

Published in final edited form as:

Biochemistry. 2010 November 2; 49(43): 9402–9412. doi:10.1021/bi1011546.

SufD and SufC ATPase activity are required for iron acquisition during in vivo Fe-S cluster formation on SufB

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Abstract

In vivo biogenesis of Fe-S cluster cofactors requires complex biosynthetic machinery to limit release of iron and sulfide, to protect the Fe-S cluster from oxidation, and to target the Fe-S cluster to the correct apo-enzyme. The SufABCDSE pathway for Fe-S cluster assembly in *E. coli* accomplishes these tasks under iron starvation and oxidative stress conditions that disrupt Fe-S cluster metabolism. Although SufB, SufC, and SufD are all required for in vivo Suf function, their exact roles are unclear. Here we show that SufB, SufC, and SufD, co-expressed with the SufS-SufE sulfur transfer pair, purify as two distinct complexes (SufBC₂D and SufB₂C₂) that contain Fe-S clusters and FADH₂. These studies also show that SufC and SufD are required for in vivo Fe-S cluster formation on SufB. Furthermore, while SufD is dispensable for in vivo sulfur transfer, it is absolutely required for in vivo iron acquisition. Finally, we demonstrate for the first time that the ATPase activity of SufC is necessary for in vivo iron acquisition during Fe-S cluster assembly.

Iron-sulfur (Fe-S) clusters are required as cofactors in a wide range of critical cellular pathways. Fe-S metalloproteins carry out diverse reactions including electron transfer and substrate binding and activation. Fe-S cluster biosynthesis requires a complex network of proteins that mobilize sulfur and iron, assemble nascent clusters, and transfer Fe-S clusters to target metalloproteins. In eukaryotes, Fe-S cluster assembly proteins are localized at multiple subcellular locations, while the mitochondria in particular play a central role in regulating Fe-S cluster metabolism (1). Prokaryotic organisms often contain multiple Fe-S cluster assembly pathways with overlapping but divergent functions depending on environmental conditions and the specific target Fe-S metalloproteins (2).

In vivo biosynthesis of Fe-S clusters is complicated by the potential oxidation of both iron and sulfide building blocks, as well as protein cysteinyl ligands, by oxygen or reactive oxygen species. The *sufABCDSE* operon is required for de novo Fe-S cluster biogenesis under iron starvation and oxidative stress conditions in *E. coli* (3–6). The SufS cysteine

[†]This work was supported by National Institutes of Health grant GM81706 and a Cottrell Scholar Award from the Research Corporation for Science Advancement (to F.W.O.) and by National Institutes of Health grant GM62524 (to M.K.J.).

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Supporting Information Available. The following Supplemental Information is available as a single PDF file free of charge via the Internet at <http://pubs.acs.org>: Supplemental Methods for protein stoichiometry measurements and in vitro Fe-S cluster reconstitution of SufBCD; Table S1, containing the stoichiometry measurements for the various complexes; Figure S1, containing the elution profile of wild-type and SufC(K40R) (His)₆-SufBCD; Figure S2, containing the UV-visible absorption spectrum of the flow-through after SufBCD concentration and the identification of low intensity bands in the (His)₆-SufBCD elution fractions; and Figure S3, containing the EPR spectrum of in vitro reconstituted [4Fe-4S]⁺ SufBCD.

desulfurase and SufE sulfur transfer protein together mobilize sulfur from free cysteine as a protein-bound persulfide (R-S-SH) that ultimately is incorporated into the Fe-S cluster as sulfide during assembly (7–9). The SufA protein is a member of the A-type Fe-S carrier protein family that transfers Fe-S clusters to target apo-enzymes (10–12). The remaining proteins, SufB, SufC, and SufD, form a stable SufBC₂D complex but the exact in vivo function of this complex is unknown (4,9).

Recent in vitro studies have shown that both SufB and the SufBC₂D complex can form a [4Fe-4S] cluster that converts to a [2Fe-2S] cluster upon exposure to oxygen (7,13). Furthermore, in vitro the SufBC₂D complex can transfer Fe-S clusters to the SufA carrier protein as well as directly to the target apo-enzyme Aconitase B (13,14). These in vitro studies suggest that the SufBC₂D complex is a novel type of Fe-S scaffold system distinct from the well-characterized IscU or NifU scaffold proteins. Furthermore, it appears that SufB is the specific Fe-S scaffold protein within the SufBC₂D complex. A key question unanswered by previous studies is the exact role of SufC and SufD in Fe-S cluster assembly. SufC is an ATPase with homology to ATPase subunits of membrane transporters, although the SufBC₂D complex is cytoplasmic (3). The basal activity of SufC alone is atypically low but SufC ATPase activity is enhanced by interacting with either SufB and SufD separately or as part of the SufBC₂D complex (15,16). The role of SufD is unknown, although the C-terminal half of SufD does share significant homology with the same region in SufB (45% sequence similarity over the C-terminal 150 residues for each protein). Despite the fact that SufB alone can easily form Fe-S clusters in vitro, deletion of any of the three components (SufB, SufC, or SufD) abolishes Suf function in vivo (3–6).

The purpose of the present study is to characterize in vivo Fe-S cluster assembly via the Suf pathway, with a focus on the in vivo role of SufC ATPase activity and the SufD protein during in vivo Fe-S cluster assembly on SufB. To address these questions we purified a polyhistidine tagged form of SufB after co-expression with SufCDSE. Using this approach, we have found that SufB, SufC, and SufD form at least two distinct complexes in vivo: SufBC₂D and SufB₂C₂. Furthermore, we determined that the absence of SufD or loss of SufC ATPase activity disproportionately diminishes iron content in the SufBCD complexes with only a small effect on sulfide content. These results indicate that SufD and SufC help mediate iron acquisition during in vivo Fe-S cluster assembly on SufB.

MATERIALS AND METHODS

Strains and Plasmids

SufS and SufE were amplified by PCR as one DNA fragment using the pGSO164 plasmid (9) as a template and primers 5'-GGGAATTCATATGATTTT TTCCGTCGACAAAGTGC GGCCGACTTTCCGGTGC-3' and 5'-GGGAATTCGGTACCTT AGCTAAGTGCAGCGGCTTTGGCGCGAATTGCGCGAATCAT-3'. The PCR product was digested with *NdeI* and *KpnI* and cloned into the corresponding sites of pETDuet-1 (Novagen), generating plasmid pFWO467. SufB, SufBC, SufBCD were amplified by PCR using the pGSO164 plasmid as a template and the following primers: SufB 5'-GGGAATTCGAATTCGTCTC GTAATACTGAAGCAACTGACGATGTAAAAAC-3' and 5'-GGGAATTCCTGCAGTTATCCG ACGCTGTGTTCAAGACTGATGGCGAGGAG-3'; SufBC 5'-GGGAATTCGAATTCGTCTC TCGTAATACTGAAGCAACTGACGATGTAAAAAC-3' and 5'-GGGAATTCCTGCAGTTACTGC TGTTCCGGTAAGCCAGCCATAACCCTGCTC-3'; SufBCD 5'-GGGAATTCGAATTCGTCTCGT AATACTGAAGCAACTGACGATGTAAAAAC-3' and 5'-GGGAATTCCTGCAGTCATCTTG CACCTCCTGGCAGCCGTTGACCGATTTCG-3'.

PCR products were digested with *EcoRI* and *PstI* and cloned into the corresponding sites of pFWO467 generating plasmids pFWO468 (His₆-SufB), pFWO469 (His₆-SufBC), and pFWO470 (His₆-SufBCD). The SufC(K40R) mutation was introduced into pGSO164 by site directed mutagenesis using the QuikChange kit (Stratagene) with primers 5'-CCAAACGGTTCGGGCAGAAGTACCTTATCGGCAACG-3' and 5'-CGTTGCCGATAAGGTA CTTCTGCCCGAACCGTTTGG-3'. The pGSO164-SufC(K40R) plasmid was used as template for PCR with the primers described above to amplify SufBCD, followed by digestion of the PCR fragment with *EcoRI* and *PstI* and ligation into the corresponding sites of pFWO467 to generate pFWO471. The sequences of all plasmid inserts were confirmed by DNA sequencing.

Protein Expression and Purification

Recombinant His₆-SufB, His₆-SufBC, and His₆-SufBCD were co-expressed with SufSE from expression vectors pFWO468, pFWO469, and pFWO470 in *E. coli* strain BL21(DE3). Cultures were grown in LB at 37 °C and induced with 100 μM of isopropyl-β-D-thiogalactoside (IPTG) when OD₆₀₀ = 0.5 – 0.6 followed by a shift to 18 °C. LB used for these expression studies was made using ultrapure water and typically contained 9 μM iron. After 18 h of induction at 18 °C, the cells were collected by centrifugation and resuspended in anaerobic buffer containing 50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 20 mM imidazole, and 1 mM phenylmethanesulphonylfluoride (PMSF) (Sigma), followed by anaerobic sonication and centrifugation to remove the cell debris. The cleared lysate was loaded onto a Ni²⁺-NTA column in line with an AKTA Prime FPLC system located completely inside an anaerobic Coy chamber. The column was washed with 5 column volumes of 50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, and 20 mM imidazole. His₆-SufB along with any interacting proteins was eluted with an increasing gradient of 20 to 500 mM imidazole. His₆-SufB or His₆-SufB₂C₂ eluted as a single peak and those fractions were pooled and concentrated anaerobically. Wild-type His₆-SufBC₂D and His₆-SufBC₂D-SufC(K40R) protein complexes eluted as two separate peaks (Fig. S1, see Results). Fractions from Peak 1 and Peak 2 were pooled separately and concentrated anaerobically. The proteins were analyzed by SDS-PAGE. To determine UV-visible extinction coefficients, EPR spin concentrations as well as iron, sulfide, and flavin ratios, the following protein molecular weights were used: His₆-SufB = 56.4 kDa. His₆-SufB₂C₂ = 168.0 kDa. wild-type His₆-SufBC₂D Peak 1 = 158.4 kDa. His₆-SufB₂C₂ Peak 2 = 168.0 kDa. His₆-SufBC₂D-SufC(K40R) Peak 1 = 158.4 kDa. His₆-SufB₂C₂-SufC(K40R) Peak 2 = 168.0 kDa.

