In Vitro Activation of Apo-Aconitase Using a [4Fe-4S] Cluster-Loaded Form of the IscU [Fe−S] Cluster Scaffolding Protein†

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ABSTRACT: Genetic experiments have established that IscU is involved in maturation of [Fe−S] proteins that require either [2Fe-2S] or [4Fe-4S] clusters for their biological activities. Biochemical studies have also shown that one [2Fe-2S] cluster can be assembled in vitro within each subunit of the IscU homodimer and that these clusters can be reductively coupled to form a single [4Fe-4S] cluster. In the present work, it is shown that the [4Fe-4S] cluster-loaded form of *A. vinelandii* IscU, but not the [2Fe-2S] cluster-loaded form, can be used for intact cluster transfer to an apo form of *A. vinelandii* aconitase A, a member of the monomeric dehydratase family of proteins that requires a [4Fe-4S] cluster for enzymatic activity. The rate of [4Fe-4S] cluster transfer from IscU to apo-aconitase A was not affected by the presence of the HscA/HscB co-chaperone system and MgATP. However, an altered form of a [4Fe-4S] cluster-containing IscU, having the highly conserved aspartate-39 residue substituted with alanine, is an effective inhibitor of wild-type [4Fe-4S] cluster-loaded IscU-directed activation of apo-aconitase A. In contrast, neither the clusterless form of IscU nor the [2Fe-2S] cluster-loaded form of IscU is an effective inhibitor of IscU-directed apo-aconitase A activation. These results are interpreted to indicate that the [2Fe-2S] and [4Fe-4S] cluster-loaded forms of IscU adopt different conformations that provide specificity with respect to the maturation of [2Fe-2S] and [4Fe-4S] centers in proteins.

Combinations of inorganic $Fe^{2+/3+}$ and acid labile S^{2-} are referred to as [Fe−S] clusters and proteins that contain such clusters are known as [Fe−S] proteins. [Fe−S] proteins are required to sustain essential life processes in most, and perhaps all, free-living organisms. The wide range of physiological processes in which [Fe−S] proteins participate, including electron transfer, substrate activation, and regulation of gene expression, can be attributed to the electronic and structural plasticity of their cognate [Fe−S] clusters (*1*, *2*). A large variety of different types of [Fe−S] clusters are found in nature, but the simplest and most abundant forms are [2Fe-2S] and [4Fe-4S] clusters. In recent years it has become clear that maturation of [Fe−S] proteins does not occur spontaneously but instead requires a complex biosynthetic system. Three different types of bacterial [Fe−S] cluster assembly systems have now been described: the Isc (iron-sulfur cluster),[1] Suf (sulfur mobilization), and Nif (nitrogen fixation) systems (*2*−*4*). In the case of *Escherichia coli*, the Isc system appears to be a generalized system involved in maturation of most [Fe−S] proteins expressed under normal conditions (*5*), whereas the Suf system operates under conditions of Fe limitation or oxidative stress (*6*−*8*). The nitrogen-fixing aerobe, *Azotobacter vinelandii*, contains an Isc system as well as the Nif system, which has a specific function in the maturation of [Fe−S] proteins involved in nitrogen fixation (*9*, *10*). One or more of the [Fe−S] cluster assembly systems identified so far, or certain combinations or remnants of these systems, can be found in all bacteria examined, although the physiological conditions under which an individual [Fe−S] cluster biosynthetic system is operational appears to be different for different organisms. A unifying feature of all [Fe−S] cluster biosynthetic systems is that they appear to include a pyridoxal phosphate dependent cysteine desulfurase, responsible for the mobilization of sulfur by formation of an active-site persulfide and a scaffold protein that is used for the preassembly of clusters prior to their delivery to target [Fe−S] proteins. The current model is that a cysteine desulfurase specifically delivers activated S to the assembly scaffold, which forms an [Fe−S] cluster when combined with Fe (*11*−*16*). Neither the specific nature of Fe delivery to the assembly scaffold within bacterial cells nor the physiological sequence of events with regard to Fe

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[1] Abbreviations: Isc, iron-sulfur cluster; Suf, sulfur mobilization; Nif, nitrogen fixation DTT, dithiothreitol; Fdx, ferredoxin; hsc, heat-shock cognate; AcnA, aconitase A; IMAC, immobilized metal affinity chromatography; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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and S delivery is well understood (*17*, *18*). Nevertheless, it is clear that the activation of S via the formation of an active-site persulfide and the participation of an assembly scaffold protein provide a biological strategy for maturation of [Fe−S] proteins without the accumulation of high concentrations of $Fe^{2+/3+}$ and S^{2-} that would be necessary for spontaneous intracellular [Fe−S] protein maturation.

Among the variety of bacterial [Fe−S] cluster biosynthetic systems that have been described, Isc has attracted considerable interest because homologs to the primary assembly components of the Isc system appear to be present in most, and perhaps all, eukaryotic organisms (*19*). The Isc system from both *E. coli* and *A. vinelandii* is encoded by a seven-gene transcriptional unit: *iscR*, *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, *fdx*, and *orf3* (*5*, *10*). Clear physiological and biochemical functions have now been established, in *E. coli* or *A. vinelandii*, for IscR, IscU, and IscS. IscR is a regulatory protein that controls expression of the transcriptional unit in response to the physiological demand for [Fe−S] cluster assembly (*20*, *21*). IscU is an [Fe−S] cluster assembly scaffold (*22*, *23*), and IscS is a cysteine desulfurase (*13*). The roles of IscA, HscA, HscB, and Fdx are less well-defined. In vitro studies indicate that IscA has the potential to function as an alternative scaffold protein (*24*−*27*) or a specific Fe donor for IscU (*17*, *28*), and recent in vivo studies indicate that IscA is likely to be operative only under oxidative stress conditions (*29*). HscA has been shown to bind to a specific LPPVK motif on IscU (*30*, *31*), and the HscA/HscB co-chaperone system has recently been shown to facilitate [2Fe-2S] cluster transfer from IscU to apo-IscFdx in an ATP-dependent reaction (*32*).

