Iron-sulfur (Fe-S) clusters are key metal cofactors of metabolic, regulatory, and stress response proteins in most organisms. The unique properties of these clusters make them susceptible to disruption by iron starvation or oxidative stress. Both iron and sulfur can be perturbed under stress conditions, leading to Fe-S cluster defects. Bacteria and higher plants contain a specialized system for Fe-S cluster biosynthesis under stress, namely the Suf pathway. In Escherichia coli the Suf pathway consists of six proteins with functions that are only partially characterized. Here we describe how the SufS and SufE proteins interact with the SufBCD protein complex to facilitate sulfur liberation from cysteine and donation for Fe-S cluster assembly. It was previously shown that the cysteine desulfurase SufS donates sulfur to the sulfur transfer protein SufE. We have found here that SufE in turn interacts with the SufB protein for sulfur transfer to that protein. The interaction occurs only if SufC is present. Furthermore, SufB can act as a site for Fe-S cluster assembly in the Suf system. This provides the first evidence of a novel site for Fe-S cluster assembly in the SufBCD complex.

Fe-S clusters perform important functions in multiple cellular processes, including respiration, gene regulation, and the trichloroacetic acid cycle (1). In vivo Fe-S biogenesis requires specific proteins such as a pyridoxal phosphate-dependent cysteine desulfurase that mobilizes sulfur from l-cysteine and an Fe-S cluster scaffold upon which the nascent cluster can be constructed prior to its transfer to an apoprotein. Homologues of these two core components can be found in numerous organisms ranging from bacteria to humans (1–3). In bacteria the basal pathway of Fe-S cluster assembly is known as IscS (iron-sulfur cluster assembly) (4, 5).

Fe-S cluster homeostasis is sensitive to disruption by reactive oxygen and reactive nitrogen species or by iron limitation (6–8). Recently it was discovered that biosynthesis of Fe-S clusters during adverse stress conditions such as iron starvation and oxidative stress requires a specialized assembly pathway different from the basal Isc system. This pathway, termed the Suf pathway (mobilization of sulfur), is regulated in response to those stress conditions (7–9). The Suf pathway is present in cyanobacteria and the chloroplast of higher plants as well as in bacteria, including human pathogens such as Yersinia pestis and Mycobacterium tuberculosis. In M. tuberculosis, the Suf pathway seems to be the primary Fe-S cluster assembly system, as deleting the suf genes is thought to be lethal to that organism (10).

Because of its importance, Suf has been the focus of intense study at the biochemical level, especially in the Gram-negative bacterium Escherichia coli. The sufABCDSE operon in E. coli encodes six proteins. SufA is homologous to IscA in the basal Isc Fe-S cluster pathway. The exact function of SufA is unknown. Although it has been suggested to act as an Fe-S scaffold, it cannot be excluded that SufA plays a regulatory role (11, 12). SufC is a soluble ATPase similar to those found associated with transporters (7). The function of the SufB and SufD proteins is not known, but the two proteins are homologous to each other. SufB and SufD interact with SufC to form the SufBCD complex, and SufB was recently shown to enhance the basal ATPase activity of SufC (13–16). SufE is a cysteine desulfurase that mobilizes sulfur from free cysteine for donation to Fe-S cluster biosynthesis (17, 18). SufE is a novel sulfur transfer protein that accepts sulfur from SufS, thereby stimulating SufS activity (14, 19, 20). The SufE-dependent stimulation of SufS is further enhanced by the SufBCD complex by an unknown mechanism (14).

Despite the recent progress in acquiring crystal structures of the SufA, SufC, SufD, SufS, and SufE proteins (21–25), a number of questions concerning their biochemical functions in vivo remain unanswered. At least two distinct multiprotein complexes form among the Suf proteins, the SufBCD complex and the SufE complex. Formation of these complexes may help
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proteins were eluted with a linear gradient of 0–1 M NaCl. Fractions containing the SufBCD complex were concentrated and separated on a Superdex 200 gel filtration column (GE Healthcare) in 25 mM Tris, pH 7.4, 150 mM NaCl.

For purification of SufC the bacterial cell pellet was resuspended in buffer A (25 mM Tris, pH 8, 50 mM NaCl, 5 mM DTT) containing 1 mM phenylmethylsulfonyl fluoride, and cells were lysed by sonication. The soluble protein fraction was obtained by centrifugation at 150,000 × g, 4 °C for 90 min. The obtained supernatant was cleared of DNA with 2% (w/v) streptomycin sulfate. The resulting supernatant was loaded on a Q-Sepharose column (GE Healthcare) equilibrated with buffer A. After extensive washing of the column, bound proteins were eluted with a linear gradient of 0–1 M NaCl. SufC-containing fractions were pooled and diluted 1:1 with buffer B (25 mM Tris, pH 7.5, 50 mM NaCl, 2 mM (NH₄)₂SO₄) before loading the protein solution on a Butyl-Sepharose column (GE Healthcare) equilibrated with buffer C (25 mM Tris, pH 7.5, 50 mM NaCl, 1 mM (NH₄)₂SO₄). After extensive washing of the column, bound proteins were eluted with a linear gradient of 1–0 mM (NH₄)₂SO₄. SufC-containing fractions were pooled, concentrated, and loaded on a Superdex 200 column (GE Healthcare) equilibrated with buffer D (50 mM Tris, pH 7.5, 150 mM NaCl).