Chemical Analyses

Iron content of purified proteins was determined colorimetrically using ferrozine as described previously (17). The acid-labile sulfide content of purified proteins was determined by a previously reported method (18). To determine the type of flavin bound to the various proteins they were denatured by boiling for 10 min, followed by incubation on ice for 5 min. Precipitated protein was removed by centrifugation at 16,000 × g for 10 min. The flavin remaining in the supernatant, which was completely oxidized, was analyzed by thin layer chromatography on silica gel 60 F254 (Merck) with Butan-1-ol/acetic acid/water (12:3:5 v/v) as the solvent system (19). FMN (MP Biomedicals) and FAD (Sigma) were run as flavin standards. Flavins were detected by their yellow color and by their fluorescence under UV light. For subsequent quantification of FADH₂/FAD bound to proteins, the spectrum of the supernatant was recorded with Beckman Coulter DU 800 spectrophotometer after removal of precipitated protein and the concentration of FAD released was calculated using the molar extinction coefficient for free FAD at 450 nm ($\epsilon = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$) (20).

Cysteine Desulfurase Activity Assay

Assays were performed using a previously reported method (9). Briefly, reactions were carried out anaerobically at 27 °C in 25 mM Tris-Cl, pH 7.4, 100 mM NaCl, using 1 μM cysteine desulfurase SufS and 4 μM SufE with or without 4 μM SufBC₂D, SufBC₂D_K40R mutant, or (His)₆-SufB₂C₂ complex.

Other Spectroscopic Methods

All samples for spectroscopic investigations were prepared under an argon atmosphere (< 5 ppm O₂) in Vacuum Atmospheres or Coy glove boxes unless otherwise indicated. UV-visible absorption were recorded in sealed anaerobic quartz cuvettes at room temperature using a Beckman or Shimadzu UV-3101 PC scanning spectrophotometer. X-band (~ 9.6 GHz) EPR spectra were recorded using an ESP-300D spectrometer (Bruker, Billerica, MA) equipped with an ER-4116 dual mode cavity and an ESR 900 flow cryostat (Oxford Instruments, Concord, MA). Spin quantifications were carried out using a 1 mM Cu(EDTA) standard and non-saturating conditions.

Reactivation of Suf Fe-S cluster assembly in crude lysates

His₆-SufBCD were co-expressed with SufSE from expression vector pFWO470 in *E. coli* strain BL21(DE3). Cell growth and protein induction was performed as described above. A cell pellet harvested from 2 L of LB was resuspended in 15 ml of buffer and lysed anaerobically as described above. 2mM L-Cysteine, 2mM Mg²⁺-ATP and/or 2mM NADPH were added to the cleared lysate. The lysate was incubated anaerobically for 2 hr at 20 °C. Following incubation, the lysate was loaded onto Ni²⁺-NTA column and His₆-SufB was purified anaerobically as described above. No exogenous iron source was added but endogenous iron was typically present at 250 μM in the cleared lysate. After removal of total protein from the crude lysate using a YM3 membrane, approximately 5 % of total iron (13 μM) remained in the flow- thru, presumably bound to low-molecular weight species.

RESULTS

SufBC₂D and SufB₂C₂, co-expressed with SufSE, purify bound to FADH₂ and a [4Fe-4S] cluster

Despite the clear evidence that SufB and the SufBC₂D complex can form a [4Fe-4S] cluster *in vitro*, purification of SufB or SufBC₂D after *in vivo* expression often results in isolation of protein bound to only low levels of iron and acid-labile sulfide that contains no distinguishable Fe-S cluster (7,9,13). Since SufB and SufBC₂D both form [4Fe-4S] clusters after *in vitro* reconstitution, these studies have not provided insight into the role of SufC and SufD during *in vivo* Fe-S cluster assembly. To clarify the *in vivo* Fe-S cluster status of the SufBC₂D complex and to characterize the function of the SufC and SufD proteins for *in vivo* Fe-S cluster assembly on SufB, we designed a novel co-expression system based on the pETDuet protein expression vector. The *sufBCD* and *sufSE* gene cassettes were cloned at two separate promoter sites within the vector. Utilizing this vector the *sufBCD* genes can be mutated without altering expression of the *sufSE* genes, which is difficult to accomplish if expressing the entire *sufABCDSE* polycistronic message. The *sufA* gene was omitted from this vector because we reasoned that Fe-S cluster accumulation in the SufBC₂D scaffold might be difficult to capture in the presence of SufA due to cluster transfer from SufBC₂D to the SufA Fe-S cluster carrier protein (14). Finally, our novel expression vector incorporates a hexahistidine tag at the N-terminus of SufB to allow for one-step anaerobic purification in order to preserve intact Fe-S clusters on SufB and/or SufBC₂D.

The chromosomally-encoded Suf system in *E. coli* is usually only induced under oxidative stress and iron starvation conditions (5,21). However, Suf can substitute for the

housekeeping Isc Fe-S cluster assembly pathway even under non-stressed conditions, especially if Suf expression levels are increased above their low basal levels (22). While it is possible that there are subtle alterations in Suf mechanism under stressed and non-stressed conditions, these previous results indicate that Suf can function under non-stress conditions to replace the Isc system. Therefore for the present study we co-expressed the SufBCD and SufSE proteins from the pETDUET vector in LB under aerobic conditions. Future studies will also examine Suf expression specifically under stress growth conditions.

Cells co-expressing SufBCD and SufSE were lysed anaerobically and (His)₆-SufB was purified anaerobically using a Ni²⁺-NTA column. Using a gradient of imidazole, (His)₆-SufB eluted in two peaks from the Ni²⁺-NTA column (Fig. S1A). Peak 1 contained approximately 85% of the total eluted protein. SufC and SufD co-purified with (His)₆-SufB in Peak 1 consistent with previous studies showing tight interactions within the complex (Fig. 1) (9). Gel filtration analysis of Peak 1 showed an apparent molecular weight consistent with a SufBC₂D complex as observed previously (data not shown) (9,13). Peak 1 was initially colored yellowish-green but the yellow color partially faded as the sample was concentrated using a filter with a 30 kDa MW retention limit. Prior to concentrating (His)₆-SufBC₂D Peak 1 showed a complex UV-visible absorption spectrum with a broad shoulder at 320 nm and features at 439 and 600 nm (Fig. 2A). However, the (His)₆-SufBC₂D Peak 1 spectrum is distinct from the spectrum of [4Fe-4S] SufBC₂D reconstituted in vitro under anaerobic conditions (compare Fig. 2A to Fig. S1C). Within 5 minutes of air exposure, the UV-visible absorption spectra of (His)₆-SufBC₂D Peak 1 showed the rapid appearance of strong maxima at 369 and 446 nm and these maxima saturated with 10 minutes of air exposure (Fig. 2A). Absorption maxima at these wavelengths are consistent with the oxidation of a flavin. Simultaneous with the appearance of the 369 and 446 nm maxima during oxygen exposure, the broad shoulder at 320 nm diminishes. The UV-visible absorption spectrum of (His)₆-SufBC₂D Peak 1 also showed a sharp feature at 414 nm that indicates the presence of an anionic flavin semiquinone species. We confirmed that (His)₆-SufBC₂D Peak 1 specifically contains FADH₂ and not FMN using thin layer chromatography (data not shown). Quantification of the flavin content of (His)₆-SufBC₂D Peak 1 indicated the presence of 1.1 FADH₂ per SufBC₂D complex immediately after purification (Table 1). During concentration of (His)₆-SufBC₂D, FADH₂ is oxidized to FAD and partially dissociates from SufBC₂D although SufBC₂D still retains about 0.8 FADH₂ following concentration. The UV-visible absorption spectrum of the yellow flow-through collected during concentration of (His)₆-SufBC₂D Peak 1 also showed absorption peaks at 374 and 448 nm although the semiquinone peak at 414 nm was not present in the flow-through (Fig. S2A). Recently Wollers et al. also have shown that SufBC₂D reversibly binds FADH₂, although the role of the flavin in cluster assembly is still unclear (13).

The second peak purified from cells expressing (His)₆-SufBCD contained 15% of the total protein isolated. Although Peak 2 contained SufB, the ratio of SufC and SufD to SufB was reduced compared to Peak 1 (Fig. 1). Quantification of the SufB, SufC, and SufD content in both peaks indicated that in Peak 2 the SufB:SufC:SufD ratios were 1:1:0.5 while Peak 1 showed the previously observed SufB:SufC:SufD ratios of 1:2:1 (Table S1) (13). Gel filtration analysis of Peak 2 showed one prominent peak, containing SufB and SufC, with an estimated molecular weight consistent with SufB₂C₂ stoichiometry and an overall SufB:SufC ratio of 1:1 (data not shown). Thus Peak 2 contains a (His)₆-SufB₂C₂ complex with only residual SufD association. Also apparent in Peak 2 were additional proteins not observed in the Peak 1 sample. These bands were identified by MS/MS sequencing analysis and found to be SufS and a small amount of partially proteolyzed SufB (Fig. S2B).

Peak 2 (His)₆-SufB₂C₂ was brown and the UV-visible absorption spectrum (Fig. 2B) showed a broad peak at 406 nm that is similar to the spectra of in vitro reconstituted

[4Fe-4S] SufB (7) or anaerobically reconstituted [4Fe-4S] SufBC₂D (Fig. S1C). Upon exposure to air, the (His)₆-SufB₂C₂ Peak 2 UV-visible absorption spectrum (Fig. 2B) decreased in overall intensity but showed sharpened peaks at 328 and 416 nm indicating conversion of the [4Fe-4S] cluster to other cluster forms. Interestingly, Peak 2 did not show dramatic increases in absorbance at 369 and 446 nm after air-exposure. Direct measurement of flavin in Peak 2 indicated only 0.4 FADH₂ bound per (His)₆-SufB₂C₂ complex, which was reduced to 0.2 FAHD₂ per complex after concentration (Table 1).

Peak 1 and Peak 2 were retained and concentrated separately under anaerobic conditions. (His)₆-SufBC₂D Peak 1 contained 1.3 Fe atoms and 1.8 S²⁻ per complex, consistent with the presence of sub-stoichiometric amounts of intact or partially degraded Fe-S clusters (Table 1). The specific type of Fe-S cluster in Peak 1 could not be easily characterized by UV-visible spectroscopy due to the presence of strong flavin absorbance features (Fig. 2). In contrast, Peak 2 (His)₆-SufB₂C₂ contained 3.2 Fe atoms and 4.2 S²⁻ atoms per complex consistent with the presence of a [4Fe-4S] cluster. Table 1 shows the average iron and acid-labile sulfide content of (His)₆-SufBCD Peak 1 and Peak 2 from triplicate measurements of three independent (His)₆-SufBCD purifications. In summary, UV-visible absorption spectroscopy and chemical analysis indicate the isolation of two distinct complexes, (His)₆-SufBC₂D, with an uncharacterized Fe-S cluster species and stoichiometric FADH₂ (Peak 1) and (His)₆-SufB₂C₂ with a [4Fe-4S] cluster and lower amounts of FADH₂ (Peak 2). The requirement for all three proteins for one equivalent of flavin binding mirrors the recent results of Wollers et al. (13).