During development of the scaffold hypothesis for [Fe−S] protein maturation, we incubated a purified sample of *A. vinelandii* IscU with catalytic amounts of IscS, together with L-cysteine and Fe^{2+}, and observed the formation of approximately one reductively labile $[2Fe\text{-}2S]^{2+}$ cluster on the homodimeric IscU protein (*22*). Similar results have since been reported with other IscU proteins, and it has also been shown that [2Fe-2S] cluster-loaded forms of IscU can be used in vitro to supply clusters for maturation of certain [2Fe-2S] cluster-containing ferredoxins (*25*, *33*−*35*). A subsequent careful analysis of an extended time course of IscS-directed cluster assembly on IscU revealed the sequential formation of a single [2Fe-2S] cluster, two $[2Fe\text{-}2S]^{2+}$ clusters, and finally, the slow formation of a single $[4Fe\text{-}4S]^{2+}$ cluster on each IscU homodimer (*23*). Importantly, formation of the $[4Fe\text{-}4S]^{2+}$ cluster-containing form was accompanied by a loss of the $2\times[2Fe\text{-}2S]^{2+}$ cluster species, suggesting that the $[4Fe\text{-}4S]^{2+}$ species is formed via reductive coupling of the two $[2Fe\text{-}2S]^{2+}$ clusters (*23*). Although such reductive coupling of paired $[2Fe\text{-}2S]^{2+}$ clusters to form $[4Fe\text{-}4S]^{2+}$ clusters has precedence in synthetic inorganic chemistry (*36*), our observations raised several important questions. First, does $[4Fe\text{-}4S]^{2+}$ cluster biosynthesis occur by reductive coupling of two $[2Fe\text{-}2S]^{2+}$ clusters and, if so, what are the in vitro and in vivo reductants? Second, can different forms of cluster-loaded IscU be used for the in vitro maturation of different types of [Fe−S] proteins? The first question was resolved in the accompanying manuscript when we found that the $2\times[2Fe\text{-}2S]^{2+}$ form of the IscU homodimer is immediately and quantitatively converted to the $[4Fe\text{-}4S]^{2+}$ form on the addition of two reducing equivalents using dithionite as an exogenous reductant (*37*). In addition, reduced IscFdx was shown to a be a competent electron donor for mediating at least partial reductive coupling, thereby providing the first experimental evidence of a potential physiological role for the Isc $[2Fe\text{-}2S]^{2+,+}$ ferredoxin (*37*). The slow formation of $[4Fe\text{-}4S]^{2+}$ clusters on IscU during IscS-mediated cluster assembly in the absence of an exogenous one-electron donor (*23*) was tentatively attributed to disulfide formation between two of the released thiolates (*37*). A redox active disulfide proximal to the cluster would be an attractive candidate for controlling cluster release. In the present work, we address the potential functional properties of *A. vinelandii* IscU by examining the capacity of the corresponding $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ cluster-loaded forms to activate *A. vinelandii* aconitase A (AcnA), a member of the dehydratase family of monomeric [4Fe-4S] cluster-containing enzymes that requires a [4Fe-4S] cluster for catalytic activity.

MATERIALS AND METHODS

Plasmids for Protein Expression. Plasmids pDB956, pDB943, pDB1303, and pDB1036 were used for heterologous expression of *A. vinelandii* IscU, IscS, HscA, and HscB, respectively. Plasmid pDB1059 was used to express a version of IscU having the Asp^{39} residue substituted with Ala (designated $IscU^{D39A}$), and pDB1300 was used to express an N-terminal version of a polyhistidine-tagged *A. vinelandii* aconitase A (AcnA). These recombinant plasmids place expression of the corresponding proteins under control of the T7 promoter. For these experiments, pT_7-7 was the vector, and *E. coli* strain BL21(DE3) was used as the host. For heterologous expression, cells harboring the appropriate plasmid were cultured in 2 liter flasks containing 500 mL of LB medium supplemented with 50 mg of ampicillin in a reciprocal shaker at 30 °C/300 rpm until they reached 160−180 Klett (red filter). Gene expression was induced by the addition of 5 g (1%) of lactose. After induction, cells were cultured for 3 h and then harvested by centrifugation. Cell pellets were frozen at −20 °C until they were used.

IscU Purification and Preparation of Cluster-Loaded IscU Samples. Purification of all of the protein samples was performed under anoxic conditions maintained using either Schlenk lines or a Coy anaerobic chamber containing 5% hydrogen gas balanced with nitrogen gas. All of the buffers were exhaustively purged with oxygen-free argon. Purification of recombinantly produced *A. vinelandii* IscU, which contains no [Fe−S] clusters in its as-isolated form, was performed as follows. Extracts were prepared by resuspending 5 g of cell pellets in 30 mL of buffer A (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol (DTT), 10% glycerol, and 4 mM $MgCl_2$), sonicated on ice for 10 min (duty cycle constant 65% and output control 7), and then centrifuged at 35 000 rpm for 25 min at 4 °C. The resulting supernatant was then applied to a 25 mL Q-Sepharose column (column diameter 1.5 cm) equilibrated with buffer A. The column was washed with buffer A until the absorbance at 280 nm reached baseline. A 60 mL linear gradient (0−1 M NaCl in buffer A) was applied at a flow rate of 2 mL min^{-1} with IscU eluting at ∼0.32 M NaCl. All of the protein samples prepared in this work could be stored in liquid nitrogen with no loss in activity.

For purification of $[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU, equal amounts of cell pellets (5 g each) containing separately expressed IscU and IscS were resuspended in 30 mL of buffer B (20 mM TEA (pH 7.8) containing 1 mM DTT, 10% glycerol, and 4 mM $MgCl_2$), then sonicated and centrifuged as described above. For assembly of $[2Fe\text{-}2S]^{2+}$ clusters, the extract (~40 mL) was incubated with 2 mM ferrous ammonium sulfate and 1 mM L-cysteine (final concentration) for 30 min at room temperature. The cell extract was then applied to a 30 mL DEAE-Sepharose column (column diameter 1.5 cm) pre-equilibrated with buffer B using a flow rate of 2 mL min^{-1}. The column was washed with buffer B until the absorbance at 280 nm reached baseline, then sequentially washed with 2 bed volumes of buffer B plus 40 mM NaCl, 1.5 bed volumes of buffer B (plus 60 mM NaCl), and one bed volume of buffer B (plus 120 mM NaCl). The $[2Fe\text{-}2S]^{2+}$ cluster-loaded form of IscU was then eluted using buffer B (plus 150 mM NaCl) at a flow rate of 3 mL min^{-1}.

Because purification of the $[2Fe\text{-}2S]^{2+}$ cluster-loaded form of $IscU^{D39A}$ using DEAE-Sepharose chromatography resulted in a very dilute sample, a modified protocol for purification of this protein was developed. For these samples, Q-Sepharose was used as the column matrix instead of a DEAE-Sepharose column. Cell extracts were applied to the Q-sepharose column pre-equilibrated with buffer A, and washed with buffer A using a flow rate of 2 mL min^{-1} until the absorbance at 280 nm reached baseline. The column was then washed with 2 bed volumes of buffer A (plus 40 mM NaCl), 1 bed volume of buffer A (plus 60 mM NaCl), and the [2Fe-2S] cluster-loaded form of $IscU^{D39A}$ eluted using buffer A (plus 150 mM NaCl).

$[4Fe\text{-}4S]^{2+}$ cluster-loaded samples of wild-type IscU or $IscU^{D39A}$ were obtained by reducing the $2\times[2Fe\text{-}2S]^{2+}$ cluster-containing samples with 1.5 reducing equivalents per $[2Fe\text{-}2S]^{2+}$ cluster using a freshly prepared stock solution of sodium dithionite (~5 mM in 50 mM Tris/HCl pH 7.4). The concentration of the dithionite solution used for these experiments was assessed by titration against benzyl-viologen.

Purification and Assay of A. vinelandii AcnA and Preparation of Apoprotein. Purification of recombinantly produced polyhistidine-tagged AcnA was performed under anoxic conditions as described above. Cell pellets (30 g) containing abundantly expressed polyhistidine-tagged AcnA were re-suspended in 60 mL binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl). The suspension was sonicated on ice for 20 min, as described above, and centrifuged at 35 000 rpm for 25 min at 4 °C. The supernatant was applied to a 50 mL Ni-charged iminodiacetic acid Sepharose (4Fast Flow) immobilized metal affinity chromatography (IMAC) column (column diameter 2.5 cm) equilibrated in binding buffer at a flow rate of 3 mL min^{-1}. The column was washed with binding buffer until the absorbance at 280 nm reached baseline. The column was then washed with binding buffer plus 50 mM imidazole, and AcnA was eluted using binding buffer plus 100 mM imidazole.