For purification of SufB and SufD the soluble protein fraction was diluted 1:1 with buffer B and the resulting protein solution was loaded on a Butyl-Sepharose column (GE Healthcare). All subsequent purification steps were the same as those for SufC purification. Purified proteins were concentrated and stored at −70 °C. The molecular mass of purified proteins was verified by mass spectrometry.

Cross-linking and Label Transfer—Purified SufE was labeled with a trifunctional cross-linker Mts-Atf-biotin (2-[N2-(4-azido-2,3,5,6-tetrafluorobenzoyl)-N6-(6-biotinamidocaproyl)-L-lysyl]ethyl methane-thiosulfonate (Pierce). This cross-linker contains a sulfhydryl-specific methane-thiosulfonate (Mts) moiety that was used to attach Mts-Atf-biotin specifically to cysteine residues in SufE. It also contains a photoactivatable tetrafluorophenyl azide moiety (Atf) (supplemental Fig. S1A). The Atf moiety will insert into carbon-hydrogen bonds within 11.1 Å of the cross-linker upon exposure to UV light. SufE at 12.6 µM was mixed with 40 µM Mts-Atf-biotin in a total reaction volume of 300 µl in phosphate-buffered saline (0.1 M, pH 7.2). After 1 h of incubation at room temperature, the non-reacted Mts-Atf-biotin was removed by Zeba Desalt spin columns (Pierce) according to the manufacturer’s protocol. Labeling reactions were carried out in the absence of ambient light to prevent premature activation of the Atf moiety. Addition of reductant to labeled SufE was able to remove the label, indicating that Mts-Atf-biotin binds SufE as expected via reducible disulfide bonds with cysteine residues. The 2 cysteine residues in SufE (Cys-17 and Cys-51) are both accessible to labeling because individual point mutants of SufE were still labeled with Mts-Atf-biotin. For label transfer assays, levels of reductant were kept low until after UV cross-linking with prey proteins to avoid premature loss of the label from SufE bait protein (see below).

Mts-Atf-biotin-labeled SufE was mixed with the other Suf proteins at a final concentration of 2 µM for each component in
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100 µl of phosphate-buffered saline with 100 µM ATP. The reactions were incubated for 1 h at room temperature. Samples were irradiated with UV light for 5 min at a distance of 10 cm using a Spectroline Model BIB-150P UV lamp (365 nm) to initiate cross-linking with the Affi-gel. After UV light exposure, 4 × LDS sample buffer (Invitrogen) with 1.2 M β-mercaptoethanol was added. The samples were separated by denaturing gel electrophoresis on 4–12% Bis-Tris gels and blotted to nitrocellulose membranes. Horseradish peroxidase-conjugated streptavidin (Pierce) was used to visualize proteins labeled with Mts-Atf-biotin.

**Surface Plasmon Resonance**—Surface plasmon resonance experiments were performed on a Biacore 3000. SufE was covalently immobilized to the carboxylated dextran matrix on a CM5 sensor chip (Biacore) via primary amino groups using the amine-coupling protocol (Biacore). Levels of SufE immobilized to the chip were 1300–1600 response units for all experiments. Purified His6-SufS, SufBCD complex, or His6-SufA was injected at various concentrations. All experiments were performed at a flow rate of 20 µl/min in HBS-EP buffer (Biacore). Binding of analyte to SufE at equilibrium was plotted against analyte concentration, and the Prism (Graphpad) program was used to create a nonlinear curve fit of the data. A Boltzmann sigmoidal model, where $Y = Y_{max} X/(K_D + X)$, provided the best fit for SufS binding to SufE based on Akaike’s Information Criterion analysis of multiple models. A Boltzmann sigmoidal model, where $Y = Bottom + (Top-Bottom)/(1 + exp((V50 - X)/Slope))$, provided the best fit for SufBCD binding to SufE.

**Interactions between Suf Proteins by Affinity Chromatography**—1 mg of pure His6-tagged SufA, SufS, SufE, or SufE(C51S) was loaded by gravity flow onto a 0.1-mL nickel-loaded metal-chelating Sepharose column (GE Healthcare) equilibrated with buffer D. Subsequently 0.5 mg of purified SufB, SufC, SufD, or SufBCD that does not carry a His6 tag were passed over these “Suf columns.” The columns were washed with 9 mL of buffer D containing 20–50 mM imidazole. Bound proteins were eluted using buffer D containing 0.5 M imidazole. These experiments were performed either aerobically or anaerobically at 20 °C. In the experiments where combinations of two proteins were used, they were mixed and preincubated for 30 min to allow any necessary complex formation to occur prior to column loading. Eluted proteins were analyzed by SDS-PAGE.