To characterize the FADH₂ and Fe-S species present in Peaks 1 and 2, we conducted a large-scale purification of (His)₆-SufBCD under anaerobic conditions in order to prepare a sample that was sufficiently concentrated for subsequent spectroscopic analysis. Initially we utilized the same linear imidazole gradient for large-scale purification of (His)₆-SufBCD that was used for the small-scale preparation. We observed elution of Peak 1 and Peak 2 similar to that from the small-scale preparations described above (data not shown). However, the run time required to maintain the proper gradient and flow rate was greater for the large column (utilized for the large-scale preparation) than for the small-scale preparation column. During the longer elution phase the Fe-S cluster content of (His)₆-SufB₂C₂ Peak 2 greatly diminished such that it was nearly undetectable after elution and concentration. To avoid this technical problem, we eluted the complexes from a large-scale preparation with a fast step gradient rather than the slow linear gradient. This modified purification protocol allowed us to prepare a concentrated sample containing both (His)₆-SufBC₂D and (His)₆-SufB₂C₂ mixed together in the same elution fractions. The Peak 1/Peak 2 mixture was frozen in liquid nitrogen either before or after addition of dithionite and both samples were analyzed by EPR spectroscopy (Fig. 3).

The EPR spectra of the purified mixture of (His)₆-SufBC₂D and (His)₆-SufB₂C₂ before and after dithionite reduction provides a preliminary assessment of the nature of the Fe-S clusters. As purified the EPR spectrum is dominated by a $g = 4.3$ resonance that is generally characteristic of adventitiously bound high-spin ($S = 5/2$) Fe(III) species (Fig. 3A). However, in this case, the breadth of the low-field component of the EPR spectrum centered at $g = 9.0$ suggests an alternative assignment. Based on spectroscopic studies of structurally synthetic complexes and “purple” aconitase, the features are characteristic of linear [3Fe-4S]⁺ clusters, which also exhibit rhombic $S = 5/2$ ground states (23,24). Confirmation and quantification of linear [3Fe-4S]⁺ clusters in as-purified SufBCD will require detailed Mössbauer and/or variable-temperature MCD studies. It is possible that this linear [3Fe-4S]⁺ cluster is an oxidative degradation product of a [4Fe-4S] cluster (as is the case in aconitase). If so, the degradation pathway for the [4Fe-4S] cluster on the SufB scaffold is distinct from that of the the [4Fe-4S] cluster on the IscU scaffold which degrades directly to the [2Fe-2S]

form with the concomitant loss of iron and sulfide without forming a stable linear $[3\text{Fe-4S}]^+$ intermediate (25). This preliminary result is therefore intriguing as both synthetic and biological linear $[3\text{Fe-4S}]^+$ clusters have been shown to convert to $[4\text{Fe-4S}]^{2+,+}$ clusters under reducing conditions in the presence of Fe(II) ion (23,26). Hence it is possible that linear $[3\text{Fe-4S}]^+$ clusters are in vivo precursors of $[4\text{Fe-4S}]$ clusters on SufB and experiments are in progress to test this hypothesis

The $g = 2.0$ region of the EPR spectrum of as-purified SufBCD is dominated by an isotopic organic radical resonance centered near $g = 2.01$ (Fig. 3A), that is readily observable without broadening at 100 K. This resonance accounts for < 0.03 spins/SufBCD complex in as purified samples, increases to 0.10 ± 0.02 spins/SufBCD complex in samples reduced with stoichiometric dithionite, and is lost in samples reduced with a 10-fold excess of dithionite. The EPR and redox properties are both indicative of a flavin semiquinone as initially suggested by the UV-visible absorption data discussed above and as recently reported for SufBC₂D bound to FADH₂ (13).

Reduction with a 10-fold excess of dithionite also results in loss of the $g = 9.0$ and 4.3 features of the $S = 5/2$ resonance and the appearance of a near-axial $S = 1/2$ resonance, $g = 2.046, 1.936,$ and 1.895 , with relaxation properties that are characteristic of a $S = 1/2$ $[4\text{Fe-4S}]^+$ cluster (Fig. 3B) (i.e. observable without broadening only below 30 K). The resonance accounts for 0.10 ± 0.03 spins/SufBCD complex and is very similar to that reported for $S = 1/2$ $[4\text{Fe-4S}]^+$ clusters in dithionite-reduced reconstituted samples of SufB, $g_{\parallel} = 2.042$ and $g_{\perp} \sim 1.93$ (7) and to the EPR spectrum of in vitro reconstituted $[4\text{Fe-4S}]^+$ SufBC₂D (Fig. S3). The low spin quantification of the as-purified sample is likely to be a consequence of having both conformations (Peaks 1 and 2) of SufBCD present in the EPR sample. While only the SufB₂C₂ Peak 2 appears to contain $[4\text{Fe-4S}]^{2+,+}$ clusters as-purified (based on the UV-visible absorption data in Fig. 2B and the iron and sulfide measurements in Table 1), SufB₂C₂ only represents about 15% of the total SufBCD protein in the sample (with the remainder being SufBC₂D Peak 1). Although we have been able to observe low-field components indicative of a high-spin ($S > 1/2$) $[4\text{Fe-4S}]^+$ clusters in the EPR spectra, we cannot rule out the possibility that the low spin quantification is also a consequence of a spin state mixture involving a heterogeneous high-spin $[4\text{Fe-4S}]^+$ cluster. In summary, the EPR spectroscopy confirms the presence of $[4\text{Fe-4S}]^{2+,+}$ clusters on as-purified SufB₂C₂ and suggests that either SufB₂C₂ or SufBC₂D may contain linear $[3\text{Fe-4S}]$ clusters that have not been previously observed in Fe-S cluster assembly systems such as *Isc* or *Nif*.

Although we were able to isolate the (His)₆-SufB₂C₂ complex bound to $[4\text{Fe-4S}]$ clusters, it is possible that formation of this novel complex is an artifact of our protein expression system. A detailed biochemical comparison of SufBC₂D and SufB₂C₂ is currently underway. However, in order to test if the (His)₆-SufB₂C₂ described here has biochemical activity consistent with Fe-S cluster assembly, we determined if as-purified (His)₆-SufB₂C₂ is able to further enhance the cysteine desulfurase activity of SufS in the presence of SufE as has been previously reported for SufBC₂D (7,9). The as-purified (His)₆-SufB₂C₂ complex enhances the activity of SufSE to a similar level as the SufBC₂D complex (Table 2), suggesting that it is a physiologically active complex rather than a purification artifact.

SufC and SufD are required for in vivo Fe-S cluster assembly on SufB

To establish the potential role of SufC and/or SufD during Fe-S cluster formation in vivo, we repeated the (His)₆-SufB purification from expression vectors for which either SufD or both SufC and SufD were deleted. This allowed us to purify the SufBC sub-complex or SufB alone. Even in the absence of SufD, SufB and SufC can still form a stable complex such that SufC co-purifies with (His)₆-SufB as a single peak (Fig. 2 and data not shown). Similar results were also reported when co-expressing SufB and SufC with a C-terminal

polyhistidine tag (16). The gel filtration elution profile showed one prominent species that eluted at a molecular weight consistent with a (His)₆-SufB₂C₂ complex and that was similar to gel filtration elution profile of Peak 2 above (data not shown). The UV-visible absorption spectra of (His)₆-SufB₂C₂ prior to concentration (Fig. 4) revealed the presence of FAD with maxima at 368 and 446 nm similar to that observed for the air-oxidized (His)₆-SufBC₂D Peak 1 (Fig. 2A) indicating that (His)₆-SufB₂C₂ purifies with FADH₂ already partially oxidized to FAD. The flavin content of the (His)₆-SufB₂C₂ sub-complex was 0.8 FAD per complex, which is double that of (His)₆-SufB₂C₂ Peak 2 (Table 1). In contrast to (His)₆-SufB₂C₂ Peak 2, the (His)₆-SufB₂C₂ sub-complex expressed without SufD did not show any features indicative of Fe-S clusters, either before or after concentration. After concentration under anaerobic conditions, (His)₆-SufB₂C₂ contained 0.2 Fe atoms and 2.3 S²⁻ atoms per sub-complex, (Table 1). For clarity we will hereafter refer to this complex (purified in the absence of SufD expression) as the (His)₆-SufB₂C₂ sub-complex to differentiate it from the SufB₂C₂ Peak 2 complex described above (purified after co-expression with SufD).

(His)₆-SufB expressed without SufC and SufD also eluted in a single peak. The UV-visible absorption spectrum of SufB showed weak but observable features including a broad shoulder at 320 nm and a broad absorption maxima at 413 nm (Fig. 4). After concentration, SufB only contained 0.1 Fe atoms and 0.9 S²⁻ atoms per monomer (Table 1) indicating negligible amounts of Fe-S cluster. SufB expressed without SufC and SufD did not contain any observable FADH₂ or FAD.

The purification studies demonstrate that, though SufB alone can be easily reconstituted with an Fe-S cluster *in vitro*, deletion of either SufD or both SufC and SufD results in significant reduction of the SufB Fe-S cluster *in vivo*. These results also indicate that the SufB₂C₂ sub-complex can bind FADH₂/FAD *in vivo* while SufB alone does not bind flavin *in vivo*. A detailed comparison of the (His)₆-SufB, (His)₆-SufB₂C₂, (His)₆-SufBC₂D Peak 1, and (His)₆-SufB₂C₂ Peak 2 samples shows that the Fe:protein ratios of (His)₆-SufB and the (His)₆-SufB₂C₂ sub-complex were 6 to 13-fold lower than Peak 1 of the (His)₆-SufBC₂D complex and 16 to 32-fold lower than (His)₆-SufB₂C₂ Peak 2 complex (Table 1). In contrast, the S²⁻:protein ratio of the (His)₆-SufB₂C₂ sub-complex was approximately the same as (His)₆-SufBC₂D Peak 1 and only 50% lower than (His)₆-SufB₂C₂ Peak 2 (Table 1). The differential depletion of iron (but not sulfide) in the (His)₆-SufB₂C₂ sub-complex indicates that SufD is likely required for *in vivo* iron acquisition during Fe-S cluster assembly on SufB. This is the first reported biochemical requirement for SufD in any step of Suf-mediated Fe-S cluster assembly.