AcnA activity was measured spectrophotometrically at 240 nm at room temperature by following the formation of *cis*-aconitate from citrate or isocitrate, using a molar absorption coefficient ϵ_{240} of 3400 mM^{-1} cm^{-1} for *cis*-aconitate (*38*).

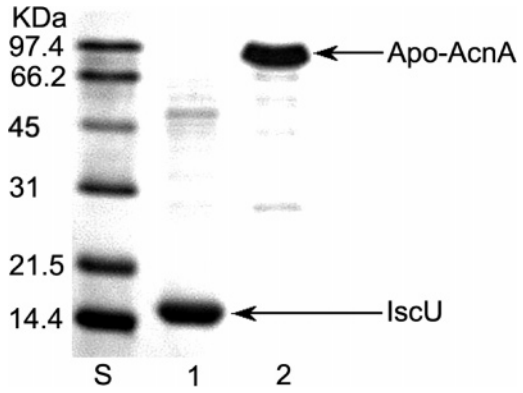

FIGURE 1: Gel electrophoretic analysis of wild-type IscU and apo-AcnA samples. Proteins were separated by 20% SDS-PAGE and stained with Coomasie brilliant blue. Lane S, molecular mass standards; Lane 1, purified IscU (12.5 μg); Lane 2, purified apo-AcnA (9.3 μg).

Assays (1 mL) were carried out in sealed anoxic cuvettes containing 900 μL of 100 mM Tris/HCl (pH 8.0) and initiated by addition of 100 μL of 200 mM citrate or isocitrate. Anaerobically reconstituted samples of *A. vinelandii* AcnA exhibited the same characteristic UV−visible spectrum as other aconitases with one [4Fe-4S] cluster per monomeric protein (*39*−*43*) and had a maximal specific activity of 25 units/mg using citrate (~100% activity) and 79 units/mg with isocitrate (~100% activity). The apo form of AcnA was obtained by incubating as-isolated AcnA with EDTA and potassium ferricyanide using the method described by Kennedy and Beinert (*39*). Apo-AcnA obtained in this way was dialyzed overnight in buffer A. Dialyzed apo-AcnA was then applied to a Q-Sepharose column (~30 mL bed volume) pre-equilibrated with buffer A using a flow rate of 2 mL min^{-1} and washed with 4 bed volumes of buffer A. Apo-AcnA was subsequently eluted in one step using buffer A plus 0.25 M NaCl.

Apo-AcnA Activation. Apo-AcnA activation mixtures (180 μL) contained 50 mM Tris/HCl (pH 7.4), 1 mM DTT, 10% glycerol, 4 mM $MgCl_2$, ~150 mM NaCl, 6.7 μM apo-AcnA, and 20.6 μM $[4Fe\text{-}4S]^{2+}$ wild-type IscU or 20.6 μM $[4Fe\text{-}4S]^{2+}IscU^{D39A}$. The molar concentration corresponds to the concentration of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU calculated using the molar absorption coefficient of $\epsilon_{390} = 14.8$ mM^{-1} cm^{-1} per $[4Fe\text{-}4S]^{2+}$ cluster (*23*). Activation mixtures were incubated at room temperature, and samples (5 μL) were taken at different time points and assayed for AcnA activity. When $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded wild-type IscU was tested for the ability to reconstitute apo-AcnA, 22.5 μM $2\times[2Fe\text{-}2S]^{2+}$ IscU was added to the reaction mixture. The molar concentration represents the concentration of $2\times[2Fe\text{-}2S]^{2+}$ IscU using a molar absorption coefficient of $\epsilon_{456} = 9.2$ mM^{-1} cm^{-1} per $[2Fe\text{-}2S]^{2+}$ cluster (*23*). The concentrations of the cluster-loaded forms of IscU available for apo-AcnA activation described above were estimated on the basis of cluster concentration, rather than protein concentration, because IscU samples used for these experiments were not completely pure (~80% estimated purity, Figure 1). As described in Results, certain samples of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU were passed over an IMAC column before being used for apo-AcnA activation assays. For these experiments, 600 μL of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU samples (in buffer A) were applied to a 700 μL uncharged IMAC column matrix that

was packed in a glass-wool plugged Pasteur pipet. $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU was eluted using buffer A. Integrity of the sample was evaluated by comparison of the UV−visible spectra of the pre- and post-column samples, which were immediately used for apo-AcnA activation assays.

Apo-AcnA Activation in the Presence of HscA/HscB/MgATP. Highly purified forms of $2\times[2Fe\text{-}2S]^{2+}$ and the $[4Fe\text{-}4S]^{2+}$ cluster-loaded forms of IscU were prepared to facilitate assessment of intact cluster transfer from IscU to apo-AcnA and the role of the HscA/HscB co-chaperone system in mediating cluster transfer. These cluster-loaded IscU samples were prepared inside a Vacuum Atmospheres glove box under an argon atmosphere (<1 ppm O_2) by overnight IscS-mediated cluster assembly on apo-IscU and cluster-bound IscU fractions were purified using a Mono Q column, as previously described (*23*). *A. vinelandii* HscA and HscB were expressed, purified, and assayed for ATPase activity as previously described (*32*). The effect of HscA/HscB/MgATP on IscU-mediated aconitase activation was addressed by incubating apo-AcnA (4 μM) with 12 μM $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU or 12 μM $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU at room temperature under anaerobic conditions in the absence and presence of 60 μM HscA and HscB, 24 mM $MgCl_2$, and 12 mM ATP. Activation mixtures were incubated at room temperature, and samples (5 μL) were taken at different time points and assayed for AcnA activity.

Mössbauer Experiments. Mössbauer experiments to address intact [4Fe-4S] cluster transfer from IscU to apo-AcnA used the highly purified samples of $[4Fe\text{-}4S]^{2+}$ IscU described in the previous section. To assess the activity of Mössbauer samples, apo-AcnA and cluster-loaded IscU were incubated in a Mössbauer cup for defined time intervals at room temperature. Prior to freezing in liquid nitrogen, 20 μL of the reaction mixture were withdrawn and analyzed for AcnA activity. In control experiments, apo-AcnA was omitted from the reaction mixture. Mössbauer spectra were recorded by using previously described instrumentation (*44*) and analyzed using the WMOSS program (Web Research). The zero velocity of the spectra refers to the centroid of a room-temperature spectrum of a metallic Fe foil.

Analytical Methods. UV−visible absorption spectra of $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ cluster-loaded samples of IscU were recorded under anoxic conditions in septum-sealed cuvettes, using a Cary 50 Bio or a Shimadzu 3101PC scanning spectrophotometer. Protein was estimated by the dye-binding method of Bradford (*45*) using the Bio-Rad protein assay kit with BSA as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (20% acrylamide) was performed as described by Laemmli (*46*), and visualization of the proteins was performed by Coomassie Blue staining (*47*). The following mass standards were used: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic acid anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Iron concentration was determined according to the method described by Fortune and Mellow (*48*), using ferrous ammonium sulfate as the standard. The time course of holo-AcnA formation at 22 °C was analyzed by fitting to second-order kinetics, on the basis of the initial concentration of apo-AcnA and $2\times[2Fe\text{-}2S]^{2+}$ or $[4Fe\text{-}4S]^{2+}$ IscU, using the Chemical Kinetics Simulator software package (IBM).

