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Native *Escherichia coli* SufA, Coexpressed with SufBCDSE, Purifies as a [2Fe−2S] Protein and Acts as an Fe−S Transporter to Fe−S Target Enzymes

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Abstract: Iron–sulfur (Fe−S) clusters are versatile biological cofactors that require biosynthetic systems in vivo to be assembled. In *Escherichia coli*, the Isc (*iscRSUA-hscBA-fdx-iscX*) and Suf (*sufABCDSE*) pathways fulfill this function. Despite extensive biochemical and genetic analysis of these two pathways, the physiological function of the A-type proteins of each pathway (IscA and SufA) is still unclear. Studies conducted in vitro suggest two possible functions for A-type proteins, as Fe−S scaffold/transfer proteins or as iron donors during cluster assembly. To resolve this issue, SufA was coexpressed in vivo with its cognate partner proteins from the *suf* operon, SufBCDSE. Native SufA purified anaerobically using this approach was unambiguously demonstrated to be a [2Fe−2S] protein by biochemical analysis and UV−vis, Mössbauer, resonance Raman, and EPR spectroscopy. Furthermore, native [2Fe−2S] SufA can transfer its Fe−S cluster to both [2Fe−2S] and [4Fe−4S] apoproteins. These results clearly show that A-type proteins form Fe−S clusters in vivo and are competent to function as Fe−S transfer proteins as purified. This study resolves the contradictory results from previous in vitro studies and demonstrates the critical importance of providing in vivo partner proteins during protein overexpression to allow correct biochemical maturation of metalloproteins.

Introduction

Iron–sulfur (Fe−S) clusters are versatile cofactors utilized for diverse biological functions, including electron transfer, substrate activation, and regulation of gene expression.[1] 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. Assembly of such clusters and maturation of iron−sulfur cluster proteins does not occur spontaneously but instead requires complex biosynthetic machineries. In *Escherichia coli*, for example this process is achieved by the Isc (*iscRSUA-hscBA-fdx-iscX*) and Suf (*sufABCDSE*) pathways.[2,3] The inorganic sulfur is supplied by a cysteine desulfurase (IscS/SufS), which catalyzes the PLP-dependent removal of sulfur from L-cysteine and donates that sulfur through sulfur shuttle proteins to a specialized scaffold protein in which the nascent Fe−S cluster is preassembled.[2] The in vivo source of iron is still unclear.

IscU homodimer is a prototype for Fe−S scaffold proteins, which are endowed with the ability to assemble either $[2Fe-2S]^{2+}$ clusters or a single $[4Fe-4S]^{2+}$ cluster[4,5] that can be directly transferred to appropriate apoproteins.[6] IscU has been extensively studied using biochemical, enzymatic, and spectroscopic methods. At the present time, its role as a scaffold protein is well-established. Each pathway also contains an A-type protein (IscA/SufA) that can also assemble Fe−S clusters in vitro and transfer those clusters to apoproteins.[7−9] *E. coli* IscA was also reported to tightly bind ferric iron ($K_{ass} = 3 \times 10^{19}$ M^{-1}) and provide iron to IscU during in vitro cluster assembly.[10] These somewhat contradictory studies led to two proposed functions for the A-type proteins: (1) as alternate Fe−S scaffolds or Fe−S transporters and (2) as Fe donors for cluster assembly

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on other scaffold proteins. The ambiguity was due, in particular, to the fact that A-type proteins were usually isolated in the apo form and that binding of iron or clusters achieved under in vitro conditions might not be physiologically relevant.

In order to produce SufA in a more physiological context for characterization study, we developed an in vivo expression system that simultaneously expresses the products of all six *suf* genes, mimicking the native situation where *sufABCDSE* are transcribed on a polycistronic mRNA. Using this expression system, we structurally and functionally characterized the native SufA by biochemical and spectroscopic approaches. We here show for the first time that SufA exists within cells in the form of an Fe−S protein containing a [2Fe−2S] cluster that can be transferred to apoprotein targets.