**Cysteine Desulfurase Activity Assays**—Assays were performed in 25 mM Tris, pH 7.5, 100 mM NaCl, 100 µM DTT in a final volume of 100 µl. Final protein concentrations were 500 nM SufS and 1.5 µM SufE, SufB, SufC, SufD, or SufBCD. Reactions were initiated by addition of 100 µM L-cysteine (final concentration) and were allowed to proceed for 20 min at 27 °C. Reactions were stopped by heating the mixtures at 99 °C for 10 min. Denatured proteins were removed by centrifugation, and the supernatant was analyzed for its alanine content using alanine-coupling protocol (Biacore). Levels of SufE immobilized to the chip were 1300–1600 response units for all experiments. Purified His6-SufS, SufBCD complex, or His6-SufA was injected at various concentrations. All experiments were performed at a flow rate of 20 µl/min in HBS-EP buffer (Biacore). Binding of analyte to SufE at equilibrium was plotted against analyte concentration, and the Prism (Graphpad) program was used to create a nonlinear curve fit of the data. A Boltzmann sigmoidal model, where $Y = Y_{max} X/(K_D + X)$, provided the best fit for SufS binding to SufE based on Akaike’s Information Criterion analysis of multiple models. A Boltzmann sigmoidal model, where $Y = Bottom + (Top-Bottom)/(1 + exp((V50 - X)/Slope))$, provided the best fit for SufBCD binding to SufE.

**Iron-Sulfur Cluster Reconstitution on SufB**—Purified SufB (135 µM) was incubated with catalytic amounts (1.5 µM) of SufS and SufE, an excess (2 mM) of L-cysteine, and a 6-fold excess (810 µM) of Fe(NH4)2(SO4)2 in the presence of 5 mM DTT at 20 °C under anaerobic conditions in a glove box. After 5 h of incubation, EDTA (270 µM) was added and after 15 min the mixture was desalted using a Nap25 column (GE Healthcare). The protein was concentrated to 125 µM and stored at −80 °C. UV-visible spectra of reconstituted SufB were recorded on a Cary 1 Bio (Varian) spectrophotometer. The iron and sulfide content of the proteins was determined as previously described (27, 28).

**Determination of Protein Concentration**—The Bio-Rad protein assay was used according to the manufacturer’s instructions using bovine serum albumin as a standard.

**Preparation of EPR Samples**—Reconstituted SufB (125 µM) was reduced by catalytic amounts of photoreduced deazaflavin during 1 h at 18 °C inside an anaerobic glove box. EPR spectra were recorded at 10 K on a Bruker EMX (9.5 Hz) EPR spectrometer equipped with an electron spin resonance (ESR) 900 helium flow cryostat (Oxford Instruments). Double integrals of the EPR signals and spin concentration were obtained through the Win-EPR software using the spectrum of a 1 mM Cu-(EDTA) standard recorded under non-saturating conditions.

**RESULTS**

**Label Transfer from SufE Shows Interactions between SufE and SufS and SufE and SufB**—To test whether transient interactions occur between SufE and the other Suf proteins we utilized the trifunctional cross-linker Mts-Atf-biotin (supplemental Fig. S1). All label transfer experiments were carried out with equal amounts of the same preparation of labeled SufE so that the same amount of Mts-Atf-biotin was present in each sample. Several previous studies have clearly demonstrated interactions between SufE and SufS, presumably to facilitate shielded sulfur transfer from SufS to SufE during Fe-S cluster assembly (14, 20).
the Mts-Atf-biotin can photocross-link to the “bait” protein itself. This intramolecular label transfer within SufE is consistent with the SufE crystal structure, which shows that Cys-17 of SufE is present at the dimer interface between two SufE monomers (29). Cys-17 can be labeled in the bait protein and would likely transfer label to the adjacent SufE monomer during cross-linking and reduction. Alternatively, the label may transfer directly to residues surrounding the labeled Cys-17 or Cys-51 within a SufE monomer. Importantly, we also observed intense label transfer to His<sub>6</sub>-SufS. In contrast, the immunoblot showed only weak biotin labeling of the bovine serum albumin control.