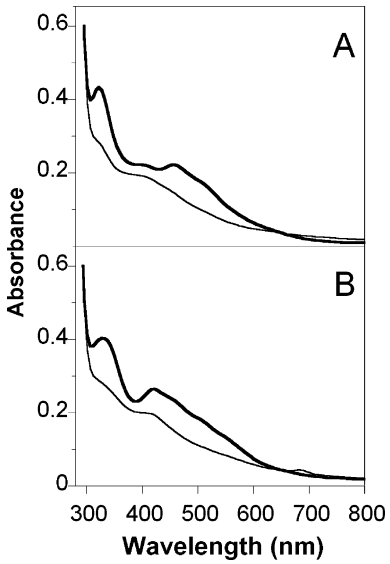

FIGURE 2: UV−visible absorption spectra of the wild-type IscU (35.7 μM; panel A) and $IscU^{D39A}$ (35.7 μM; panel B): as-isolated (thick line), reduced using 1.5 reducing equivalents of dithionite (thin line). The spectra were recorded in sealed anaerobic cuvettes.

RESULTS

[4Fe-4S] Cluster-Loaded Form of IscU Can Activate Apo-Aconitase. Our experimental strategy involved preparation of an inactive apo-form of AcnA and testing whether or not the $2\times[2Fe\text{-}2S]^{2+}$ cluster- or $[4Fe\text{-}4S]^{2+}$ cluster-loaded forms of IscU could be used to activate apo-AcnA catalytic activity. Figure 1 shows the typical purity of IscU and apo-AcnA samples used in these experiments and Figure 2A shows the UV−visible absorbance of the $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ cluster forms used in typical activation experiments. In these experiments, the $[2Fe\text{-}2S]^{2+}$ cluster-loaded form of IscU represents the IscS-directed [Fe−S] cluster assembly fraction that contains predominantly two $[2Fe\text{-}2S]^{2+}$ clusters per IscU homodimer, as judged by absorption spectra and the ability to undergo reductive coupling to yield $[4Fe\text{-}4S]^{2+}$ clusters (*37*). The $[4Fe\text{-}4S]^{2+}$ cluster-loaded form of IscU was prepared by reductive coupling of the $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded sample by addition of dithionite to provide 1.5 reducing equivalents per $[2Fe\text{-}2S]^{2+}$ cluster. The number of reducing equivalents required for cluster conversion in these samples was dictated by reductive titrations of $2\times[2Fe\text{-}2S]^{2+}$ IscU samples monitored by absorption spectroscopy. Quantitative comparison with the absorption spectra of homogeneous and heterogeneous IscU samples with Mössbauer-defined $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ cluster content (*23*, *37*) indicates that the $2\times[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ IscU samples used for aconitase activation studies contained >90% $[2Fe\text{-}2S]^{2+}$ clusters and >70% $[4Fe\text{-}4S]^{2+}$ clusters, respectively.

In our initial apo-AcnA activation experiments, different concentrations of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU samples were mixed with a fixed concentration of apo-AcnA for 30 min and assayed for recovery of AcnA activity. The results of these experiments (Figure 3) show approximately linear activation of apo-AcnA with a maximal activity obtained with a ≥3.1-fold excess of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU homodimer relative to apo-AcnA. A time course for the activation of apo-AcnA when $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU was added in 3.1-fold excess relative to apo-AcnA is shown in Figure 4A, which reveals that activation nears completion

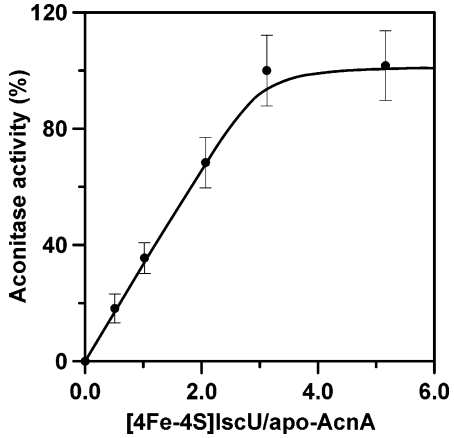

FIGURE 3: Titration of apo-AcnA activation with increasing amounts of $[4Fe\text{-}4S]^{2+}$ IscU. The concentration of apo-AcnA was kept constant at 6.7 μM, and the concentration of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU was varied as indicated on the *x* axis. After 30 min, aliquots were withdrawn and assayed for AcnA activity. Details of the components contained in the [Fe−S] cluster transfer mixture, apo-AcnA activation, and AcnA activity assays are described under Materials and Methods.

after about 30 min under these conditions. Analysis on the basis of the initial concentrations of $[4Fe\text{-}4S]^{2+}$ IscU and apo-AcnA and assuming second-order kinetics indicate a second-order rate constant of 11 000 ± 2000 M^{-1} min^{-1} at 22 °C. Direct evidence for $[4Fe\text{-}4S]^{2+}$ cluster assembly on polyhistidine-tagged apo-AcnA using $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU was also apparent by the appearance of the characteristic $[4Fe\text{-}4S]^{2+}$-cluster absorption spectrum in samples of aconitase separated from the reaction mixture using a Ni-charged IMAC column (data not shown).

In contrast to the results with $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU, a 3.4-fold excess of $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU homodimer failed to provide any significant activation (Figure 4A). Moreover, $[2Fe\text{-}2S]^{2+}$ clusters were not transferred to apo-aconitase during these experiments as evidenced by the absence of any chromophore in the aconitase separated from the reaction mixture after a 30 min incubation using the Ni-charged IMAC column. However, on addition of 1.5 reducing equivalents per $[2Fe\text{-}2S]^{2+}$ cluster to the $2\times[2Fe\text{-}2S]^{2+}$ IscU/apo-AcnA activation mixture, aconitase activation ensues with a time course analogous to that obtained with $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (data not shown). Because reductive coupling of the $[2Fe\text{-}2S]^{2+}$ clusters on $2\times[2Fe\text{-}2S]^{2+}$ IscU to form the $[4Fe\text{-}4S]^{2+}$ cluster has been shown to occur immediately on addition of dithionite, see accompanying manuscript (*37*), and $[2Fe\text{-}2S]^{2+}$ clusters are not transferred from $2\times[2Fe\text{-}2S]^{2+}$ IscU to apo-aconitase, these results unambiguously demonstrate that only the $[4Fe\text{-}4S]^{2+}$-loaded form of IscU is effective for assembly of the $[4Fe\text{-}4S]^{2+}$ cluster on apo-AcnA.

Activation of Apo-AcnA Does Not Result from Free Fe and S^{2-}. The accompanying manuscript shows that dithionite induces rapid and quantitative reductive degradation of isolated $[2Fe\text{-}2S]^{2+}$ clusters as well as reductive coupling of adjacent $[2Fe\text{-}2S]^{2+}$ clusters assembled on the IscU homodimer (*37*). In addition, IscU-bound $[4Fe\text{-}4S]^{2+}$ clusters were shown to be readily degraded by excess reductant or exposure to O_2 (*37*). Given that AcnA can be activated by the simple addition of Fe and S^{2-}, it was therefore important to establish that the activation reported here is not the result of free Fe