Experimental Section

Bacterial Growth and Purification of [2Fe−2S] SufA. The strains used to purify SufA were Top10 pGSO164 and Top10 pGSO164 ($SufD^{H128A}$), which contain the entire *suf* operon under the control of an arabinose-inducible promoter. The strains were grown in 25 mL of LB + Ampicillin (100 $\mu g/\mu L$) overnight. A 10 mL aliquot of this was used to inoculate 1 L of LB + Ampicillin media. The cells were allowed to grow at 37 °C until the midlog phase, and then 10 mL of 20% sterile L-arabinose was added per liter of media to induce expression of the *sufABCDSE* operon. The cells were induced for 3 h and then collected by centrifugation and frozen at −80 °C. A modified freeze−thaw procedure was used to purify SufA anaerobically. Briefly, the cell pellet was thawed on ice and resuspended in Buffer A containing 25 mM Tris (pH 7.5) with protease inhibitor cocktail (Pierce) and 5 mM βME. The pellet was refrozen at −80 °C for 1 h. The freeze−thaw cycle was repeated at least twice. All steps in the freeze−thaw procedure were carried out in a Coy anaerobic chamber containing a 95% nitrogen/5% hydrogen gas mixture. The freeze−thaw extract was centrifuged at 20000g for 25 min at 4 °C in an anaerobically sealed tube. The supernatant was then loaded on a Q-Sepharose anion exchange column, equilibrated with Buffer A, and eluted with a linear gradient of 1 M NaCl in Buffer A. These steps were carried out using an FPLC instrument outside of the Coy chamber, but all of the buffers were extensively nitrogen-sparged to reduce dissolved oxygen prior to use. The fractions containing SufA (as determined by SDS-PAGE) were red. SufA fractions were collected and concentrated in a YM10 centricon (Amicon) according to the manufacturer's protocol.

Preparation of ^{57}Fe-Labeled SufA for Mössbauer Studies. The Top10 pGSO164 ($SufD^{H128A}$) strain was used to purify ^{57}Fe-labeled SufA protein. The cells were grown in a similar manner as described above except that the LB was enriched with ^{57}Fe. ^{57}Fe (Cambridge Isotopes) was resuspended in dilute HCl and added to a final concentration of 10 μM twice: at culture inoculation and at the time of induction with 0.2% L-arabinose. The protein purification was performed as described above. After concentration, holoSufA was frozen in a Mössbauer cup using liquid nitrogen and stored at 77 K.

Fe−S Cluster Transfer to Ferredoxin and Spore Photoproduct Lyase. *E. coli* apoferredoxin (apo-Fdx) and the apo form of *Bacillus subtilis* spore photoproduct lyase (apo-SPL) were expressed and purified in our laboratory as previously described.[11,12] Apo-Fdx (180 μM) or apo-SPL (80 μM) was incubated anaerobically with native SufA (0.4 iron and sulfur atoms per monomer) in 200 μL of 0.1 M Tris-HCl (pH 8)/50 mM KCl buffer in order to provide two iron and sulfur atoms per Fdx or four iron and sulfur atoms per SPL. After 1 h of incubation under anaerobic conditions, SufA was separated from SPL on a Ni-NTA column, on which SPL was retained since it contains a His tag at its N-terminus. SufA was recovered in the run-through fraction during extensive washing with 0.1 M Tris-HCl (pH 8)/50 mM KCl buffer, whereas SPL was collected in the 400 mM imidazole fraction. Fe−S transfer to SPL was monitored by assaying the corresponding imidazole fraction for iron and sulfur content and for its UV−vis spectrum. In the case of ferredoxin, Fe−S transfer was followed by monitoring the UV−vis absorption in the 300−600 nm region and by electron paramagnetic resonance (EPR) analysis of the SufA−Fdx protein mixture after reduction with 2 mM dithionite.