Furthermore, the (His)₆-SufB₂C₂ sub-complex and the (His)₆-SufB₂C₂ Peak 2 are qualitatively different. While the (His)₆-SufB₂C₂ sub-complex expressed in the absence of SufD contained no detectable Fe-S cluster, the (His)₆-SufB₂C₂ Peak 2 complex, co-expressed with SufD appears to contain a [4Fe-4S] cluster (Table 1, Fig. 2B, and Fig. 3B). This difference indicates that SufD is absolutely required *in vivo* for efficient Fe-S cluster assembly on SufB. Interestingly, SufD appears to dissociate from SufBC at some point during or after assembly of the [4Fe-4S] cluster and is replaced by a second SufB monomer. At present we cannot determine if SufD dissociates on its own (to be replaced by a single SufB) or as part of a SufCD sub-complex (to be replaced by a SufBC sub-complex). Previous reports have clearly shown that SufC₂D₂ can form an independent complex that is stable enough for structural characterization (16,27). Our present purification strategy utilizing (His)₆-SufB would not be able to capture an independent SufCD sub-complex and does not directly address this mechanistic question. However, these results provide the first indication of a separate role for SufB₂C₂ after complete cluster assembly.

Characterizing the role of SufC ATPase activity for Fe-S cluster assembly

Previous studies have shown that SufC is required *in vitro* for SufBC₂D enhancement of SufS cysteine desulfurase activity and for SufE-SufB interactions during sulfur transfer (7). Part of SufC's role in these processes may be to stabilize SufB and/or induce conformational changes in SufB to improve its interaction with SufSE or SufA. To evaluate the role of SufC ATPase activity *in vivo* without disrupting the integrity of the SufBC₂D or SufB₂C₂ complexes, the conserved lysine at position 40 in the Walker A motif of the SufC ATPase was substituted by an arginine within the SufBCD expression plasmid to generate SufC(K40R). Based on the analogous mutation in the MalK ATPase, the SufC K40R mutation allows ATP binding but blocks ATP hydrolysis thereby locking SufC into the ATP-bound form (28). ATPase assays were performed to confirm that the purified SufBC₂D-SufC(K40R) complex lacks ATPase activity (data not shown).

The (His)₆-SufBC₂D-SufC(K40R) complex also eluted as two peaks just as observed with wild-type SufBCD (Fig. S1B). SufC(K40R) and SufD co-purified with (His)₆-SufB in Peak 1 during the anaerobic purification in an apparent SufBC₂D complex as observed for wild-type SufBCD, confirming that the SufC(K40R) mutation does not disrupt complex stoichiometry (Fig. 5). Upon initial purification the UV-visible spectrum of (His)₆-SufBC₂D-SufC(K40R) Peak 1 looks nearly identical to that of wild-type (His)₆-SufBC₂D Peak 1 with a broad shoulder at 320 nm and maxima at 439 and a lower maxima at 600 nm (Fig. 6A). After 10 minutes air exposure, the UV-visible absorption spectrum of (His)₆-SufBC₂D-SufC(K40R) Peak 1 (Fig. 6A) showed maxima at 375 and 448 nm that were nearly identical to that of the wild-type (His)₆-SufBC₂D Peak 1 exposed to air for 10 minutes (Fig. 2A). Both spectra are consistent with the oxidation of FADH₂ to FAD. The (His)₆-SufBC₂D-SufC(K40R) Peak 1 contained 1 FADH₂ per complex. After anaerobic concentration, (His)₆-SufBC₂D-SufC(K40R) Peak 1 still contained 0.8 FADH₂ per complex (Table 1). (His)₆-SufBC₂D-SufC(K40R) Peak 1 only contained 0.2 Fe atoms per complex, an 8-fold reduction in iron content relative to the wild-type (His)₆-SufBC₂D Peak 1 (Table 1). However, the sulfide content of (His)₆-SufBC₂D-SufC(K40R) Peak 1 (1.3 S²⁻ atoms per complex) was only reduced 40% relative to the wild-type (His)₆-SufBC₂D Peak 1 (Table 1). The reduction in iron and sulfide parallel that observed for SufB or the SufB₂C₂ sub-complex indicating that disruption of SufC ATPase activity impairs Fe-S cluster assembly on SufB to an equal extent as the absence of SufD or the absence of both SufC and SufD (Table 1). It appears that disruption of Fe-S cluster assembly in the mutant complex is specifically linked to disrupted iron acquisition rather than perturbed sulfur transfer (Table 1). To independently confirm that the loss of cluster content in the (His)₆-SufBC₂D-SufC(K40R) mutant complex is not attributable to disrupted sulfur transfer, we tested if SufBC₂D-SufC(K40R) can still enhance the cysteine desulfurase activity of SufS in the presence of SufE. Since the mutant complex enhances SufSE activity equally as well as the wild-type SufBC₂D complex (Table 2), the SufC(K40R) mutation likely does not directly perturb sulfur transfer.

SDS-PAGE analysis of Peak 2 revealed that levels of SufC and SufD protein were reduced compared to Peak 1 just as was observed for wild-type (His)₆-SufB₂C₂ Peak 2 (Fig. 5 and Table S1). The UV-visible absorption spectra of (His)₆-SufB₂C₂-SufC(K40R) Peak 2 showed weak Fe-S cluster features with a broad but low intensity maxima at 413 nm (Fig. 6B). After anaerobic concentration, (His)₆-SufB₂C₂-SufC(K40R) Peak 2 had an 8-fold reduction in iron content (0.4 Fe atoms per complex) compared to the wild-type (His)₆-SufB₂C₂ Peak 2 (Table 1). However, the sulfide content of (His)₆-SufB₂C₂-SufC(K40R) Peak 2 was only reduced 3.7-fold compared to wild-type (His)₆-SufB₂C₂ Peak 2 (1.1 S²⁻ atoms per complex). In contrast to wild-type (His)₆-SufB₂C₂ Peak 2, (His)₆-SufB₂C₂-SufC(K40R) Peak 2 contained only trace amounts of FADH₂ (0.1 per complex). These results indicate that the SufC ATPase activity is required for *in vivo* Fe-S cluster formation

on SufB. Loss of SufC ATPase activity leads to a significant decrease in the iron content of (His)₆-SufBC₂D Peak 1 and (His)₆-SufB₂C₂ Peak 2 with a less severe reduction of sulfide content.

To directly test if SufC ATPase activity is required for in vivo Suf function, SufC Lys40 was mutated to Arg in the pGSO164 plasmid, which contains the entire *sufABCDSE* operon under the control of an arabinose-inducible promoter. We determined if the SufC(K40R) mutant plasmid could rescue the growth defect of the Δ *sufABCDSE* strain under iron starvation conditions using the ferrous iron chelator 2,2'-dipyridyl (Fig. 7). The wild-type pGSO164 plasmid was able to fully rescue the Δ *sufABCDSE* strain under iron starvation conditions (Fig. 7). In contrast, the SufC(K40R) mutation completely abolished the ability of the pGSO164 to rescue the Δ *sufABCDSE* strain, confirming that SufC ATPase activity is absolutely required for Fe-S cluster biosynthesis during iron starvation stress in *E. coli* (Fig. 7).

The SufBCDSE cluster assembly pathway can be activated in cell lysates by the addition of ATP and NADPH

The SufB₂C₂ complex isolated after co-expression with SufD (Peak2) contains 3.2 Fe per complex, consistent with 0.40 [4Fe-4S] clusters bound to each SufB monomer in the complex. The isolated SufBC₂D complex contains 1.3 Fe per complex, which equates to 0.33 [4Fe-4S] clusters per SufB monomer in the complex. If we assume that SufB in both complexes may bind a [4Fe-4S] cluster in vivo as has been shown in vitro (7,13), it appears that both complexes contain less than their full theoretical complement of [4Fe-4S] cluster. The lack of complete cluster binding by SufB suggests that one or more substrates required for cluster assembly are limiting in our in vivo expression system.

The three confirmed substrates required by Suf are L-cysteine for SufSE sulfur donation, ATP for SufC ATPase activity, and an iron source. Therefore, we attempted to reactivate the Suf assembly pathway in cell lysates from cells co-expressing SufBCD and SufSE by adding exogenous L-cysteine and ATP to freshly prepared cell lysates under anaerobic conditions. Due to the potential role of FADH₂ in the Suf system, exogenous NADPH was also added to serve as source of reducing equivalents for redox cycling of FAD/FADH₂. Since the in vivo iron donor is unknown, no exogenous iron source was added. Only endogenous iron donors/sources already present in the cell lysate could be used for cluster assembly. After incubating the lysates with various combinations of L-cysteine, ATP, and/or NADPH for 120 minutes, (His)₆-SufB was purified under anaerobic conditions as described for the other purifications above. Two distinct complexes (SufBC₂D and SufB₂C₂) were isolated during the purification and the approximate ratios of protein in both complexes did not significantly change (data not shown).

After incubation with only exogenous L-cysteine in the lysate, the UV-visible absorption spectrum of purified SufBC₂D (Figure 8A) showed a spectrum similar to SufBC₂D purified without incubation with L-cysteine (Figure 2A). After incubation with L-cysteine + NADPH, there was a slight increase in the overall intensity of the broad 430 nm peak (Figure 8A). After incubation with L-cysteine + ATP, the SufBC₂D spectrum showed a further increase in the 430 nm peak, an increase in the 320 nm shoulder, and a broad peak appearing at 620 nm (Figure 8A). After incubation with L-cysteine + ATP + NADPH, the SufBC₂D spectrum looked quite similar to that obtained with just L-cysteine + NADPH, except for a slight increase in the 320 nm shoulder feature.