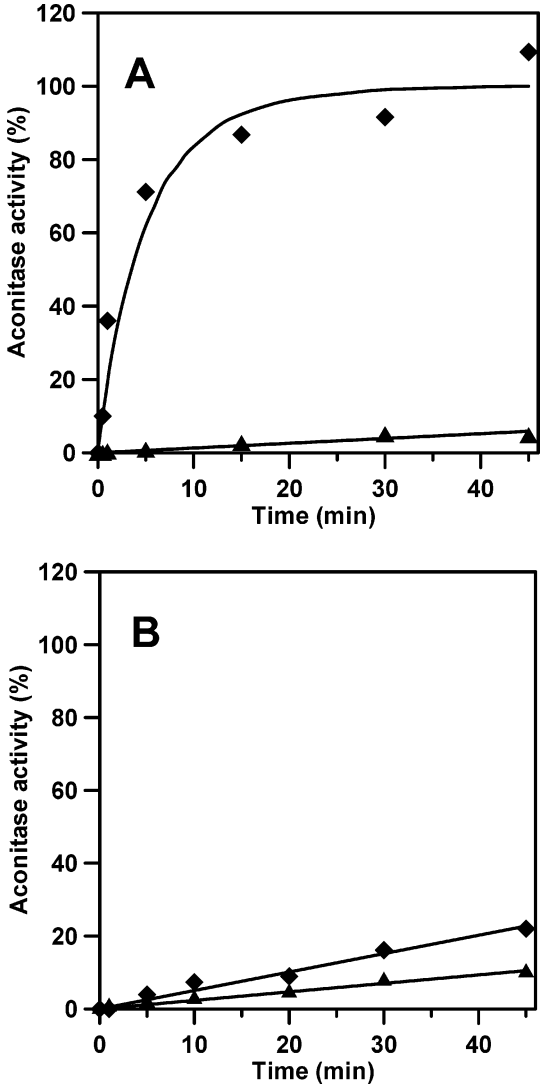

FIGURE 4: Activation of apo-AcnA activity using $2\times[2Fe\text{-}2S]$ and $[4Fe\text{-}4S]$ cluster-loaded forms of wild-type and D39A IscU. (A) Apo-AcnA (6.7 μM) was incubated with 20.6 μM $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (◆), or 22.5 μM $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU (▲) at room temperature under anaerobic conditions. Aliquots containing 3 μg AcnA were withdrawn after 30 s, 1, 5, 15, 30, and 45 min, and AcnA activity was immediately measured. Solid lines are best fits to second-order kinetics with rate constants of 11 000 M^{-1} min^{-1} for $[4Fe\text{-}4S]^{2+}$ IscU and 60 M^{-1} min^{-1} for $2\times[2Fe\text{-}2S]^{2+}$ IscU. (B) Apo-acnA (6.7 μM) was incubated with 20.6 μM $[4Fe\text{-}4S]^{2+}$ cluster-loaded $IscU^{D39A}$ (◆), or 22.5 μM $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded $IscU^{D39A}$ (▲) at room temperature under anaerobic conditions. Aliquots containing 3 μg AcnA were withdrawn after 1, 5, 10, 20, 30, and 45 min. AcnA activity was immediately measured. Solid lines are best fits to second-order kinetics with rate constants of 150 M^{-1} min^{-1} for $[4Fe\text{-}4S]^{2+}$ $IscU^{D39A}$ and 70 M^{-1} min^{-1} for $2\times[2Fe\text{-}2S]^{2+}$ $IscU^{D39A}$.

and S^{2-} released owing to oxidation or reduction of the sample. In this regard, it should be emphasized that the anion exchange chromatography step used to prepare the $2\times[2Fe\text{-}2S]^{2+}$ cluster-loaded form removes excess free Fe and S^{2-} present in the original [Fe−S] cluster biosynthetic cocktail. We have also previously shown in experiments using the nitrogen-fixation specific [Fe−S]-cluster biosynthetic scaffold NifU that excess Fe can be effectively removed from [Fe−S]-cluster biosynthetic reactions by passing the reaction cocktail over an uncharged IMAC column after cluster assembly has been completed. This procedure results in the

effective chelation of all detectable free Fe, whereas Fe sequestered on the biosynthetic scaffold passes through the column with the attendant [Fe−S] cluster intact (*49*). When $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU samples were passed over an IMAC column to remove any free Fe, they retained ~80% of the capacity to activate apo-AcnA when compared to the normal activation process. The small loss of activity may reflect removal of adventitious Fe^{2+} and/or minor oxidative degradation during sample processing. Either way, we conclude that the $[4Fe\text{-}4S]^{2+}$ cluster assembled on IscU is responsible for the majority, and possibly all, of the aconitase activation.

Activation of Apo-AcnA Occurs through Direct Cluster Transfer. Confirmation that activation of apo-AcnA occurs via direct [4Fe-4S] cluster transfer was provided by spectroscopically monitored activation studies using rigorously purified IscU samples with analytically and spectroscopically defined clusters and adventitiously bound Fe content. The $2\times[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ IscU samples used for these studies were prepared by strictly anaerobic IscS-mediated cluster assembly on IscU followed by purification of cluster-bound IscU fractions using a Mono Q column (*23*). Mössbauer studies of these samples invariably indicated homogeneous $[2Fe\text{-}2S]^{2+}$ or $[4Fe\text{-}4S]^{2+}$ cluster composition with ≤5% of the Fe present as adventiously bound Fe^{2+} species, see Figure 5 and refs 23 and 37). Figure 6 shows the time course of apo-AcnA activation using these highly purified forms of cluster-bound IscU in the absence (panel A) and presence (panel B) of a 5-fold excess of the HscA/HscB co-chaperones and a 1000-fold excess of MgATP. The results add three important pieces of information. First, the presence of HscA/HscB/MgATP has no effect on the inability of $2\times[2Fe\text{-}2S]^{2+}$ IscU to mediate $[4Fe\text{-}4S]^{2+}$ assembly on apo-AcnA. Second, the second-order rate constant for $[4Fe\text{-}4S]^{2+}$ cluster transfer in the absence of HscA/HscB/MgATP ($8700 \pm 800\ M^{-1}\ min^{-1}$) is the same, within experimental error, as that observed with less-purified samples of $[4Fe\text{-}4S]^{2+}$ IscU ($11\,000 \pm 2000\ M^{-1}\ min^{-1}$). Hence, adventitiously bound Fe^{2+} and S^{2-} cannot be responsible for the observed cluster assembly on apo-AcnA. Third, the absence of any significant enhancement in the rate of $[4Fe\text{-}4S]^{2+}$ cluster transfer in the presence of HscA/HscB/MgATP (second-order rate constant $= 7000 \pm 800\ M^{-1}\ min^{-1}$) indicates that the co-chaperone system does not play a role in facilitating $[4Fe\text{-}4S]^{2+}$ cluster transfer from IscU to apo-AcnA. In contrast, recent in vitro studies indicated that $[2Fe\text{-}2S]^{2+}$ cluster transfer from $1\times[2Fe\text{-}2S]^{2+}$ IscU to apo-IscFdx was enhanced 20-fold by HscA/HscB in an ATP-dependent reaction.

Mössbauer spectroscopy was used to assess if $[4Fe\text{-}4S]^{2+}$ cluster incorporation on apo-AcnA involves direct cluster transfer from $[4Fe\text{-}4S]^{2+}$ IscU or breakdown of the $[4Fe\text{-}4S]^{2+}$ cluster on IscU followed by reassembly of the breakdown products, $Fe^{2+/3+}$ and S^{2-}, on apo-AcnA. Figure 5 shows Mössbauer spectra of a 1:1 mixture of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU and apo-AcnA after incubation at room temperature for 30 min as well as zero time and 30 min controls with buffer added instead of apo-AcnA. Each spectrum has been simulated as a sum of $[4Fe\text{-}4S]^{2+}$ (blue), $[2Fe\text{-}2S]^{2+}$ (red), and mononuclear Fe^{2+} (green) components using the parameters previously established for $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ clusters assembled on IscU (*23*, *37*), see Table 1.