Activation of Aconitase A Using Native SufA. All of the following procedures were performed anaerobically in the glovebox at 18 °C. Aconitase A (AcnA) (0.2 nmol), in its apo form, was incubated in 50 mM Tris-HCl (pH 7.6) containing 5 mM dithiothreitol (DTT) with a 10-fold molar excess of the native SufA (0.4 iron and sulfur atoms per monomer) in order to provide four iron and four sulfur atoms per AcnA. After 15 min of incubation, aconitase activity was assayed using the procedure of Gardner and Fridovitch[13] by monitoring the formation of NADPH through the increase in absorbance at 340 nm. For that, AcnA−SufA proteins were added to 0.6 mM $MnCl_2$, 25 mM citrate, 0.5 unit of isocitric dehydrogenase, 0.25 mM $NADP^+$, and 50 mM Tris-HCl (pH 7.6) in a final volume of 100 μL. The 100% activity corresponded to the activity of the chemically reconstituted AcnA (recAcnA) prepared by incubating apo-AcnA with a 4-fold molar excess of ferrous iron and sulfur for 30 min in the presence of 5 mM DTT (5 $\mu mol\ mg^{-1}\ min^{-1}$). The values reported for the specific activity of AcnA (SufA−recAcnA, recAcnA, and apo-AcnA) correspond to the average of three independent experiments.

Cluster Transfer in the Presence of BPS. Apo-AcnA (0.2 nmol) was incubated anaerobically in 50 mM Tris-HCl (pH 7.6)/5 mM DTT solution with either [2Fe−2S] SufA (providing 4 equiv of Fe and S atoms per apo-AcnA) or a 4-fold molar excess of Fe^{2+} and S^{2-}. Bathophenanthroline sulfonate (BPS) was added at the same time as iron and sulfide (or [2Fe−2S] SufA) in both cases, and the mixtures were incubated for 15 min, after which aconitase activity was measured as described above.

Iron, Sulfide, And Protein Analysis. The protein concentration was measured using Bradford's assay with 2 mg/mL BSA as the standard.[14] Beinert's assay was used for sulfide estimation, and both the ferrozine assay and ICP−AES were used for iron estimation.[15,16]

Spectroscopic Methods. UV−vis spectra were recorded with a Cary 1 Bio spectrophotometer (Varian). EPR spectra were recorded on a Bruker EMX (9.5 GHz) EPR spectrometer equipped with an ESR 900 helium flow cryostat (Oxford Instruments). Double integrals of the EPR signals and spin concentration were obtained with the Win-EPR software using the spectrum of a 200 μM Cu(EDTA) standard recorded under nonsaturating conditions. Mössbauer spectra were recorded at 4.2 K on either a weak-field spectrometer using a permanent magnet of 50 mT or a strong-field spectrometer furnished with a Janis CNDT/SC SuperVaritemp cryostat equipped with an 8 T superconducting magnet. Both spectrometers were operated in constant-acceleration mode in a transmission geometry. The spectra were analyzed using the program WMOSS (WEB Research). The isomer shifts are reported with reference to the centroid of a room-temperature spectrum of a metallic Fe foil. Resonance Raman spectra of frozen samples held at 15 K were recorded using a Jobin-Yvon U1000 double-additive monochromator equipped with a front-illuminated LN_2-cooled CCD detector. The excitation wavelength was obtained from the 441.6 nm line of a HeCd laser (Liconix). The spectral resolution was 4 cm^{-1} under these experimental conditions.

Results

SufA Is a [2Fe−2S] Protein. Following coexpression of the SufABCDSE proteins in *E. coli*, SufA was purified anaerobically.

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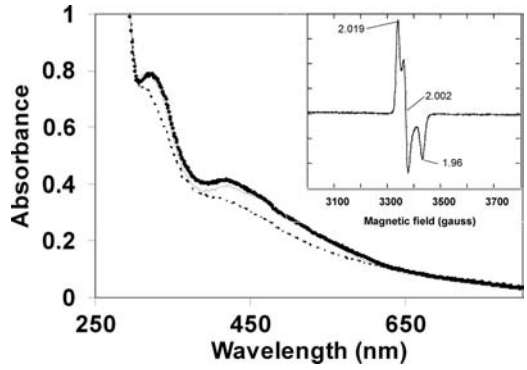

Figure 1. UV−vis spectra of SufA isolated from anaerobic anion exchange chromatography of freeze−thaw lysates from the SufABCDSE(SufD^{H128A}) expression system in 0.1 M Tris-HCl (pH 8)/50 mM KCl buffer, before (bold trace) and after (dashed trace) anaerobic incubation for 60 min with 5 mM DTT and then after re-exposure to air for 1 min (thin trace). Inset: EPR spectrum of SufA reduced with 5 mM DTT (microwave power, 0.1 mW; modulation amplitude, 1 mT; receiver gain, 2×10^4; temperature, 9.5 K).