The UV-visible absorption spectrum of purified SufB₂C₂ complex after incubation with L-cysteine in the lysate (Figure 8B) looked similar to SufB₂C₂ purified without incubation with L-cysteine (Figure 2B), including the broad peak at 420 nm. After incubation with L-

cysteine + ATP, the SufB₂C₂ spectrum increased substantially in intensity (Figure 8B). The 420 nm peak doubled in intensity and showed a slight sharpening. Incubation with L-cysteine + ATP also resulted in the appearance of new features in the SufB₂C₂ spectrum at approximately 520 and 615 nm (Figure 8B). After incubation with L-cysteine + ATP + NADPH, the SufB₂C₂ spectrum further increased in intensity such that the 420 nm absorption was nearly triple that observed after addition of L-cysteine alone. The 420 nm peak also continued to sharpen in contrast to the broader 420 nm peak observed after addition of L-cysteine alone. The 520 nm feature was still present in the spectrum while the 620 nm absorption feature shifted slightly to approximately 600 nm. The dramatic changes in the UV-visible absorption spectra of SufB₂C₂ suggest that ATP is able to reactivate cluster assembly in the cell lysate resulting in an increase in the Fe-S cluster content of SufB₂C₂. Addition of ATP + NADPH further increases cluster assembly on SufB₂C₂ although NADPH alone causes only a modest change in the spectrum (Figure 8B).

Chemical analysis of SufBC₂D and SufB₂C₂ purified from the various lysates closely agreed with the trends observed in the UV-visible absorption spectra. Addition of L-cysteine, ATP and/or NADPH had little net effect on the iron, acid-labile sulfide, or FAD content of SufBC₂D (Table 3). In contrast, addition of L-cysteine + ATP increased the iron content of SufB₂C₂ from \approx 3.0 Fe atoms per complex to 6.4 Fe atoms per SufB₂C₂ (Table 3). The acid-labile sulfide content of SufB₂C₂ similarly increased from 3.3 S²⁻ per complex to 6.8 S²⁻ per complex. Addition of L-cysteine + ATP + NADPH further increased iron content to 7.6 Fe per SufB₂C₂ and sulfide content to 7.8 S²⁻ per SufB₂C₂. FAD content of SufB₂C₂ was not significantly altered by the various additions (Table 3). The UV-visible absorption spectra and chemical analysis show that addition of L-cysteine + ATP + NADPH to the cell lysate was able to reactivate the Suf system leading to an approximate doubling of cluster content on SufB₂C₂ such that each SufB subunit contains sufficient iron and sulfide to form a [3Fe-4S] or [4Fe-4S] cluster. There was no significant net change in SufBC₂D iron and sulfur content in response to the same treatment; although it is worth noting that transient changes in SufBC₂D may not be apparent in these endpoint measurements (after 120 minutes incubation) and might occur at intermediate time points in the experiment.

Since Fe-S cluster accumulates in SufB₂C₂ when the system is reactivated in a cell lysate, this result provides additional support for the model that SufB₂C₂ is the mature cluster-containing complex. This result also indicates that ATP + NADPH are sufficient to reactivate iron acquisition using endogenous iron sources found in the cell lysate and is consistent with the selective depletion of iron content in the SufC_K40R mutant complexes which lack ATPase activity. The results suggest that ATP and/or a source of reducing equivalents (NADPH) may be somewhat limiting in our in vivo expression experiments thereby limiting the total level of in vivo Fe-S cluster assembly on the Suf proteins.

DISCUSSION

SufC and SufD are critical for in vivo Fe-S cluster biosynthesis

Our results clearly demonstrate the importance of SufC and SufD for in vivo Fe-S cluster assembly on SufB. Despite the relative ease of reconstituting [4Fe-4S] SufB in vitro (7), disruption of SufC or SufD by deletion or point mutation abolishes most in vivo cluster assembly on SufB (Fig. 4 and Table 1). These studies also establish that at least two distinct complexes can exist in vivo, SufBC₂D (Peak 1) and SufB₂C₂ (Peak 2). The SufB₂C₂ complex was purified previously after co-expressing SufB and SufC-(His)₆ but this sub-complex was not analyzed for cluster content or flavin binding (16). Surprisingly, if SufB and SufC are purified separately and then mixed in vitro, a novel complex with SufBC₂ stoichiometry is generated (13), suggesting there is a post-transcriptional mechanism to ensure proper complex formation in vivo.

Based on spectroscopic and chemical analysis, we isolated SufBC₂D that contains FADH₂ and a sub-stoichiometric amount of Fe-S cluster species (Peak 1) and SufB₂C₂ that lacks most SufD and has lower levels of FADH₂ but contains a [4Fe-4S] cluster (Peak 2). The increased iron and sulfide present in SufB₂C₂ Peak 2 logically places this conformational intermediate downstream of the SufBC₂D conformation in Peak 1. These results indicate that SufD is required for in vivo iron acquisition to complete the Fe-S cluster in the SufBC sub-complex but during cluster assembly or upon cluster maturation, SufCD dissociates from SufBC, possibly allowing the formation of SufB₂C₂ (Scheme 1). This hypothesis is supported by our results showing that the SufB₂C₂ sub-complex (expressed in the absence of SufD) cannot mature to the [4Fe-4S] form in vivo, largely because in vivo iron acquisition is blocked (Table 1 and Fig. 4). This result also argues against a post-assembly role for SufD in Fe-S cluster transfer. If SufD is utilized for Fe-S cluster transfer, one would predict that the cluster content of SufB₂C₂ would remain unchanged or perhaps increase in the absence of SufD due to lack of Fe-S cluster transfer to target enzymes. Instead we obtained the opposite result showing that lack of SufD blocks de novo Fe-S cluster formation in SufB₂C₂.

The reproducible co-purification of SufS with SufB₂C₂ (Fig. 1 and Fig. S2B) suggests that in vivo sulfur donation actually occurs between SufSE and SufB₂C₂. This hypothesis is supported by previous studies showing that SufBC is the minimum complex necessary for SufB to interact with SufE and for stimulation of SufSE cysteine desulfurase activity while SufD is not required (7). All current in vitro and in vivo data indicate that SufD and ATP hydrolysis by SufC are not utilized during sulfur transfer to SufB (9,29). Scheme 1 shows one model consistent with this data where the Suf scaffold system cycles between SufB₂C₂-SufSE (for sulfur acquisition) and SufBC₂D (for iron acquisition) until the mature 2 × [4Fe-4S] SufB₂C₂ complex is achieved. Since sulfide donation appears to still occur despite a block in iron acquisition (caused by loss of SufC ATPase activity or loss of SufD), this supports the “sulfur first” model of stepwise Fe-S cluster assembly (30,31). The purification results show that <80% of the SufBCD scaffold system is in the SufBC₂D conformation under steady state expression conditions, suggesting that iron acquisition by SufBC₂D is the rate limiting step of cluster assembly in vivo. While this model awaits further testing at the biochemical level, it is consistent with phylogenetic analysis showing that only SufB and SufC strictly co-occur among Archaeal and Eubacterial genomes while SufD is sometimes lacking (6). The SufB₂C₂ complex may therefore constitute the core scaffold complex while SufD is an adaptor protein used for iron acquisition in specific organisms or under specific environmental conditions.

It was previously shown that cluster assembly on the IscU scaffold protein proceeds via reductive coupling of 2 × [2Fe-2S] clusters to generate a [4Fe-4S] cluster (25). Interestingly, we did not observe stable [2Fe-2S] intermediate forms of SufBCD during our purification. Instead we obtained preliminary evidence for the presence of a linear [3Fe-4S] cluster bound to SufBCD. At present we cannot definitively assign the linear [3Fe-4S] cluster to either SufBC₂D or SufB₂C₂. We also cannot determine if the linear [3Fe-4S] cluster is a degradation product derived from a cuboidal [4Fe-4S] cluster or a bona fide intermediate during Fe-S cluster assembly. If the linear [3Fe-4S] cluster is a stable intermediate formed during assembly of the [4Fe-4S] cluster on SufB₂C₂, this would indicate that the Suf pathway uses a novel cluster assembly mechanism compared to the well-characterized Isc system.

SufC ATPase activity and SufD may work together for in vivo iron acquisition

The iron content of all SufBCD complexes is reduced if SufC lysine 40 is mutated to arginine. Furthermore, addition of ATP to cell lysates expressing the wild-type SufBCDSE proteins can reactivate the Suf system leading to a doubling of the Fe-S cluster content of

SufB₂C₂ (Figure 8, Table 3). These results indicate that SufC ATP hydrolysis is likely used for iron acquisition during *in vivo* Fe-S cluster assembly by the Suf pathway. Since the absence of SufD diminishes the iron content of the SufB₂C₂ sub-complex to a similar extent as the SufC K40R mutation reduces iron content in SufBC₂D Peak 1 and SufB₂C₂ Peak 2 (Table 1), we propose that SufC ATPase activity works in concert with SufD to provide iron for *in vivo* Fe-S cluster assembly by the Suf pathway.

The need to expend energy for iron acquisition (in the form of ATP hydrolysis) is logical as the Suf pathway functions under iron starvation conditions where bioavailable iron is limiting. Similarly, during hydrogen peroxide stress most of the labile iron pool available for cofactor biosynthesis is sequestered in iron storage proteins, especially the dodecameric ferritin-homologue Dps, in order to minimize Fenton chemistry (32). Therefore Suf must accumulate bioavailable iron against a considerable concentration gradient created by iron sequestration into storage proteins in order to carry out *in vivo* Fe-S cluster assembly. In contrast to the defects in iron acquisition, the SufC K40R mutation or omission of SufD reduced *in vivo* sulfur donation to SufB to a much less extent (Table 1). The partial reduction in sulfide content could result from aborted cluster assembly due to reduced iron acquisition or from subtle structural changes in the SufB₂C₂ sub-complex or SufBC₂D complex. Our current results suggest that ATP hydrolysis helps drive *in vivo* iron acquisition by SufBC₂D but they do not rule out additional roles for SufC in cluster transfer or binding to target apo-proteins. Further experiments are necessary to delineate the functional differences between SufBC₂D and SufB₂C₂ and SufC ATPase activity may very well have differential roles in each complex.

As noted above, the isolation of FADH₂ SufBC₂D using our expression system parallels other recent studies (13). In addition, we show here that the SufBC₂D-SufC(K40R) Peak 1 complex (which is blocked for ATP hydrolysis) can still bind one equivalent of FADH₂ just as wild-type SufBC₂D. In contrast, the flavin content of the SufB₂C₂-SufC(K40R) Peak 2 complex decreased from 0.4 to 0.1 per complex (Table 1). Currently it is not clear if FADH₂ is actually required for *in vivo* Fe-S cluster assembly by the Suf pathway. Reducing equivalents donated by FADH₂ may be used *in vivo* for iron acquisition, especially to reduce Fe³⁺ to Fe²⁺ to release it from iron storage proteins or siderophores. Wollers et al. demonstrated that FADH₂ SufBC₂D can reduce Fe³⁺ to Fe²⁺ in small chelates (ferric citrate) and ferric iron binding proteins (CyaY), although free flavin was more efficient than FADH₂ SufBC₂D for reducing ferric citrate (13). Alternatively, FADH₂ may help reduce persulfide (S⁰) to generate a bridging sulfide (S²⁻) or could drive reductive coupling of [2Fe-2S] clusters during cluster assembly. Future studies are necessary to clarify the role of FADH₂ (if any) in the Suf pathway.