Next, labeled SufE was mixed with various combinations of His<sub>6</sub>-SufS, the SufBCD complex, and His<sub>6</sub>-SufA (Fig. 1B). SufE interacted with SufS and SufB, resulting in a high level of label transfer to those proteins (Fig. 1B, lanes 1 and 3). In contrast, only small amounts of label could be observed on SufD, SufC, or SufA. The same pattern of interactions between SufE and the other Suf proteins was also observed if SufE C51S or SufE C17S single mutant proteins were used as bait, indicating neither cysteine is required for binding to SufS and SufB (data not shown). The same level of label transfer was observed after 2 or 60 min of incubation time before the irradiation step, indicating that the interaction occurs within a short time frame. We also performed another critical control experiment in which the labeled SufE was incubated with the prey proteins but the mixture was not cross-linked by UV light. In the absence of UV cross-linking, we observed no label transfer from SufE to prey proteins, excluding a nonspecific Mts-Atf-biotin transfer mechanism via disulfide bond exchange caused by exposed cysteine thiol groups in SufS or SufBCD (data not shown). Surprisingly, the presence of His<sub>6</sub>-SufA in the label transfer assay seems to diminish interactions between SufE and both His<sub>6</sub>-SufS and SufB (Fig. 1B, compare lane 1 to lane 4 and lane 3 to lane 5). At present the significance of this effect is unclear. Interestingly, SufE was able to interact directly with SufB even in the absence of SufS, indicating that SufB likely contains a SufE-specific binding site (Fig. 1B, lane 1).

Suf Interactions by Column Co-purification—We next used a column chromatography co-purification assay to determine whether SufS or SufA interact with the SufBCD complex. In this assay, His<sub>6</sub>-tagged SufA, SufS, or SufE was loaded on nickel-charged metal-chelating Sepharose columns and the SufBCD complex (with no tag) was passed over these “Suf columns”. After extensive washing, bound proteins were eluted with imidazole and elution fractions were analyzed by SDS-PAGE. Using this method we observed that SufBCD was not retained on columns that were charged with His<sub>6</sub>-SufA or His<sub>6</sub>-SufS. In contrast, SufBCD was found in the elution fraction of a column charged with His<sub>6</sub>-SufE (Fig. 2A). Under the same conditions SufBCD did not bind to an empty nickel-charged metal-chelating Sepharose column (data not shown). These results indicate that SufBCD does not interact with SufA or SufS, or at least that interactions are not strong enough to be detected by our column chromatography method. However, these experiments show that SufBCD interacts with SufE in agreement with the results of the label transfer experiments. Neither label transfer nor co-purification assays showed any interaction between His<sub>6</sub>-SufA and the other Suf proteins. To determine whether

Therefore, as an initial test of this method, we labeled SufE as bait protein and performed the label transfer with His<sub>6</sub>-SufS (Fig. 1A). After UV cross-linking and reduction, an immunoblot performed using a horseradish peroxidase-streptavidin conjugate detected intense biotin labeling of SufE, indicating...

![Figure 1A](image-url) Label transfer from SufE to His<sub>6</sub>-SufS. Labeled SufE was incubated with equimolar amount of bovine serum albumin or His<sub>6</sub>-SufS followed by UV light exposure to initiate cross-linking and label transfer. **Top panel**, an SDS-PAGE gel of cross-linked and reduced samples stained with Coomassie. **Bottom panel**, identical samples immunoblotted with an horseradish peroxidase-streptavidin conjugate (α-Biotin). B, label transfer with SufE as bait protein. Labeled SufE was incubated with equimolar amounts of His<sub>6</sub>-SufA, SufBCD complex, and His<sub>6</sub>-SufS followed by UV light exposure to initiate cross-linking and label transfer. **Top panel**, an SDS-PAGE gel of cross-linked and reduced samples stained with Coomassie. **Bottom panel**, identical samples immunoblotted with an horseradish peroxidase-streptavidin conjugate (α-Biotin). Note that bands for SufS and SufD are very close to each other due to almost similar molecular masses. Intense bands in lanes 2, 3, and 5 of the bottom panel (below SufB) correspond to SufS with label and not to SufD.
the presence of the His$_6$ tag on SufA was responsible for this lack of interaction, an affinity co-purification assay was performed with His$_6$-SufE loaded on the column and untagged SufA passed over the column. No interaction was observed between untagged SufA and SufE (data not shown).

**SufE-SufBCD Interaction Does Not Depend on Intermolecular Disulfide Bridges**—To investigate whether the observed interaction between SufE and SufBCD requires formation of intermolecular disulfide bonds, we repeated the co-purification assays in an anaerobic chamber using degassed buffers. Under these anaerobic conditions the SufE-SufBCD interaction was still observed (supplemental Fig. S2). Additionally, we repeated the experiment using a His$_6$-SufE protein in which the reactive nucleophilic cysteine residue Cys-51 (14, 19) was mutated to serine (SufE C51S) or alternatively a His$_6$-SufE protein that had been treated with iodoacetamide in order to block free cysteine residues by alkylation (successful alkylation was verified by mass spectrometry). For SufE C51S (Fig. 2B) as well as for alkylated SufE (supplemental Fig. S2) the interaction with SufBCD was still observed. All these experiments show that the SufE-SufBCD interaction does not depend on the formation of intermolecular disulfide bridges between exposed thiols on the SufE and SufBCD proteins. Rather, the interaction is mediated via non-covalent forces between specific protein binding surface interfaces.