The SufA-containing fraction was red, and its UV−vis spectrum displayed absorption bands at $\lambda_{max} = 330$, 420, and 460 nm (Figure S1B in the Supporting Information), as previously reported for reconstituted $[2Fe-2S]^{2+}$ SufA from *Erwinia chrysanthemi*.[9] During a systematic screen to mutate His residues of SufD, we observed that the fraction containing SufA was a deeper red in the SufABCDSE expression system with a SufD^{H128A} mutation than in the wild-type SufABCDSE expression system. Indeed, SufA purified from the SufABCDSE(SufD^{H128A}) expression system showed more intense light-absorption bands and higher iron and sulfur content (0.6 iron and 0.7 sulfur per SufA monomer) (Figure 1 and Figure S1B in the Supporting Information). To firmly define the type of cluster on isolated native SufA, this preparation was then used for further biochemical, spectroscopic (EPR, resonance Raman, and Mössbauer spectroscopy), and functional characterization. The origin of the effect of the SufD^{H128A} mutation is the subject of a separate study.

Gel filtration chromatography revealed that SufA migrates as a single peak at an apparent molecular weight of 30.2 kDa, which is close to the expected molecular weight of a SufA dimer (26.6 kDa) (not shown). EPR analysis revealed that reduction with 5 mM DTT generates a paramagnetic SufA protein characterized by an $S = {}^1/_2$ EPR signal (Figure 1, inset) whose g values, temperature dependence, and microwave power saturation properties are consistent with those of a $[2Fe-2S]^+$ cluster. The EPR signal integrates to only 10% of the total iron.

The fact that the cluster was not destroyed during treatment with DTT is supported by (i) the UV−vis spectrum of SufA after reduction (Figure 1), (ii) an almost full restoration of the initial absorption bands after a short re-exposure to air (Figure 1), and (iii) the conservation of Fe and S after chromatography of the reduced SufA over a desalting column (data not shown).

The resonance Raman spectrum of SufA recorded at 15 K (Figure S2 in the Supporting Information) shows intense bands at 286 and 402 cm^{-1} and additional bands at 350, 382, and 393 cm^{-1}. The band at 286 cm^{-1} is diagnostic of $[2Fe-2S]^{2+}$ clusters and is not seen for $[4Fe-4S]^{2+}$ clusters or mononuclear iron sites.[17] The Mössbauer spectrum for ^{57}Fe-labeled SufA recorded at 4.2 K in a 50 mT magnetic field (Figure 2A) consists of an intense symmetric quadrupole doublet (gray solid line in Figure 2A) and a weak asymmetric quadrupole doublet (dashed line in Figure 2A). The Mössbauer parameters determined for the intense doublet ($\Delta E_Q = 0.53 \pm 0.03$ mm/s and $\delta = 0.28 \pm 0.02$ mm/s) are indicative of a $[2Fe-2S]^{2+}$ cluster with cysteinyl ligands, while that of the weak doublet ($\Delta E_Q = 2.68$ mm/s and $\delta = 1.22$ mm/s) suggest nonspecifically bound ferrous ions. Moreover, the spectrum recorded in a strong applied field of 6 T (Figure 2B) shows that the intense doublet detected in the weak-field spectrum arises from a diamagnetic system. This observed diamagnetism is consistent with the $S = 0$ ground state expected for a $[2Fe-2S]^{2+}$ cluster containing two antiferromagnetically coupled ferric ions. Detailed analysis of the data showed that the intense doublet accounts for 89% of the total Fe absorption. Thus, the Mössbauer data clearly show that a majority (i.e., 89%) of the Fe atoms in purified SufA is present in the form of $[2Fe-2S]^{2+}$ clusters, with the remaining Fe atoms (11%) in nonspecifically bound ferrous ion form.