In summary, we present both *in vitro* and *in vivo* evidence that the SufBCD scaffold system can form [3Fe-4S] and [4Fe-4S] clusters *in vivo* and that SufC ATPase activity and SufD are required for iron acquisition during *in vivo* Fe-S cluster assembly. Our results also suggest that distinct sub-complexes form among the SufB, SufC, and SufD proteins during cluster assembly *in vivo* and that the mature [4Fe-4S] cluster is bound to the SufB₂C₂ complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

Isc iron-sulfur cluster

Suf	sulfur mobilization
ATP	adenosine triphosphate
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate

Acknowledgments

We thank Dr. C. E. Outten and Dr. R. Drevland for helpful comments on the manuscript.

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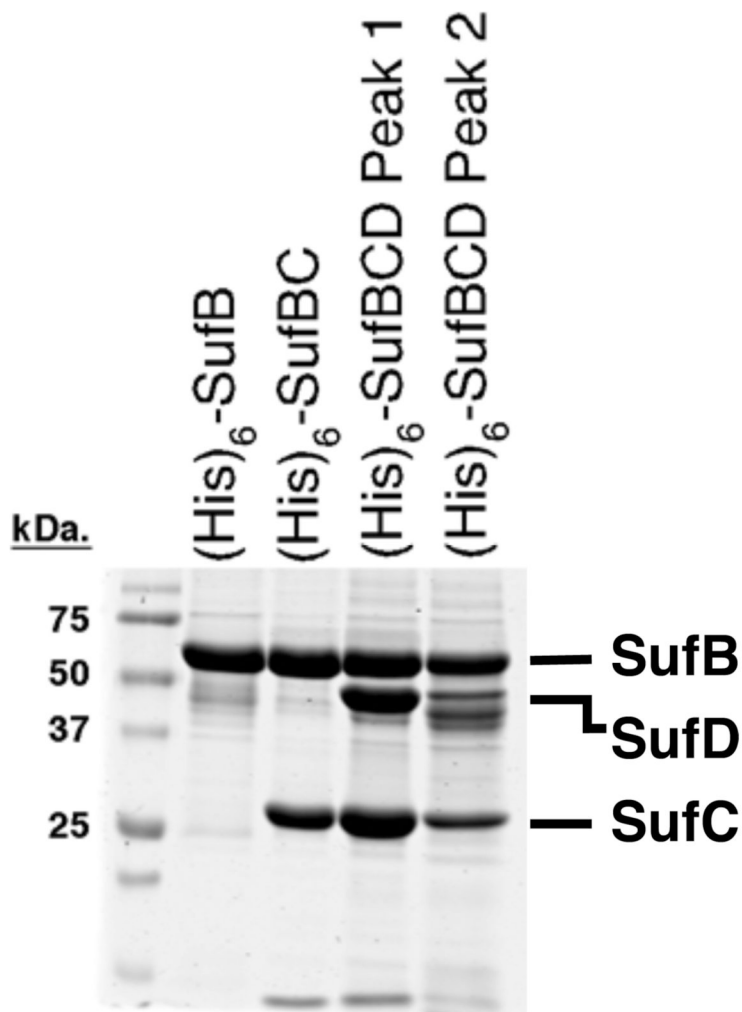


Figure 1. SDS-PAGE analysis of proteins and complexes purified using (His)₆-SufB expressed from pFWO468, pFWO469, and pFWO470. Samples from each anaerobic purification were separated on a 12% SDS-PAGE gel and stained with Coomassie blue stain.

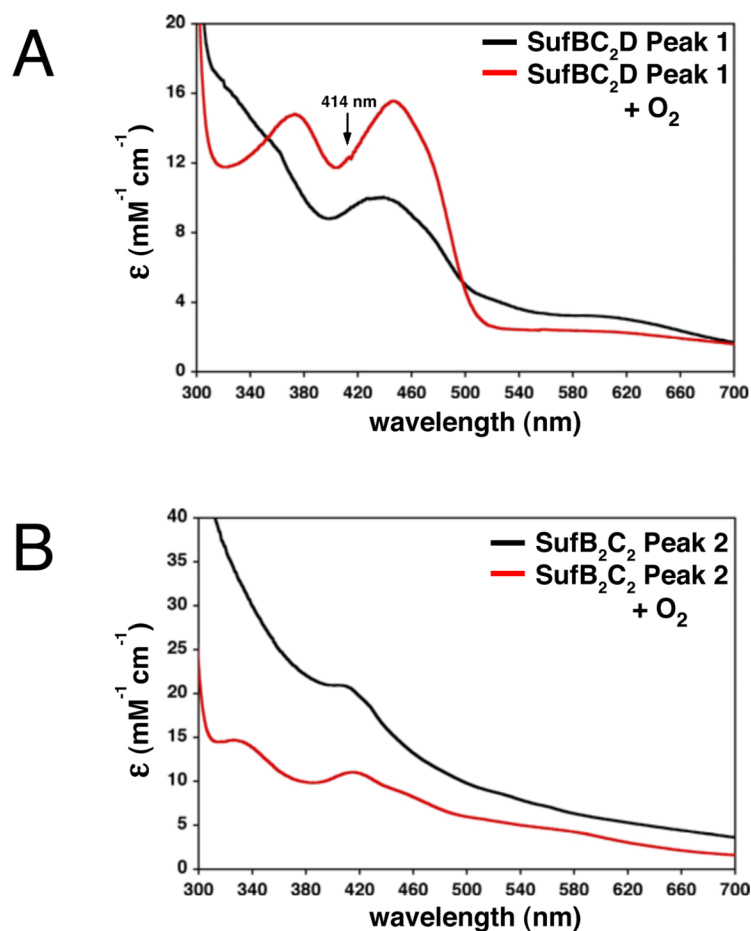


Figure 2. Analysis of in vivo purified SufBCD complexes. (A) UV-visible absorption spectra of (His)₆-SufBC₂D Peak 1 upon initial anaerobic purification (black trace) and after subsequent 10 min exposure to air (red trace). (B) UV-visible absorption spectra of (His)₆-SufB₂C₂ Peak 2 upon initial anaerobic purification (black trace) and after subsequent 10 min exposure to air (red trace). Note that SufS and SufE also were co-expressed with SufBCD in these experiments.

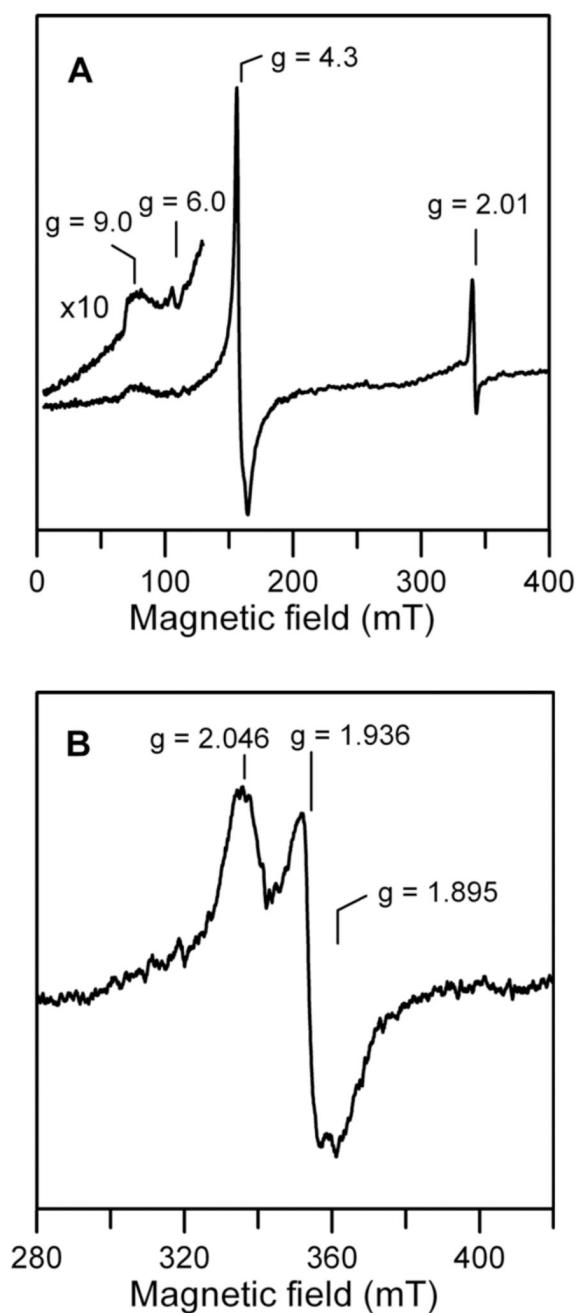


Figure 3.

EPR spectra of a mixture of purified $(\text{His})_6\text{-SufBC}_2\text{D}$ and $(\text{His})_6\text{-SufB}_2\text{C}_2$ before (A) and after (B) anaerobic reduction with a 10-fold excess of sodium dithionite. Samples contained 0.20 mM $(\text{His})_6\text{-SufBCD}$. EPR conditions: temperature, 5.6 K (A) and 10.6 K (B); microwave power, 4 mW (A) and 10 mW (B); modulation amplitude, 0.65 mT (A and B); microwave frequency, 9.5948 GHz (A) and 9.5939 (B). The $g = 6.0$ component in spectrum A results from a minor high-spin ferric heme impurity.