**SufC Is Required for SufB to Interact with SufE**—Finally, to determine whether the SufE-SufBCD interaction requires each of the three proteins of the SufBDC complex or whether SufE can form a complex with the individual SufB, SufC, or SufD proteins, we added the three individual proteins SufB, SufC, and SufD and also different combinations of SufB + SufC, SufB + SufD, and SufC + SufD to a His$_6$-SufE affinity column. We observed that a mixture of SufB + SufC interacts with His$_6$-SufE. However, combinations of SufB + SufD or SufC + SufD did not interact with His$_6$-SufE (Fig. 2B). The individual SufB, SufC, and SufD proteins also did not interact with His$_6$-SufE (supplemental Fig. S2). Therefore, we can conclude that SufE either binds at an interface consisting of SufB and SufC or that SufC binding to SufB induces a conformational change in SufB that allows it to contact SufE.

**Surface Plasmon Resonance Measurement of SufE Interactions**—Because of the qualitative nature of the label transfer and affinity column techniques, we utilized surface plasmon resonance to better quantify the interactions between SufE and the SufBDC complex. SufE was covalently immobilized for these studies while SufA, SufBCD, or SufS was added in solution as the analyte. Equilibrium binding analysis was performed over a range of Suf protein concentrations, and the level of binding to SufE at equilibrium for each concentration was determined (supplemental Fig. S3). Plots of the resulting equilibrium binding isotherms for SufS, SufBCD, and SufA and their respective nonlinear curve fits are shown in Fig. 3. Binding of SufS to SufE showed a $K_D$ of 0.36 ± 0.05 μM (Fig. 3A). Binding of SufBCD to SufE showed a $K_D$ of 2.78 ± 0.52 μM (Fig. 3B). In
contrast, no measurable interaction between SufA and SufE was observed over the concentration range of SufA used for these studies (0.01–10 μM) (Fig. 3B). Surface plasmon resonance revealed that SufE interacts more strongly with SufS than with SufBCD because the $K_D$ for SufE-SufS was ~10 times lower than that of SufE-SufBCD (Fig. 3). This result is consistent with previous reports that SufS and SufE co-purify when expressed together in vivo and show stable interactions via the yeast two-hybrid assay (19).

**SufBC Is Required for Enhancement of SufS Activity**—After discovering that SufE and SufBC interact, we next determined whether this interaction plays a functional role in the Suf system. Previously, it was reported that the SufBCD complex can enhance the cysteine desulfurase activity of SufSE by an unknown mechanism (14). To determine which of the proteins of SufBCD is responsible for stimulation of SufSE cysteine desulfurase activity, we measured cysteine desulfurase activity of the SufSE complex in the presence of SufB, SufC, SufBC, and SufBCD. We observed enhancement of the cysteine desulfurase activity of SufSE in the presence of SufB, SufC, SufBC, and SufBCD. We observed enhancement of the cysteine desulfurase activity of SufSE in the presence of SufBCD as reported previously (14) and in the presence of SufBC. When the cysteine desulfurase activity of SufSE was measured in the presence of SufB or SufC alone there was no detectable stimulating effect by these proteins (supplemental Fig. S4). Because the enhancement of SufSE cysteine desulfurase activity requires the minimal complex of SufBC, this suggests that SufSE enhancement may be tied directly to the interaction between SufBC and SufE.

**A Role for SufE-SufBC Interactions in Sulfur Transfer**—One mechanism by which SufBC could enhance SufS-SufE activity would be to act as a sulfur acceptor from SufS or SufE. To test this hypothesis that sulfur atoms are actually deposited on SufB or SufC or both, we performed a SufSE-SufBCD cysteine desulfurase reaction and analyzed SufBCD by ESI mass spectrometry for the addition of sulfur atoms (Fig. 4). As a reference, unreacted SufBCD was also subjected to ESI mass spectrometry. The mass spectrum of the unreacted SufBCD complex exhibits three peaks for the individual proteins, with masses at 54613 ± 5 Da for SufB (Fig. 4A), 27583 ± 1 Da for SufC, and 46692 ± 3 Da for SufD. This result is in agreement with the theoretical masses of the proteins based on primary sequences (54745, 27582, and 46823 Da, respectively) minus the 131 Da mass of N-terminal methionines for SufB and SufD. After incubating SufBCD with SufSE and cysteine to initiate the sulfur transfer reaction, mass spectrometry analysis showed that the molecular masses of SufC and SufD did not change. In contrast, we found that the peak representing native SufB almost disappeared and instead several smaller peaks at masses of 54613 + $n$32 Da appeared with $n$ = 1–7 (Fig. 4B). These peaks correspond to the addition of up to seven sulfur atoms onto SufB. Reduction of this mixture with DTT converts SufB back to its native form (Fig. 4C). These results indicate that cysteines on SufB are covalently modified with per- and/or polysulfide species during the SufSE cysteine desulfurase reaction. Interestingly, when SufBCD was incubated with SufS alone and cysteine we did not observe any sulfur addition on SufB. Instead, the mass spectrum of SufB displayed only two peaks, one at 54613 Da corresponding to native SufB and a second at 54733 Da (supplemental Fig. S5A). This second peak corresponds to the addition of one molecule of cysteine to SufB during the incubation with SufS and cysteine. Because this peak disappeared upon addition of DTT, we conclude that the linkage was a disulfide bridge between free cysteine and SufB. Next, SufE was preincubated with SufS and cysteine in order to preload sulfur on SufE. Sulfur-loaded SufE was then freed from cysteine to block further desulfurase activity and added to SufBCD at a SufE:SufBCD molar ratio of 1:2 or 1:1. Under these conditions, we observed the addition of a single sulfur atom to SufB (peaks at masses 54610 and 54639 Da) (supplemental Fig. S5B). Together these experiments demonstrate that SufB, and not SufC or SufD, is able to function as a sulfur acceptor. They also show that the sulfur transfer to SufB requires SufE as a shuttle because incubation of SufBCD with SufS alone did not result in sulfur-loaded forms of SufB.