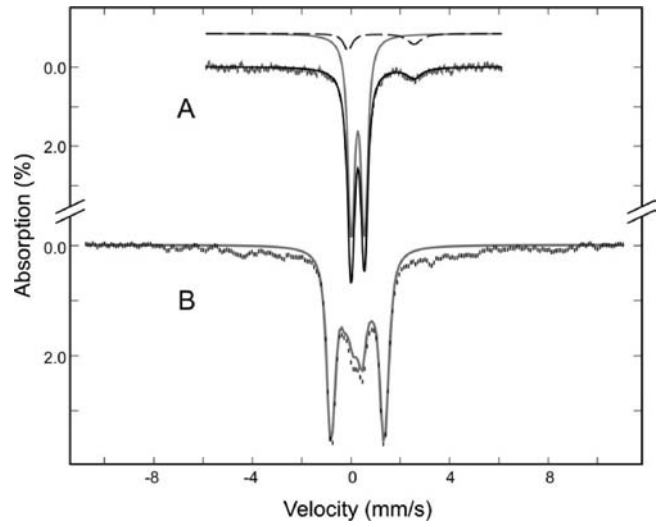

Figure 2. Mössbauer spectra of ^{57}Fe-labeled SufA purified from the SufABCDSE(SufD^{H128A}) expression system recorded at 4.2 K in magnetic fields of (A) 50 mT and (B) 6 T. The data can be decomposed into a major spectral component (gray solid lines) arising from a diamagnetic $[2Fe-2S]^{2+}$ cluster and a minor component (dashed line in A) corresponding to nonspecifically bound Fe^{II}.

All of these spectroscopic data indicate that native SufA coexpressed with SufBCDSE is a [2Fe−2S] protein. In view of the iron and sulfur content, it may be estimated that in our best as-isolated preparations, ∼50% of the SufA dimers contain a [2Fe−2S] cluster.

SufA Is an [Fe−S] Transport Protein. To investigate whether native SufA has the ability to transfer its cluster to target proteins (as was previously demonstrated in vitro with chemically reconstituted SufA protein), apo-Fdx from *E. coli* was used as a cluster-acceptor protein. The holoprotein of Fdx contains a [2Fe−2S] cluster that exhibits unique spectroscopic properties, allowing easy monitoring of the cluster transfer reaction. Apo-Fdx was incubated anaerobically with sufficient [2Fe−2S] SufA to provide a 2-fold molar excess of Fe and S with respect to apo-Fdx. The UV−vis spectrum of the SufA−Fdx mixture after 60 min of incubation indicated formation of holo-Fdx with λ_{max} at 415 and 460 nm[11] (Figure 3). Subsequent reduction of the SufA−Fdx mixture with an excess of dithionite confirmed the formation of holo-Fdx, as unambiguously indicated by the appearance of an absorption band at 550 nm (indicated by an arrow in Figure 3) characteristic of reduced $[2Fe-2S]^+$ Fdx,[8] with concomitant disappearance of the 415 and 460 nm bands (Figure 3).

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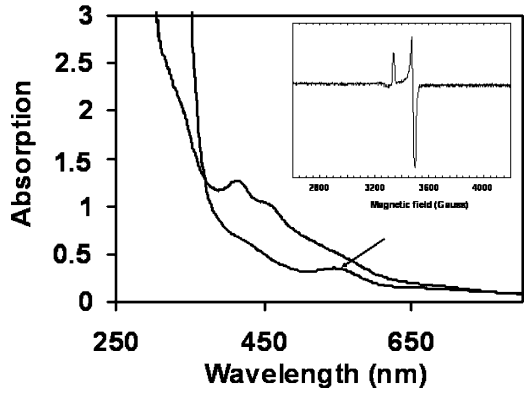

Figure 3. [2Fe−2S] cluster transfer from SufA to apoferredoxin, as indicated by the UV−vis spectra of the protein mixture after 1 h of incubation before (upper trace) and after (lower trace) reduction with 2 mM dithionite. The arrow indicates the absorbance at 550 nm. Inset: EPR spectrum of the protein mixture after reduction with dithionite (temperature, 10 K; microwave power, 0.1 mW; receiver gain, 2×10^4; modulation amplitude, 1 mT).