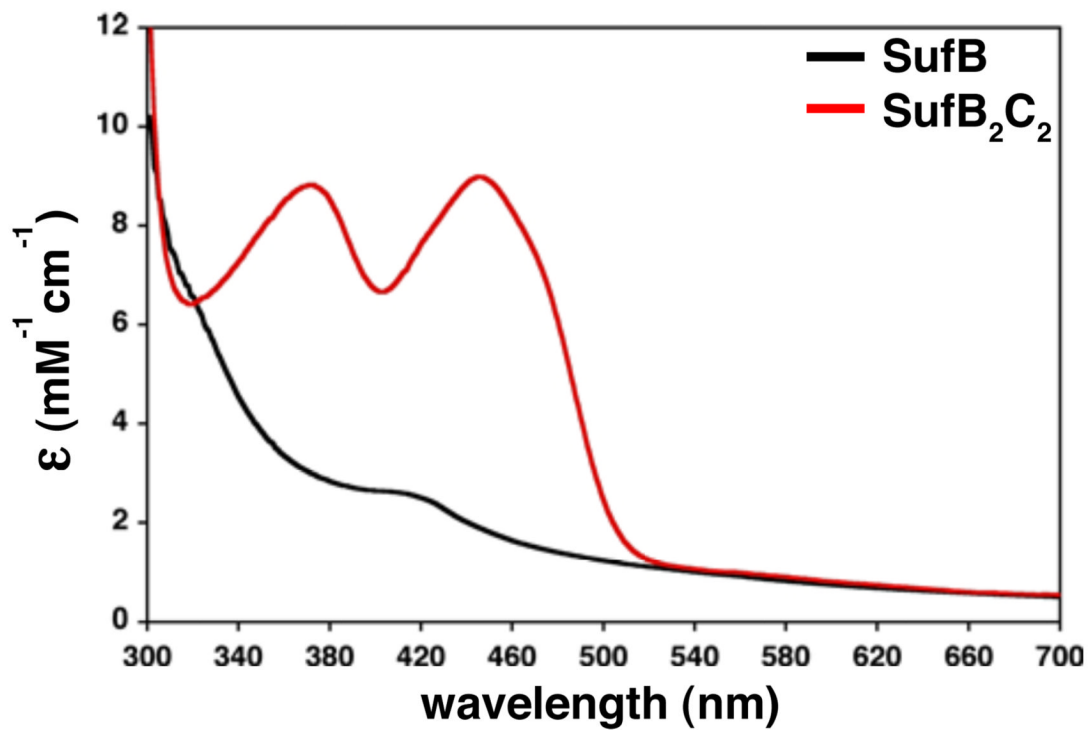


Figure 4. UV-visible absorption spectra of (His)₆-SufB (black trace) and (His)₆-SufBC (red trace) upon initial anaerobic purification. These proteins were co-expressed with SufS and SufE but SufD was not present in either expression vector.

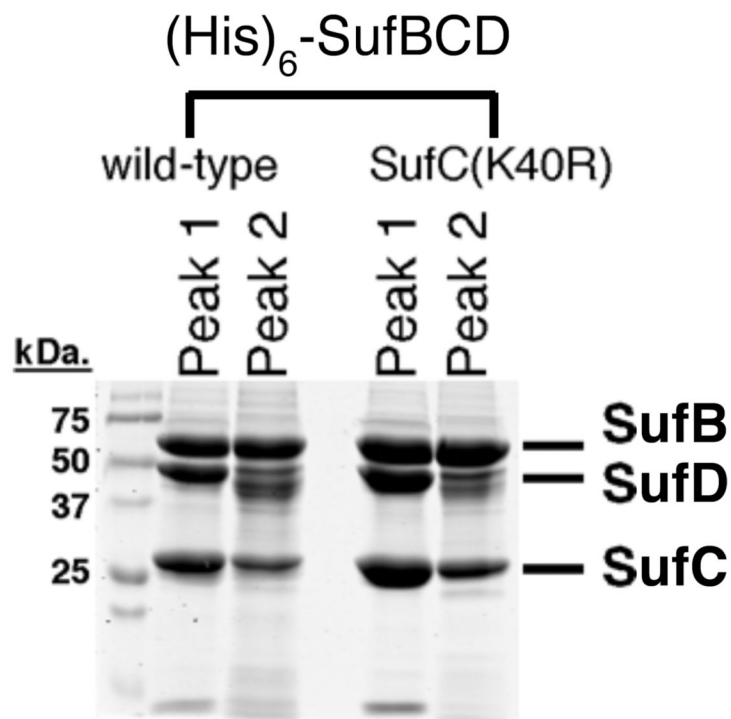


Figure 5. SDS-PAGE analysis of proteins and complexes purified using $(\text{His})_6\text{-SufB}$ expressed from pFWO470 and pFWO471 (containing the SufC K40R mutation). Samples from each anaerobic purification were separated on a 12% SDS-PAGE gel and stained with Coomassie blue stain.

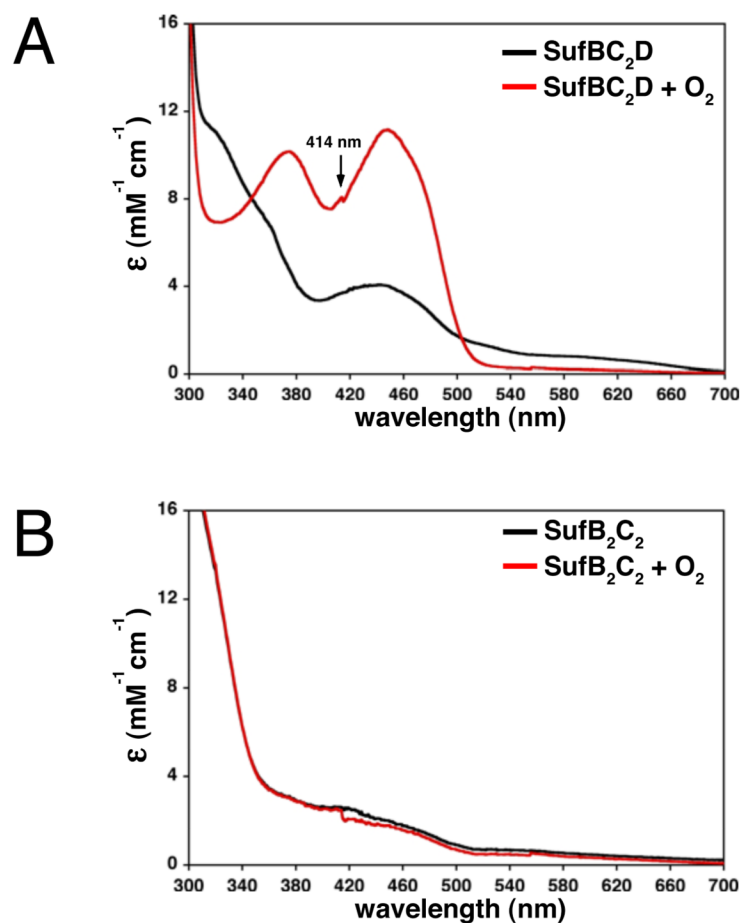


Figure 6. UV-visible absorption spectra of anaerobically purified $(\text{His})_6\text{-SufBC}_2\text{D}$ Peak 1 (A) and $(\text{His})_6\text{-SufB}_2\text{C}_2$ Peak 2 (B) complexes containing the SufC(K40R) point mutation. Black traces are spectra taken upon initial anaerobic purification and red traces are spectra of the same samples taken after subsequent 10 min exposure to air. Note that SufS and SufE also were co-expressed with SufBCD in these experiments.

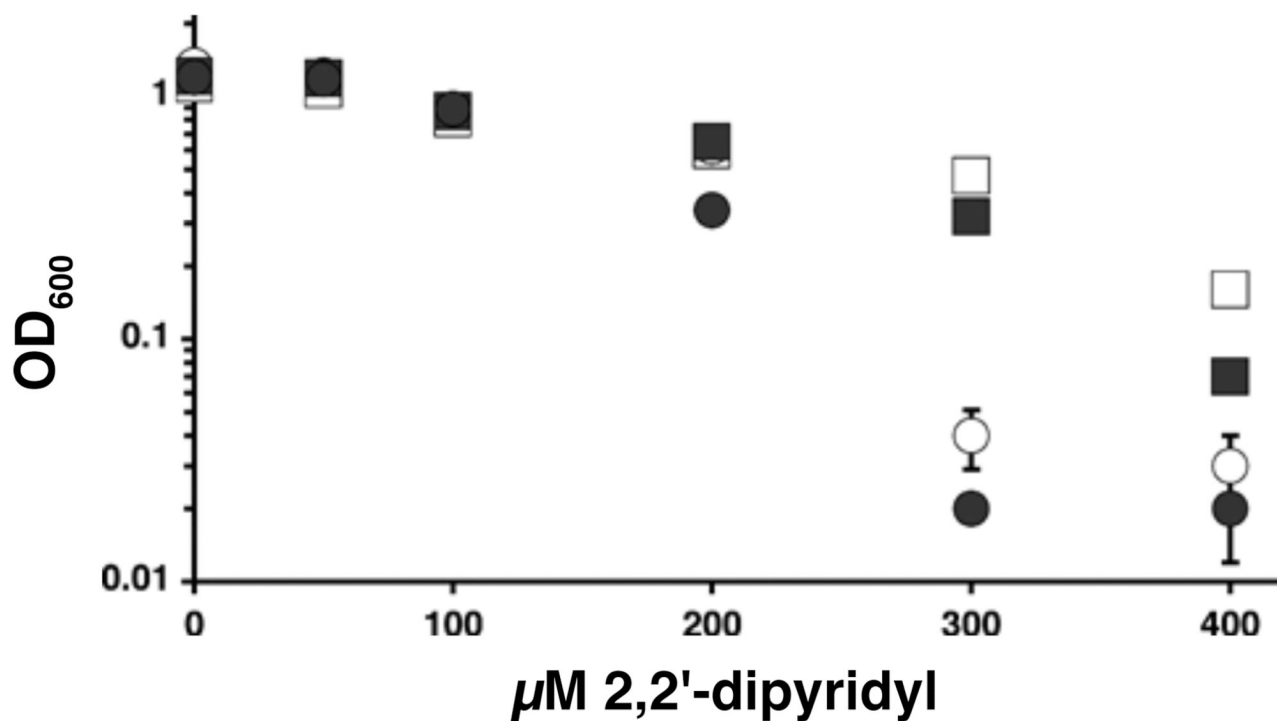


Figure 7.

Complementation of the $\Delta sufABCDE$ mutation in trans with pGSO164 and pGSO164 SufC(K40R) plasmids. The following *E. coli* strains were diluted to the same starting cell density into M9 / gluconate media containing increasing amounts of 2,2'-dipyridyl: Wild-type (open squares), $\Delta SufC(K40R)$ (open circles), $\Delta sufABCDE$ pGSO164 (filled squares), and $\Delta sufABCDE$ pGSO164-SufC(K40R) (closed circles). Final growth was measured as OD₆₀₀ after 14 hrs at 37 °C. The arabinose-regulated promoter in pGSO164 has significant basal expression and arabinose addition was not required for complementation.