**FIGURE 4.** ESI mass spectrometry of SufB. **A**, ESI mass spectrum of SufB within the unreacted SufBCD complex. **B**, ESI mass spectrum of SufB within the SufBCD complex after incubation of SufBCD (100 μM) with SufSE (5 μM) and cysteine (5 mM). **C**, ESI mass spectrum of an identical sample as in panel B after addition of 10 mM DTT.
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**SufB Is an Iron-Sulfur Cluster Protein**—Because SufB can accept sulfur transferred from SufE and SufB contains numerous cysteine residues (13 total with 4 highly conserved), it is reasonable to propose that SufB could be an iron-sulfur cluster protein. We investigated this hypothesis by incubating the apoform of SufB with iron and sulfide under anaerobic conditions. Iron was provided by addition of a 6-molar excess of a ferrous iron salt, and sulfide was provided by addition of catalytic amounts of SufSE, the physiological sulfur donor system, with an excess of cysteine. Reconstituted by this method, SufB is brown in color and its UV-visible spectrum is characteristic of a [4Fe-4S]$^{2+}$ cluster with only one absorption band around 420 nm (Fig. 5A). Iron and sulfide measurements revealed the presence of comparable amounts of iron and sulfide with 3.6 iron and 5 sulfide atoms/SufB monomer, further supporting the assignment of a [4Fe-4S] cluster. Upon exposure to air the color of reconstituted SufB quickly became more reddish and the UV-visible spectrum changed with the appearance of additional absorption bands around 320, 450, and 560 nm, which likely indicates a conversion of the [4Fe-4S] cluster to a [2Fe-2S] cluster. In a similar SufB reconstitution reaction in which only SufS was present as the cysteine desulfurase, cluster formation proceeded much more slowly than in the presence of SufE (only 30% cluster formation after 5 h compared with samples with both SufS and SufE). To verify the presence of an iron-sulfur cluster and accurately determine its nature, anaerobically reconstituted SufB was characterized using EPR spectroscopy. Reduction of reconstituted SufB with catalytic amounts of photoreduced deazaflavin under anaerobic conditions led to a rapid bleaching of the protein solution with disappearance of the 420-nm band (Fig. 5A). During this reaction the initial EPR silent protein was converted to a S = 1/2 species, characterized by an axial EPR signal with g values at g = 2.042 and 1.93 (Fig. 5B). Temperature dependence and microwave power saturation properties of the signal were in agreement with a [4Fe-4S]$^{3+}$ cluster (data not shown). The signal integrated to 60% of total iron. Upon exposure of the tube to air for 10 min the EPR signal totally disappeared, indicating oxidation of the cluster to an EPR-silent form or loss of the cluster.

**DISCUSSION**

Previous studies have established that stable interactions occur in the SufBCD and SufSE multiprotein complexes of the Suf pathway (14–16, 19). Here we demonstrate that in fact the five proteins may constitute a single functional complex, as shown in Fig. 6. This conclusion derives from the observation of (i) a weak but significant ($K_{D} = 2.78 \mu M$) interaction between SufE and SufBCD and (ii) specific sulfur transfer from SufE to SufB and (iii) stimulation of the cysteine desulphurase activity of SufSE by SufBC. In contrast, we found no evidence for SufA (the remaining component of the operon) binding to SufE or the SufBCD complex.