An EPR signal from the reduced sample was also observed, and its *g* values were distinct from those of SufA and matched those of holo-Fdx[11] (Figure 3, inset). With the assumption of complete reduction, EPR quantification indicates that 80% of the [2Fe−2S] clusters present in SufA were transferred to Fdx. In contrast, after 60 min of incubation with a 2-fold molar excess of Fe and S in the form of ferrous iron and sulfide salts in the absence of SufA, only 6% of the Fdx protein was converted into holo-Fdx.

To investigate whether SufA could also provide Fe−S clusters to [4Fe−4S] target enzymes, native SufA was anaerobically incubated with apo-SPL or apo-AcnA, two well-characterized [4Fe−4S] proteins.[12,18] Following incubation, both apo-SPL and apo-AcnA were converted into mature [4Fe−4S] holoproteins, as shown by spectroscopy and activity assays, respectively, with concomitant formation of apo-SufA. A reducing agent, DTT, was absolutely required during this maturation process, as expected for a process converting ferric ions present in $[2Fe-2S]^{2+}$ clusters into a mixture of ferric and ferrous ions in $[4Fe-4S]^{2+}$ clusters. In addition, no cluster transfer from [2Fe−2S] SufA to apo-SPL or apo-AcnA could be observed when DTT was omitted from the reaction mixture or when apo-SPL or apo-AcnA were first pretreated with DTT and then desalted anaerobically over a G-25 column to remove the DTT before the transfer reaction. The results for apo-SPL and apo-AcnA are presented in Figures 4 and 5, respectively.

In the case of apo-SPL, incubation with native SufA in the presence of 5 mM DTT led to a change in the UV−vis spectrum of SufA with incubation time, with a decrease of the broad absorption in the 500−700 nm region and disappearance of the bands at 460 and 330 nm. The final UV−vis spectrum is similar to that of a reconstituted holo-SPL containing a $[4Fe-4S]^{2+}$ cluster, which has one major broad absorption band at 420 nm (Figure 4A). Using a His-tagged SPL protein allowed purification through a Ni-NTA affinity column, and under these conditions, SPL was shown to contain ~3.2−3.3 iron and sulfur per monomer. In the absence of DTT or when apo-SPL pretreated with DTT was used as a target, no change in the UV−vis spectrum of SufA was observed over the same incubation time and no iron and sulfur could be detected in the His-tagged SPL fraction after separation onto a Ni-NTA column, showing that no cluster transfer took place

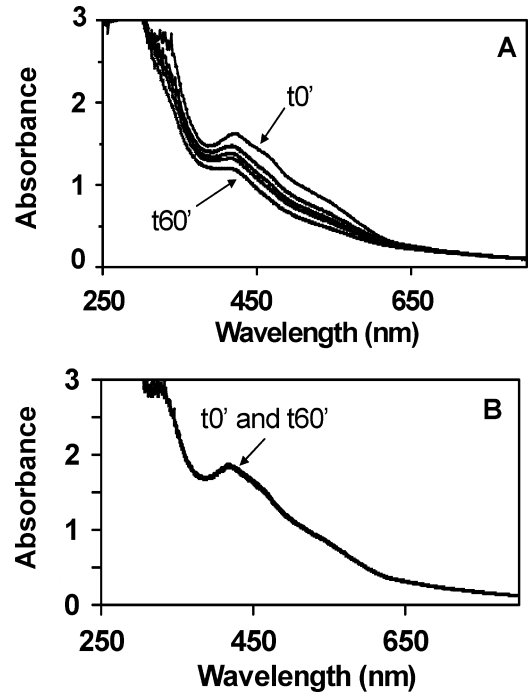

Figure 4. Iron−sulfur cluster transfer from native [2Fe−2S] SufA to apo-SPL. Apo-SPL (80 μM) was incubated with a 10-fold molar excess of native SufA (0.4 iron and sulfide per monomer) in 0.1 M Tris-HCl (pH 8)/50 mM KCl buffer in the (a) presence or (B) absence of 5 mM DTT. The reaction was monitored by UV−vis absorption spectroscopy before separation onto a Ni-NTA column.