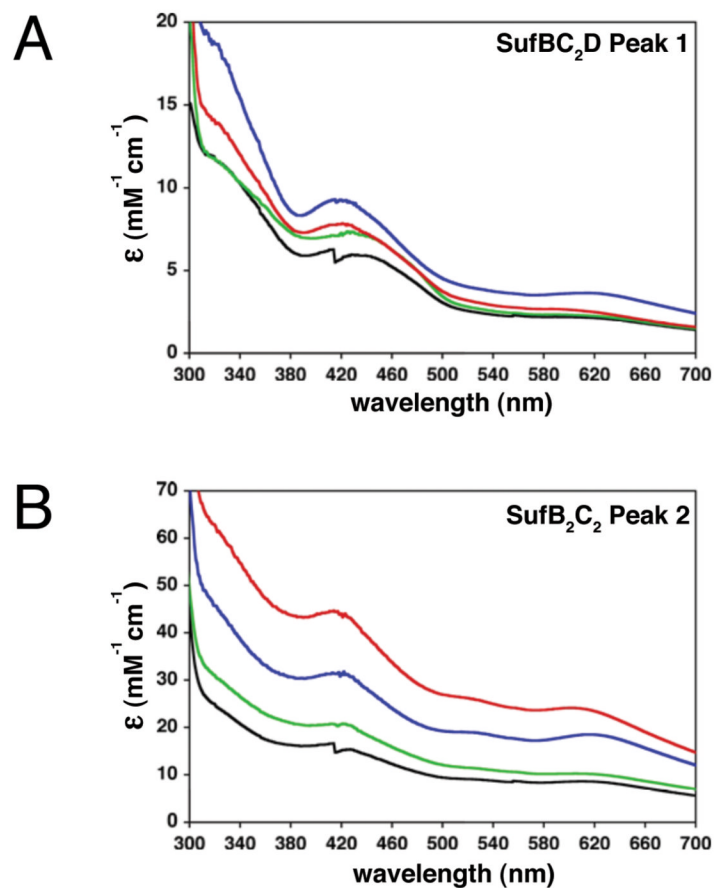
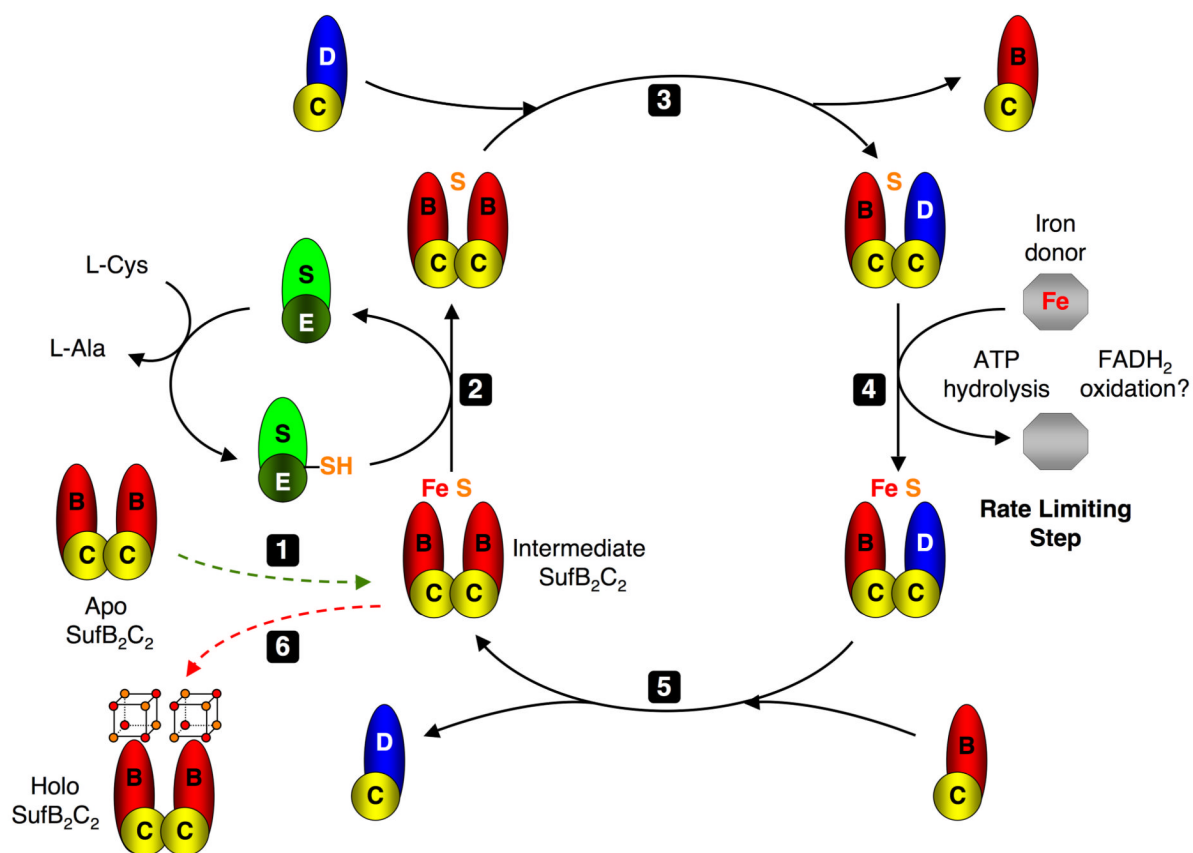


Figure 8. UV-visible absorption spectra of anaerobically purified $(\text{His})_6$ -SufBC₂D Peak 1 (A) and $(\text{His})_6$ -SufB₂C₂ Peak 2 (B) complexes. Suf complexes were purified after 120 min incubation in crude cell lysates containing 2 mM each of: L-cysteine (black trace), L-cysteine + NADPH (green trace), L-cysteine + ATP (blue trace), or L-cysteine + ATP + NADPH (red trace). Note that SufS and SufE also were co-expressed with SufBCD in the crude cell lysates used for these experiments.



Scheme 1.

Model for in vivo Suf Fe-S cluster assembly. Sulfur is mobilized from L-cysteine by SufSE and transferred to apo SufB₂C₂ (Steps 1 and 2). SufBC interact with SufCD in the SufBC₂D complex for iron acquisition, ATP hydrolysis, and possibly for FADH₂ oxidation (Steps 3 and 4). A SufB₂C₂ intermediate containing sub-stoichiometric iron and sulfide begins another cycle (Step 5). After multiple cycles, SufB₂C₂ forms 2 × [4Fe-4S] clusters and exits the cycle for cluster transfer (Step 6). The exact mechanism of SufD/SufB association and dissociation during the reaction cycle is unknown but we show the cycle proceeding through exchange of SufB₁C₁ and SufC₁D₁ heterodimer intermediates. A second interlocking cycle could be occurring simultaneously with exchange of SufB₁C₁ and SufC₁D₁ intermediates connecting the two cycles (not shown for simplicity). Green arrow shows entry of apo SufB₂C₂ into the cycle. Red arrow indicates maturation of SufB₂C₂ into the 2 × [4Fe-4S] form.

Table 1

Chemical Analysis of Purified Protein or Complex

Protein Purified ^a	Fe:Protein ^b	S ²⁻ :Protein ^b	FAD: Protein ^c	Cluster Assembly Step ^d
Suffi	0.1 ± 0.01	0.9 ± 0.1	–	–
SufB ₂ C ₂ sub-complex	0.2 ± 0.08	2.3 ± 0.6	0.8 ± 0.01	2
SufBC ₂ D Peak 1	1.3 ± 0.7	1.8 ± 0.5	1.1 ± 0.04	4
Suffi ₂ C ₂ Peak 2	3.2 ± 0.01	4.2 ± 0.5	0.4 ± 0.05	5
SufBC ₂ D-SufC(K40R) Peak 1	0.2 ± 0.04	1.3 ± 0.1	1.0 ± 0.01	3
Suffi ₂ C ₂ -SufC(K40R) Peak 2	0.4 ± 0.01	1.1 ± 0.6	0.1 ± 0.03	2

^a All proteins were purified from cells also co-expressing SufS and SufE (see text for details). See Materials and Methods for molecular weights used to determine ratios of protein to iron and sulfide.

^b Average of triplicate measurements of samples from three independent purifications.

^c Average of two independent purifications (see Materials and Methods for details).

^d Number refers to specific cluster assembly step outlined in Scheme 1. Protein complexes are the products of the indicated step in the assembly cycle.

Table 2

Enhancement of SufS Cysteine Desulfurase Activity

Proteins ^a	SufS Specific Activity ^b
SufS	6.6 ± 0.2
SufS + SufE	31.6 ± 0.4
SufS + SufE + SufBC ₂ D	79.5 ± 5.1
SufS + SufE + SufB ₂ C ₂	59.5 ± 2.2
SufS + SufE + SufBC ₂ D-SufC(K40R)	81.2 ± 5.2

^aSufS was present at 1 μM in all samples. SufE and SufBC(D) complexes were added at 4 μM.

^bSpecific Activity is nmole S²⁻/min/mg SufS. The average of triplicate measurements is shown.

Table 3

Chemical Analysis of Purified Complex After Suf Reactivation in Cell Lysates.

↓ Complex Purified ^a	Addition→	L-cys	L-cys + NADPH	L-cys + ATP	L-cys + ATP + NADPH
SufBC ₂ D Peak 1	Fe:Complex ^b	1.7 ± 0.01	1.4 ± 0.3	1.8 ± 0.07	1.2 ± 0.03
	S ²⁻ :Complex ^b	2.1 ± 0.8	2.2 ± 0.2	2.3 ± 0.6	2.4 ± 0.9
	FAD:Complex ^b	1.0 ± 0.1	1.0 ± 0.02	1.3 ± 0.02	1.1 ± 0.4
SufB ₂ C ₂ Peak 2	Fe: Complex ^b	3.0 ± 0.07	3.1 ± 0.5	6.4 ± 0.04	7.6 ± 0.17
	S ²⁻ :Complex ^b	3.3 ± 0.4	3.4 ± 0.9	6.8 ± 0.7	7.8 ± 1.0
	FAD:Complex ^b	0.4 ± 0.04	0.5 ± 0.3	0.4 ± 0.05	0.6 ± 0.3

^aAll proteins were purified from cells also co-expressing SufS and SufE (see text for details). See Materials and Methods for molecular weights used to determine ratios of protein to iron and sulfide.^bAverage of triplicate measurements of samples.