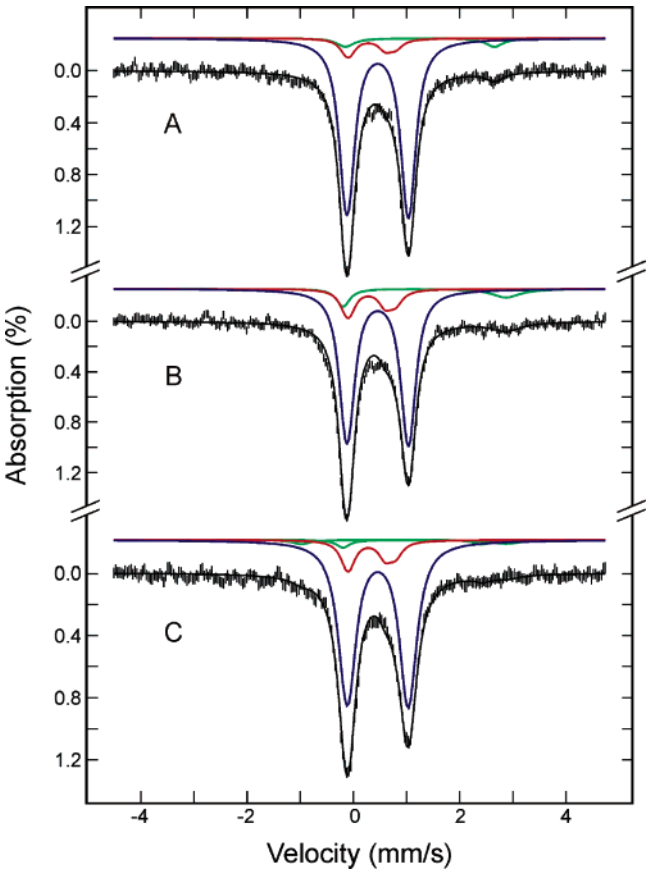

FIGURE 5: Mössbauer studies of apo-AcnA activation using stoichiometric $[4Fe\text{-}4S]^{2+}$ IscU. (A) $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (119 μM in $[4Fe\text{-}4S]^{2+}$ clusters) at zero time, (B) $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (119 μM in $[4Fe\text{-}4S]^{2+}$ clusters) and 120 μM apo-AcnA incubated at room temperature for 30 min, and (C) $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (119 μM in $[4Fe\text{-}4S]^{2+}$ clusters) incubated at room temperature for 30 min. The Mössbauer spectra were recorded at 4.2 K and simulated as a sum of $[4Fe\text{-}4S]^{2+}$ (blue), $[2Fe\text{-}2S]^{2+}$ (red), and mononuclear Fe^{2+} (green) components with the black line corresponding to the composite simulation. The percent compositions obtained for the samples are listed in Table 1.

In accord with the titration of apo-AcnA activation with increasing amounts of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU, see Figure 3, the 1:1 Mössbauer sample exhibited ~24% of maximal aconitase activity. Hence, approximately 24% of the $[4Fe\text{-}4S]^{2+}$ clusters in the Mössbauer sample are expected to be associated with aconitase rather than IscU. However, in view of the similarity in the Mössbauer parameters for the aconitase and IscU $[4Fe\text{-}4S]^{2+}$ clusters (*23*, *37*, *50*), and the anticipated low percentage of aconitase $[4Fe\text{-}4S]^{2+}$ clusters, it is not meaningful to attempt quantitative deconvolution of the $[4Fe\text{-}4S]^{2+}$ component into IscU and aconitase $[4Fe\text{-}4S]^{2+}$ clusters. Nevertheless, the Mössbauer data clearly support intact $[4Fe\text{-}4S]^{2+}$ cluster transfer from IscU to apo-aconitase. In particular, the lack of increase in the total amount of mononuclear Fe^{2+} species in the $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU control samples, after incubating at room temperature for 30 min, indicates that the IscU $[4Fe\text{-}4S]^{2+}$ clusters are not significantly degraded over the time course of the activation. Hence, cluster breakdown to yield $Fe^{3+/2+}$ and S^{2-} cannot account for the observed activation of apo-AcnA. A small increase in the Fe^{2+} component is observed in the sample containing apo-AcnA, and this is attributed to inefficiency in the cluster transfer process, which has yet to

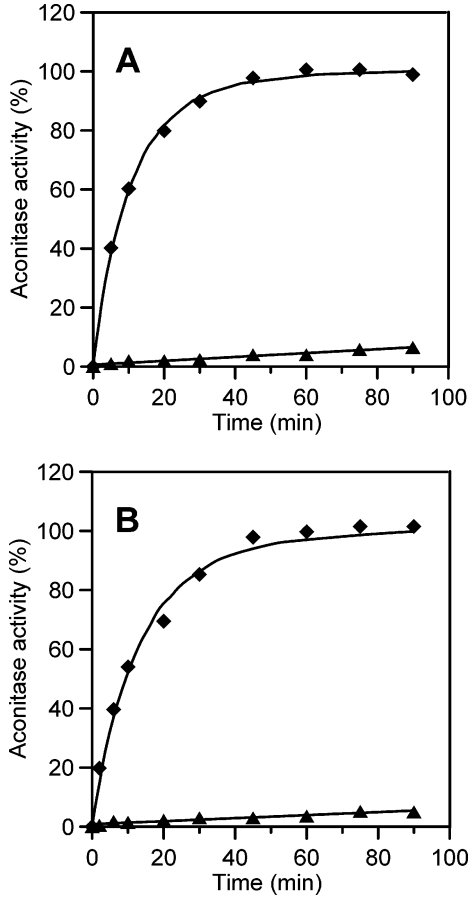

FIGURE 6: Activation of apo-AcnA activity using 2×[2Fe-2S] and [4Fe-4S] cluster-loaded forms of wild-type IscU in the absence and presence of HscA/HscB/MgATP. (A) Apo-AcnA (4 μM) was incubated with 12 μM $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (◆), or 12 μM 2×$[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU (▲) at room temperature under anaerobic conditions. Aliquots containing 3 μg AcnA were withdrawn after 5, 10, 20, 30, 45, 60, 75, and 90 min. AcnA activity was immediately measured. Lines are best fits to second-order kinetics with rate constants of 8700 M^{-1} min^{-1} for $[4Fe\text{-}4S]^{2+}$ IscU and 70 M^{-1} min^{-1} for 2×$[2Fe\text{-}2S]^{2+}$ IscU. (B) Apo-AcnA (4 μM) was incubated with 12 μM $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU (◆) or 12 μM 2×$[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU (▲) at room temperature under anaerobic conditions in the presence of 60 μM HscA and HscB, 24 mM $MgCl_2$, and 12 mM ATP. Aliquots containing 3 μg AcnA were withdrawn after 5, 10, 20, 30, 45, 60, 75, and 90 min. AcnA activity was immediately measured. Lines are best fits to second-order kinetics with rate constants of 7000 M^{-1} min^{-1} for $[4Fe\text{-}4S]^{2+}$ IscU and 60 M^{-1} min^{-1} for 2×$[2Fe\text{-}2S]^{2+}$ IscU.

be fully optimized. After a 30 min incubation at room temperature inside the glove box, both the control without apo-AcnA and the sample with apo-AcnA show a similar small increase (4−5% of the total Fe) in the amount of $[2Fe\text{-}2S]^{2+}$ clusters. This result is attributed to the extreme oxidative lability of $[4Fe\text{-}4S]^{2+}$ clusters assembled on IscU (*23*, *37*) and demonstrates that the previously observed O_2-induced conversion of $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$ clusters (*37*) is not enhanced by the presence of apo-AcnA.