**SufE-SufBCD Interactions**—Our results show that SufE interacts with SufBCD regardless of the presence of SufS. We also observed that SufE interacts with both SufS and SufBCD when SufS is present in the incubation mixture. The independent nature of SufE interactions with SufS and SufBCD suggests a model in which sulfur transfer during Fe-S cluster assembly proceeds linearly from SufS to SufE and then from SufE to SufB (Fig. 6). This model is supported by (i) label transfer from SufE to SufB, but not to SufC or SufD; (ii) selective sulfur transfer from SufE to SufB, and not to SufC or SufD, and no sulfur transfer from SufS to SufB; and (iii) the requirement of SufE for SufBCD enhancement of SufS cysteine desulphurase activity. Because SufE in which the active Cys-51 is changed to a serine is also able to interact with SufBCD and because the SufE-SufBCD interaction still occurs under anaerobic conditions, we conclude that formation of the SufE-SufBCD complex does not require Cys-51 or the formation of any disulfide linkages.

Interestingly, SufB alone is not able to interact with SufE. Instead, SufB must be in a complex with SufC to be competent for SufE binding. Furthermore, enhancement of SufSSE cysteine desulphurase activity also requires a minimum complex of SufBC. This requirement provides an exquisite regulatory mechanism to ensure that sulfur transfer is coordinated with complex formation between SufB and the SufC ATPase. It is interesting to note that, in yeast, cytosolic Fe-S cluster assembly depends on soluble P-loop ATPases containing Fe-S clusters such as Cfd1 and Nbp35 (3). SufBC may be the functional
equivalent of Cfd1 or Nbp35 in the bacterial and plant Suf systems. Recent work has shown that SufB can stimulate the intrinsic ATPase activity of SufC (13). Thus sulfur transfer to SufB from the SufSE pair might occur simultaneously with SufB stimulation of SufC ATPase activity. Coordination of these two events may be important for further sulfur transfer to other proteins, Fe-S cluster assembly, or Fe-S cluster transfer to target apoprotein (see below). Alternatively, SufC ATPase activity could be required for iron acquisition, a step that also would be carefully coordinated with sulfur donation. Our data suggest SufC may bind to SufB and induce a conformation change that allows subsequent binding of SufE. This change could occur by creating a new SufBC interface for SufE binding or SufC may function as an allosteric regulator. These interactions imply formation of a large complex consisting of SufSEBCD (Fig. 6) and suggest that the Suf sulfur transfer pathway is well protected with no exposure to the solvent. Such protected sulfur transfer could be a key adaptation of Suf for Fe-S cluster assembly during oxidative stress.

SufB as a Sulfur Shuttle or a Scaffold?—We have shown that SufE interacts with SufB to transfer sulfur. The exact site of sulfur transfer on SufB is not yet known but likely involves one or several of the 13 cysteine residues present in SufB. At present it is not clear whether SufB is the terminal sulfur acceptor during cluster assembly or whether SufB could further transfer sulfur to other proteins such as SufA or an apoenzyme (Fig. 6). One possible scenario is that sulfur transfer to SufB is needed to form a novel type of Fe-S cluster protein. Indeed, the N terminus of SufB from E. coli and its close relatives does contain a putative Fe-S cluster motif (CXXCCXXX) that could be the site of Fe-S cluster assembly. However, this motif is not highly conserved in SufB from more divergent organisms so its importance in cluster assembly is unclear. Even though SufD is homologous to SufB (21), the presence of an Fe-S cluster in SufD is doubtful because it does not contain a CXXCCXXX motif and only one of its 3 cysteine residues is strictly conserved. Our finding that SufB can assemble a [4Fe-4S] cluster favors this hypothesis where sulfur transfer to SufB accompanies Fe-S cluster formation in SufB. The function of the iron-sulfur cluster in SufB remains to be determined. For example, SufB could serve as an alternative scaffold protein or it could also fulfill some redox functions. If SufB should be a scaffold protein, some Suf machineries, e.g. that of E. coli, would contain two scaffolds, namely SufA and SufB. If this should be the case, the physiological conditions under which each one is functional have yet to be defined. One key observation is that none of the known scaffold proteins, such as SufA, IscA, or IscU, is able to stabilize a S = 1/2 reduced cluster (12, 30–32). Therefore, if it is an Fe-S cluster scaffold, SufB would be a unique scaffold protein because its cluster is easily reduced into a stable [4Fe-4S]+ state. Given this difference between other scaffolds and SufB, the features of the SufB Fe-S cluster are more consistent with a redox function until future studies clarify its role.

All of the possibilities outlined above remain to be addressed by future characterization of the Suf protein-protein complexes and by future experiments to determine the sites of assembly and the functional roles of Fe-S clusters in the Suf pathway. We have begun to unravel the network of interactions that take place between the components of the Suf pathway to better define the biochemical details of Suf Fe-S cluster assembly.

