subunits in IscU, and this is consistent with the cluster stoichiometry based on absorption and analytical data presented above.

Table 1. Fe−S Stretching Frequencies (cm^{-1}) and Assignments for the $[Fe_2S_2]^{2+}$ Centers Assembled in *A. vinelandii* IscU and NifU-1 and Present in *A. vinelandii* NifU and Human Ferrochelatase

mode (D_{2h})[a]	IscU	NifU-1[b]	NifU[c]	ferrochelatase[d]
$B_{2u}{}^b$	425	~420	417	420
$A_g{}^b$	406	401	393	398
$B_{3u}{}^b$	367	364	356	365
$A_g{}^t$	353	349	342	350
$B_{1g}{}^b$	328	320	314	320
$B_{3u}{}^t$	296	294	288	295

[a] Symmetry labels under idealized D_{2h} symmetry for an $Fe_2S_2{}^bS_4{}^t$ unit, where S^b and S^t indicate bridging and terminal (cysteinyl) S, respectively. [b] Taken from Yuvaniyama et al.[8] [c] Taken from Fu et al.[7a] [d] Taken from Crouse et al.[15]

The reductive lability of the $[Fe_2S_2]^{2+}$ cluster assembled in IscU was demonstrated by three distinct types of experiment. First, anaerobic reduction with stoichiometric dithionite resulted in complete and oxidatively irreversible bleaching of the visible absorption. Second, EPR studies gave no indication of the formation of a $S = {}^1/_2$ $[Fe_2S_2]^{+}$ cluster on reduction, even in samples frozen within 2 s of dithionite addition. Third, the iron released on dithionite reduction was quantitatively accounted for using ferrous ion chelators such as ferrozine or α,α'-dipyridyl.

Evidence that IscS and IscU are able to interact with each other was obtained in two different ways. First, gel exclusion chromatography was used to show that an equimolar mixture of isolated IscS dimer and isolated IscU dimer results in formation of a weakly bound $\alpha_2\beta_2$ heterotetramer (data not shown). Second, the thiol-specific cross-linking reagent dibromobimane could be used to cross-link one subunit of IscS to one subunit of IscU, see Supporting Information. The results of these experiments are consistent with the hypothesis that persulfides catalytically formed on IscS[2] can be specifically transferred to IscU for cluster assembly through association of the two proteins.

The results indicate a general mechanism for Fe−S cluster biosynthesis in which IscU provides a scaffold for IscS-mediated assembly of $[Fe_2(\mu_2\text{-}S)_2]$ cores. Structurally and electronically these units constitute the fundamental building blocks of biological $[Fe_2S_2]$, $[Fe_3S_4]$, and $[Fe_4S_4]$ centers.[1] Hence, our current working hypothesis is that these units are transferred intact into apoproteins. The Fe−S cluster biosynthesis gene cluster that is widely conserved in prokaryotes, encodes for several proteins whose functions are currently unknown, but are good candidates for mediating such transfer. The most likely candidates are the IscA protein that contains three conserved cysteine residues and the heat shock proteins HscA and HscB that bear sequence homology to the molecular chaperones.[2] Determining how the Fe^{3+} ion is acquired by IscU and how the $[Fe_2S_2]$ cores assembled in IscU are released and transferred into apoproteins present fascinating challenges for future studies.

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Supporting Information Available: Figure S1, denaturing polyacrylamide gel electrophoresis evidence for dibromobimane cross-linking of IscU and IscS.

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(16) The possibility that the anomalously high frequencies of the predominantly Fe−S^t stretching modes of the $[Fe_2S_2]^{2+}$ centers assembled in IscU and NifU-1 are a consequence of partial oxygenic ligation cannot be ruled out. Mutagenesis studies involving 2Fe ferredoxins indicate that the $A_g{}^t$ and $B_{3u}{}^t$ Fe−S stretching modes in the IscU and NifU-1 lie within the ranges established thus far for $[Fe_2S_2]^{2+}$ centers with one serinate ligand, i.e., 332−354 cm^{-1} and 289−302 cm^{-1}, respectively.