Inhibition of Apo-AcnA Activation by a [4Fe-4S] Cluster-Loaded Form of IscU^{D39A}. The NifU protein has been identified as an [Fe−S] cluster scaffold protein specifically required for maturation of [Fe−S] cluster-containing nitrogenase components. NifU is a modular protein with the N-terminal module exhibiting a high degree of sequence conservation when compared to IscU. We have previously shown that [4Fe-4S] clusters can be assembled in vitro on recombinantly produced full-length NifU or the N-terminal NifU module (designated NifU-1). Such cluster-bound forms of NifU and NifU-1 can be used effectively to activate an apo form of the nitrogenase Fe protein, which requires a [4Fe-4S] cluster for its role in nitrogenase catalysis. In addition, substitution of the NifU-1 Asp^{37} residue with Ala^{37} was shown to result in an altered form of NifU-1 upon which [4Fe-4S] clusters are assembled, but it cannot be effectively used to activate apo-Fe protein (*3*, *49*, *51*). In the present work, we substituted the analogous IscU Asp^{39} residue with Ala and investigated the consequences of this substitution on the capacity for [Fe−S] cluster formation and apo-AcnA activation. As shown in Figure 2B, the IscU^{D39A} substituted protein retains the capacity to serve as a scaffold for in vitro $[2Fe\text{-}2S]^{2+}$ cluster assembly, and this form can be readily converted to a form that primarily contains $[4Fe\text{-}4S]^{2+}$ clusters by reductive coupling on addition of 1.5 reducing equivalents per $[2Fe\text{-}2S]^{2+}$ cluster. Neither the $[2Fe\text{-}2S]^{2+}$ cluster- nor the $[4Fe\text{-}4S]^{2+}$ cluster-loaded form of IscU^{D39A} is able to effectively supply clusters for apo-AcnA activation (Figure 4B).

Table 1: Percent Composition of Components in the Mössbauer Spectra of the Samples Used to Investigate Apo-AcnA Activation Using $[4Fe\text{-}4S]^{2+}$ Cluster-Loaded IscU[a]

reaction mixture	component[b]	percent
spectrum A	$[4Fe\text{-}4S]^{2+}$ (blue)	86
$[4Fe\text{-}4S]^{2+}$ IscU	$[2Fe\text{-}2S]^{2+}$ (red)	9
at zero time	Fe^{2+} (green)[c]	5
spectrum B	$[4Fe\text{-}4S]^{2+}$ (blue)	78
$[4Fe\text{-}4S]^{2+}$ IscU + apo-AcnA (1:1)	$[2Fe\text{-}2S]^{2+}$ (red)	14
after 30 min incubation	Fe^{2+} (green)[d]	8
at room temperature		
spectrum C	$[4Fe\text{-}4S]^{2+}$ (blue)	82
$[4Fe\text{-}4S]^{2+}$ IscU	$[2Fe\text{-}2S]^{2+}$ (red)	13
after 30 min incubation	Fe^{2+} (green)[e]	5
at room temperature		

[a] See Figure 5. [b] Mössbauer parameters used for the $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ components are those established previously for the $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ clusters assembled on IscU (*23*, *37*). [c] Mössbauer parameters $\Delta E_Q = 2.8$ mm/s and $\delta = 1.25$ mm/s were used. [d] $\Delta E_Q = 3.1$ mm/s and $\delta = 1.34$ mm/s. [e] Two Fe^{2+} components of similar percentages were detected with parameters $\Delta E_Q = 3.1$ mm/s and $\delta = 1.34$ mm/s for component 1 and $\Delta E_Q = 3.4$ mm/s and $\delta = 0.72$ mm/s for component 2.

Because [4Fe-4S] cluster-loaded IscU^{D39A} is unable to support apo-AcnA activation, it was also of interest to ascertain whether or not the cluster-loaded form of IscU^{D39A} is an inhibitor of wild-type IscU-directed activation of apo-AcnA. The results of these experiments are summarized in Figure 7 and show that the $[4Fe\text{-}4S]^{2+}$ cluster form of IscU^{D39A} is an effective inhibitor of apo-AcnA activation. Namely, an equimolar mixture of $[4Fe\text{-}4S]^{2+}$ cluster-loaded forms of wild-type IscU and IscU^{D39A} yields about only 50% of the activation when compared to $[4Fe\text{-}4S]^{2+}$ cluster-loaded wild-type IscU activation (compare lanes A and B in Figure 7). In contrast, neither as-isolated wild-type IscU (no clusters assembled) nor the $[2Fe\text{-}2S]^{2+}$-loaded forms of either IscU or IscU^{D39A} show a marked inhibition of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU activation of apo-AcnA (compare lanes A, C, D, and E in Figure 7).

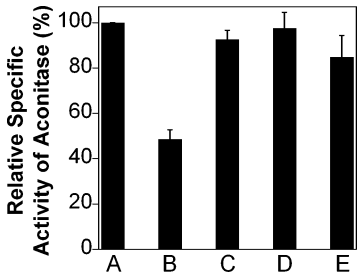

FIGURE 7: Specific inhibition of apo-AcnA activation by the [4Fe-4S]$^{2+}$ cluster-loaded form of IscU^{D37A}. (A) Activation of 6.7 μM of apo-AcnA using 20.6 μM of [4Fe-4S]$^{2+}$ cluster-loaded wild-type IscU, (B) same as in A except that the activation cocktail also included 20.6 μM [4Fe-4S]$^{2+}$ cluster-loaded IscU^{D39A}, (C) same as in A except that the activation cocktail also included 41.2 μM apo-IscU, (D) same as in A except the activation cocktail also included 20.6 μM [2Fe-2S]$^{2+}$ cluster-loaded wild-type IscU, and (E) same as in A except the activation cocktail included 20.6 μM [2Fe-2S]$^{2+}$ cluster-loaded IscU^{D39A}.

DISCUSSION

Our previous studies of *A. vinelandii* IscU demonstrated that two [2Fe-2S]$^{2+}$ clusters can be assembled per homodimer and that these clusters can react in a slow process to form one [4Fe-4S]$^{2+}$ cluster per homodimer (*23*). The accompanying manuscript shows that this slow reductive coupling process can be instantly achieved by addition of one reducing equivalent of dithionite per [2Fe-2S]$^{2+}$ cluster in strictly anaerobic reductive titrations of the 2×[2Fe-2S]$^{2+}$ cluster-containing form of IscU (*37*). Moreover, reduced IscFdx, a [2Fe-2S]$^{2+,+}$ ferredoxin that is co-transcribed with other members of the Isc biosynthetic machinery, was shown to be a competent electron donor for mediating at least partial reductive coupling (*37*). Hence, in vitro studies suggest that IscU has the potential to be present in both the [2Fe-2S]$^{2+}$ and the [4Fe-4S]$^{2+}$ cluster-loaded forms in the cell. The objective of this work was therefore to assess if both cluster-loaded forms of IscU are competent for maturation of Fe−S proteins.