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REFERENCES
1. Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005) Annu.
Rev. Biochem. 74, 247–281
2. Fontecave, M., Choudens, S. O., Py, B., and Barras, F. (2005) J. Biol.
Inorg. Chem. 10, 713–721
3. Lill, R., and Muhlenhoff, U. (2006) Annu. Rev. Cell Dev. Biol. 22, 457–486
4. Takahashi, Y., and Nakamura, M. (1999) J. Biochem. (Tokyo) 126, 917–926
5. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) J. Biol.
Chem. 273, 13264–13272
6. Flint, D. H., Tuminello, J. F., and Emptage, M. H. (1993) J. Biol.
Chem. 268, 22369–22376
7. Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001)
Mol. Microbiol. 39, 960–972
8. Ollagnier, F. W., Djaman, O., and Storz, G. (2004) Mol. Microbiol. 52, 861–872
9. Takahashi, Y., and Tokumoto, U. (2002) J. Biol. Chem. 277, 28380–28383
10. Huet, G., Daffe, M., and Saves, I. (2005) J. Bacteriol. 187, 6137–6146
11. Palasubramanian, R., Shen, G., Bryant, D. A., and Golbeck, J. H. (2006)
J. Bacteriol. 188, 3182–3191
12. Ollagnier-de Choudens, S., Nachin, L., Sanakis, Y., Loiseau, L., Barras, F.,
and Fontecave, M. (2003) J. Biol. Chem. 278, 17993–18001
13. Eccleston, I. F., Petrovic, L., Davis, C. T., Rangachari, K., and Wilson, R. J.
(2006) J. Biol. Chem. 281, 8571–8578
14. Ollagnier, F. W., Wood, M. J., Munoz, F. M., and Storz, G. (2003) J. Biol.
Chem. 278, 45713–45719
15. Rangachari, K., Davis, C. T., Eccleston, I. F., Hirst, E. M., Saldanha, J. W.,
Strath, M., and Wilson, R. J. (2002) FEBS Lett. 514, 225–228
16. Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) EMBO J. 22,
427–437
17. Mihara, H., Fujii, T., Kato, S., Kurihara, T., Hata, Y., and Esaki, N. (2002)
J. Biochem. (Tokyo) 131, 679–685
18. Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) J. Biochem.
(Tokyo) 127, 559–567
19. Loiseau, L., Ollagnier-de Choudens, S., Nachin, L., Fontecave, M.,
and Barras, F. (2003) J. Biol. Chem. 278, 38352–38359
20. Ollagnier-de Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E.,
and Fontecave, M. (2003) FEBS Lett. 555, 263–267
21. Badger, J., Sauder, J. M., Adams, J. M., Antonsamy, S., Bain, K., Bergseid,
M. G., Buchanan, S. G., Buchanan, M. D., Batyenko, Y., Christopher, J. A.,
Ettang, S., Eroshkina, A., Feil, I., Furlong, E. B., Gajiwala, K. S., Gao, X., He,
D., Hendle, J., Huber, A., Hoda, K., Kearns, P., Kissing, C., Laubert, B.,
Lewis, H. A., Lin, J., Loomis, K., Lörimer, D., Louie, G., Maletic, M., Marsh,
C. D., Miller, L., Molinari, J., Muller-Dieckmann, H. J., Newman, J. M.,
Noland, B. W., Pagarigan, B., Park, F., Peat, T. S., Post, K. W., Radijojic, S.,
Ramos, A., Romero, R., Rutter, M. E., Sanderson, W. E., Schwinn, K. D.,
Tressler, J., Winhoffen, J., Wright, T. A., Wu, L., Xu, J., and Harris, T. J.
(2005) Proteins 60, 778–796
22. Fujii, T., Maeda, M., Mihara, H., Kurihara, T., Esaki, N., and Hata, Y. (2000)
Biochemistry 39, 1263–1273
23. Kitaoka, S., Wada, K., Hasegawa, Y., Minami, Y., Fukuyama, K., and Taka-
hashi, Y. (2006) FEBS Lett. 580, 137–143
24. Liu, G., Li, Z., Chiang, Y., Acton, T., Montelione, G. T., Murray, D., and
Szyperski, T. (2005) Protein Sci. 14, 1597–1608
25. Wada, K., Hasegawa, Y., Gong, Z., Minami, Y., Fukuyama, K., and Taka-
hashi, Y. (2005) FEBS Lett. 579, 6543–6548
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26. Yoshida, A., and Freese, E. (1970) Methods Enzymol. 17, 176–181
27. Beinert, H. (1983) Anal. Biochem. 131, 373–378
28. Fish, W. W. (1988) Methods Enzymol. 158, 357–364
29. Goldsmith-Fischman, S., Kuzin, A., Edstrom, W. C., Benach, J., Shastry, R., Xiao, R., Acton, T. B., Honig, B., Montelione, G. T., and Hunt, J. F. (2004) J. Mol. Biol. 344, 549–565
30. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) Biochemistry 39, 7856–7862
31. Agar, J. N., Zheng, L., Cash, V. L., Dean, D. R., and Johnson, M. K. (2000) J. Am. Chem. Soc. 122, 2136–2137
32. Krebs, C., Agar, J. N., Smith, A. D., Frazzon, J., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2001) Biochemistry 40, 14069–14080