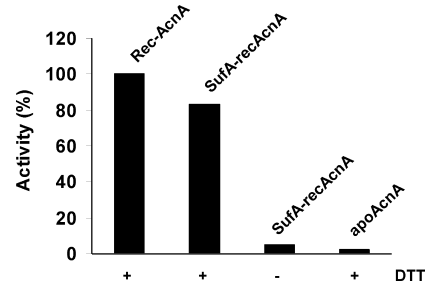

Figure 5. Activation of apo-AcnA by [2Fe−2S] SufA (SufA−recAcnA). Apo-AcnA (0.2 nmol) was incubated anaerobically with [2Fe−2S] SufA (10 equiv) in 50 mM Tris-HCl (pH 7.6) for 15 min, and the aconitase activity was assayed as described in the Experimental Section. As a control, 0.2 nmol of either pure chemically reconstituted AcnA (recAcnA) or apoprotein (apo-AcnA) were also assayed for aconitase activity. In all cases, proteins (recAcnA, SufA−recAcnA, and apo-AcnA) were added to 0.6 mM $MnCl_2$, 25 mM citrate, 0.1 mg isocitric dehydrogenase (0.5 unit), and 0.25 mM $NADP^+$ in the presence (+) or absence (−) of 5 mM DTT in a 100 μL final volume, and the formation of NADPH was monitored by UV−vis absorption spectroscopy.

under these conditions (Figure 4B). In the case of apo-AcnA (Figure 5), an activity assay was used to monitor the assembly of the [4Fe−4S] cluster during incubation with [2Fe−2S] SufA. Activation of AcnA (80% yield) by native SufA was observed after 15 min of incubation in the presence of DTT, whereas less than 5% activation occurred when DTT was omitted from the reaction mixture, an activity close to that found with apo-AcnA (Figure 5). All of these studies clearly show that native SufA has the ability to transfer its Fe−S cluster to both [2Fe−2S] and [4Fe−4S] apoproteins, a function expected for an Fe−S scaffold protein. However, transfer for [4Fe−4S] apoproteins specifically requires a reducing agent such as DTT.

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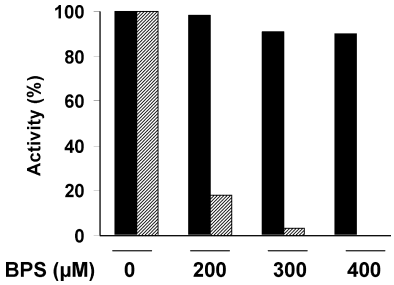

Figure 6. Intact cluster transfer from SufA. Apo-AcnA (0.2 nmol) was incubated anaerobically in 50 mM Tris-HCl (pH 7.6)/5 mM DTT solution with either [2Fe−2S] SufA (providing 4 equiv of Fe and S atoms per apo-AcnA) (black bars) or a 4-fold molar excess of Fe^{2+} and S^{2-} (hatched bars). Increased amounts of BPS were added at the same time as iron and sulfide (or [2Fe−2S] SufA) in both cases, and the mixtures were incubated for 15 min, after which the aconitase activity was measured. The values reported for the activity of AcnA correspond to the average of three independent experiments.

Intact Cluster Transfer from SufA. In order to determine whether the [4Fe−4S] clusters assembled in AcnA arose from intact cluster transfer from native SufA or through spontaneous self-assembly using Fe and sulfide ions transiently released in solution from the [2Fe−2S] cluster of SufA, we monitored cluster assembly in AcnA by measuring aconitase activity during anaerobic reaction with SufA in the presence of an iron chelator. BPS proved suitable because it did not inhibit aconitase activity and did not chelate iron out of native SufA at the concentrations used in the experiments (data not shown). BPS should inhibit the cluster transfer reaction only in the case of a transient disassembly of the SufA cluster but not if the mechanism implies a direct transfer. As shown in Figure 6, BPS indeed efficiently inhibited apo-AcnA activation by iron and sulfide. In contrast, when iron and sulfur were provided by SufA, almost no inhibition was observed at BPS concentrations up to 400 μM. These results thus strongly support a direct cluster transfer mechanism during maturation of AcnA by SufA.