A variety of physiological studies using plasmid expression systems and mutants deficient in individual Isc components have indicated that the Isc biosynthetic machinery is required for the maturation of both [2Fe-2S] and [4Fe-4S] cluster-containing [Fe−S] proteins (*5*, *29*, *52*). Two possible processes can be considered for the maturation of [Fe−S] proteins. One possibility is that the [2Fe-2S]$^{2+}$ cluster-loaded form of IscU is responsible for assembly of both [2Fe-2S] and [4Fe-4S] centers. In this case, the [2Fe-2S] cluster-containing target proteins would receive their cognate clusters from a single transfer event from [2Fe-2S]$^{2+}$ cluster-loaded IscU. Indeed, it has already been shown that the [2Fe-2S]$^{2+}$ cluster-loaded form of IscU can be used in vitro to supply [2Fe-2S] clusters for maturation of [2Fe-2S] ferredoxins (*33*−*35*, *53*) and that the rate of cluster transfer is enhanced by the Isc HscA/HscB co-chaperone system in an ATP-dependent process (*32*). In contrast, maturation of [4Fe-4S] proteins would require a more complicated sequential or concerted delivery of two [2Fe-2S]$^{2+}$ clusters from the IscU scaffold. A sequential or concerted transfer of [2Fe-2S]$^{2+}$ clusters from IscU to form [4Fe-4S] clusters on target proteins is further complicated because this would require not only two separate or simultaneous transfer events but also a reductive coupling event. It is difficult to imagine how a target protein, such as AcnA, that contains only three cluster-coordinating thiolate ligands could accommodate two [2Fe-2S]$^{2+}$ clusters during the maturation process, or alternatively, how reducing equivalents could be supplied to a scaffold-target complex during concerted delivery of two [2Fe-2S]$^{2+}$ cluster units.

The second possibility is that separate [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ cluster-loaded forms of IscU are respectively used for the maturation of [2Fe-2S] and [4Fe-4S] centers in proteins. Results reported here provide support for this model because the [4Fe-4S]$^{2+}$ cluster-loaded form of IscU, but not the [2Fe-2S]$^{2+}$ cluster-loaded form, can be used to activate apo-AcnA, which requires an intact [4Fe-4S] cluster for its catalytic activity. This model is an attractive one because maturation of both [2Fe-2S] and [4Fe-4S] proteins can be expected to proceed through a simple cysteine thiol exchange mechanism between the IscU cluster-donating biosynthetic scaffold and free thiols of the target protein, thereby resulting in intact cluster transfer. This concept is also compatible with the idea that free thiols within target proteins might serve as a recognition signature for IscU-mediated cluster delivery. Furthermore, the controlled distribution of IscU between [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ cluster-loaded forms could provide an effective way to appropriately populate cluster-loaded IscU for maturation of different types of [Fe−S] proteins.

If [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ cluster-loaded forms of IscU are specifically destined for different types of [Fe−S] proteins, it seems reasonable to expect that such specificity would be endowed by different conformational states of the different types of cluster-loaded forms of IscU. Work reported here provides evidence that different forms of IscU do have different conformations. Namely, the inactive [4Fe-4S]$^{2+}$ cluster-loaded form of IscU^{D39A} is an inhibitor of in vitro apo-AcnA activation, whereas neither the cluster-free form nor the [2Fe-2S]$^{2+}$ cluster-loaded form is an effective inhibitor of apo-AcnA activation. Conformational differences between [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ cluster-loaded forms of IscU may also account for differences in the ability of the HscA/HscB co-chaperone system to enhance the rate of cluster transfer in the presence of MgATP. No rate enhancement was observed for [4Fe-4S]$^{2+}$ cluster transfer from IscU to apo-AcnA, whereas a 20-fold rate enhancement was observed for [2Fe-2S]$^{2+}$ cluster transfer from IscU to apo-IscFdx (*32*). HscA has been shown to interact with a LPPVK motif adjacent to the putative cluster binding site on IscU (*30*, *31*, *54*, *55*). Hence, conformational differences between the [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ cluster-loaded forms of IscU that affect the interaction of HscA with the IscU LPPVK motif may be responsible for lack of HscA/HscB enhancement of the rate of [4Fe-4S] cluster transfer.

What is the specific function of the IscU Asp^{39} residue? The crystallographic and NMR solution structures of Zn-bound, monomeric forms of *H. influenzae* IscU and *S. pyogenes* SufU show that the three proposed cluster-coordinating cysteine ligands (Cys^{37}, Cys^{63}, Cys^{106}), together with Asp^{39}, are solvent exposed and are all located relatively close to each other (*54*, *55*). Thus, Asp^{39} might: (i) function as a cluster-assembly ligand, (ii) have a direct role in promoting interaction with target proteins, or (iii) function to destabilize clusters attached to IscU in order to promote effective delivery. None of these possibilities are mutually exclusive. Evidence reported here that both [2Fe-2S]$^{2+}$ and

$[4Fe\text{-}4S]^{2+}$ clusters can be assembled on the $IscU^{D39A}$ scaffold, yet neither form can be used to activate apo-AcnA, favors a dominant role for the Asp^{39} in cluster transfer, rather than assembly of [Fe−S] clusters on the assembly scaffold. The fact that the $[4Fe\text{-}4S]^{2+}$ cluster-loaded form of $IscU^{D39A}$ is an effective inhibitor of apo-AcnA activation further indicates that Asp^{39} does not have an essential role in protein−protein interactions required for cluster transfer. This conclusion is also supported by recently reported work on the NifU scaffold, where it was shown that substitution of the analogous Asp residue in NifU (Asp^{37}) with Ala, results in both in vivo and in vitro loss of function without a loss in the capacity for serving as an [Fe−S] cluster assembly scaffold (*51*). However, inhibition of apo-Fe protein activation by NifU-Ala^{37} has not yet been tested.

Data presented in this work show that an approximately 3-fold excess of $[4Fe\text{-}4S]^{2+}$ cluster-loaded IscU is necessary to achieve in vitro activation of apo-AcnA, which is completed in about 30 min. However, given that [Fe−S] proteins are abundantly produced in *A. vinelandii* cells and that IscU has recently been shown to function catalytically in the assembly of [2Fe-2S] clusters in apo-IscFdx in *Escherichia coli* (*34*), it is evident that the in vitro results reported here cannot be considered to faithfully reproduce the cellular process. Furthermore, recent in vivo studies clearly indicate a direct role for IscU, HscA, HscB, and IscFdx in maturation of aconitase in *A. vinelandii* (*29*). Hence, it seems likely that the in vitro system is lacking a key component that is physiologically important. For example, the system may be lacking an intermediate [4Fe-4S] cluster chaperone, a general protein folding chaperone such as GroEL, which has been shown to play a role in aconitase maturation (*56*), or components necessary to optimize the conformation of $[4Fe\text{-}4S]^{2+}$ IscU for efficient cluster transfer. Indeed, the observed 3:1, [4Fe-4S] IscU to apo-AcnA stoichiometry is most readily interpreted in terms of only one-third of the $[4Fe\text{-}4S]^{2+}$ IscU being competent for cluster transfer, and different conformations may relate to the redox status of the cysteine thiols released during $[4Fe\text{-}4S]^{2+}$ cluster formation. We are currently exploring the possibility of a redox-active disulfide on cluster-loaded forms of IscU and the involvement of GroEL and/or specific cluster chaperone proteins in facilitating IscU-mediated assembly of the $[4Fe\text{-}4S]^{2+}$ cluster on apo-AcnA.

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