Discussion

All of the results reported here, namely the spectroscopic characterization and the Fe and S analysis, unambiguously demonstrate that SufA as-isolated from *E. coli* cells harbors a [2Fe−2S] cluster. Both the Mössbauer and resonance Raman data are in agreement with complete cysteinyl coordination of the cluster. Since there are only three cysteine residues per SufA monomer, this suggests that one [2Fe−2S] cluster is located at the dimer interface, consistent with structural studies.[19,20] The presence of a $[2Fe-2S]^{+}$ cluster was also demonstrated by EPR spectroscopy after reduction with DTT. It is interesting to mention that this is the first report of an A-type Fe−S scaffold protein in the one-electron-reduced state.

Most of the Fe−S proteins involved in Fe−S biogenesis (such as IscU, IscA, SufA, and SufB) are usually purified in the apo form when they are expressed without their cognate partners. Since protein−protein interactions have been shown to carefully regulate the activity of the individual proteins, the maturation of metal centers would likely depend on the presence of correct partner proteins.[21,22] Furthermore, Fe−S biogenesis pathways are subject to complex in vivo regulation in response to the demand for Fe−S cluster protein maturation. Thus, analysis of Fe−S assembly proteins presents a number of technical challenges due to the dynamic aspects of the in vivo cluster assembly process. In the present work, detection of a cluster in the isolated SufA was possible only because of coexpression of the full *suf* operon. These cellular conditions probably lead to saturation of in vivo target proteins with Fe−S clusters, thus allowing accumulation of labile intermediates in proteins, like SufA, that are involved in Fe−S cluster biogenesis. The fact that the best preparations here nevertheless were substoichiometric in terms of [2Fe−2S] clusters might also be due to partial loss of the labile clusters during purification.

Our results also demonstrate that SufA can transfer Fe−S clusters to both [2Fe−2S] and [4Fe−4S] apoproteins, as shown in the case of Fdx, SPL, and AcnA. This supports the notion that SufA has a cluster scaffold or cluster shuttle protein function, with a labile cluster that can be transferred to apoprotein targets. Interestingly, in vitro assembly of [4Fe−4S] clusters in AcnA or SPL proceeded only when DTT, a reducing agent, was present in the reaction mixture, in agreement with the requirement of two electrons during formation of a [4Fe−4S] cluster from two [2Fe−2S] clusters. The detailed mechanism of formation of a [4Fe−4S] cluster in aconitase from [2Fe−2S] clusters from SufA is an interesting issue that will be addressed in future studies. Recently, reduced Fdx was shown to donate electrons for the conversion of two [2Fe−2S] clusters to one [4Fe−4S] cluster within the IscU from *Azotobacter vinelandii*.[4] Whether such a reductive coupling mediated by DTT occurs on SufA prior to cluster transfer or on the target protein after transfer is an open question requiring further investigation. What we have clearly demonstrated here is that cluster assembly in target proteins does not occur through a transient disassembly of the [2Fe−2S] cluster in native SufA into free Fe and S in solution. Indeed, a strong iron chelator did not inhibit cluster transfer from SufA to AcnA, supporting a direct intact cluster transfer mechanism and confirming previous reports using reconstituted holoSufA or holoIscA proteins.[9]

All of these results now firmly establish that SufA functions as an Fe−S scaffold or Fe−S transporter protein in vivo rather than as a Fe donor for delivery of only Fe during cluster assembly. Furthermore, our results emphasize that in vivo Fe−S cluster formation is a dynamic process that involves complex interactions among all of the proteins encoded by the *suf* operon.

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Supporting Information Available: Comparison of anion exchange chromatography elution profiles and optical spectra of SufA purified from the wild-type SufABCDSE and SufABCDSE ($SufD^{H128A}$) expression systems, and the resonance Raman spectrum of native $^{57}Fe-S$ SufA. This material is available free of charge via the Internet at http://pubs.acs.org

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