Functional Dynamics Revealed by the Structure of the SufBCD Complex, a Novel ATP-binding Cassette (ABC) Protein That Serves as a Scaffold for Iron-Sulfur Cluster Biogenesis*

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ATP-binding cassette (ABC)-type ATPases are chemo-mechanical engines involved in diverse biological pathways. Recent genomic information reveals that ABC ATPase domains/subunits act not only in ABC transporters and structural maintenance of chromosome proteins, but also in iron-sulfur (Fe-S) cluster biogenesis. A novel type of ABC protein, the SufBCD complex, functions in the biosynthesis of nascent Fe-S clusters in almost all Eubacteria and Archaea, as well as eukaryotic chloroplasts. In this study, we determined the first crystal structure of the Escherichia coli SufBCD complex, which exhibits the common architecture of ABC proteins: two ABC ATPase components (SufC) with function-specific components (SufB-SufD protomers). Biochemical and physiological analyses based on this structure provided critical insights into Fe-S cluster assembly and revealed a dynamic conformational change driven by ABC ATPase activity. We propose a molecular mechanism for the biogenesis of the Fe-S cluster in the SufBCD complex.

The ATP-binding cassette (ABC)3 is a ubiquitous, universally conserved ATPase domain/subunit historically defined as the nucleotide-binding domain of an ABC transporter. ABC transporters comprise a large and diverse family of membrane-spanning proteins that transport various substances, ranging from ions to proteins, across membranes (1–5). With the availability of complete genomes and the refinement of bioinformatic tools, it has become apparent that ABC type ATPase domains are present not only in ABC transporters but also in a variety of nontransporter proteins, the most well known examples of which are the structural maintenance of chromosome (SMC) proteins involved in chromosome segregation/condensation and DNA repair (6–8). Although the SMC proteins, like the ABC transporters, have attracted great interest because its members are implicated in various human diseases, there are additional types of nontransporter ABC proteins. Here, we focus on a novel type of ABC protein, the SufBCD complex, whose ABC ATPase components (SufC) segregate in a different clade from those of transporters and SMC proteins (Fig. 1).

The SufBCD complex is a component in the Suf machinery that is responsible for de novo iron-sulfur (Fe-S) cluster biogenesis. This machinery is phylogenetically diverse and is present in photosynthetic organisms such as higher plants, as well as in Eubacteria and Archaea (9,10). Fe-S clusters act as cofactors of various Fe-S proteins that are essential for maintaining fundamental biological processes such as respiratory and photosynthetic electron transfer and regulation of gene expression (11,12). Fe-S cluster biogenesis requires a complex network of proteins that mobilize sulfur and iron, assemble nascent clusters, and transfer the assembled clusters to Fe-S proteins. The Suf machinery in Escherichia coli is composed of six proteins encoded by the sufABCDSE operon. The SufS cysteine desulfurase and SufE sulfur shuttle protein act together to provide sulfur for construction of nascent Fe-S clusters (13,14). SufA is an Fe-S carrier protein that transfers Fe-S clusters to target proteins.

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The atomic coordinates and structure factors (codes 5AWF and 5AWG) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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3 The abbreviations used are: ABC, ATP-binding cassette; SMC, structural maintenance of chromosome; Fe-S, iron-sulfur; r.m.s.d., root mean square deviation; RCT, random conical tilt; SAXS, small angle x-ray scattering; ANS, 1-anilinonaphthalene-8-sulfonate; DACM, N-(7-dimethylamino-4-methylcoumarinyl)-maleimide.
apo-proteins (15). The remaining proteins, SufB, SufC, and SufD, have attracted a great deal of attention because deletion of any of them abolishes Suf function in vivo (10, 16, 17). SufB and SufD are homologues and show some sequence similarity (17% identity and 37% similarity). SufB accepts sulfur transferred from SufE (14), and SufD may play a role in iron acquisition (18). SufB, SufC, and SufD interact with each other, and three distinct states have been reported: the SufBCD ternary complex, the SufBC subcomplex (18, 19), and the SufCD subcomplex (19, 20). Recent in vitro reconstitution studies suggested that the SufBCD complex can serve as the scaffold for nascent Fe-S cluster assembly (21–24). It is clear that the SufBCD complex plays a central role in the Suf pathway, but the molecular mechanism underlying Fe-S cluster formation in the SufBCD complex remains enigmatic.

Although SufC is a member of the ABC ATPase superfamily and exhibits ATPase activity, the role of ATPase activity in Fe-S cluster biogenesis is currently unclear (21–23). In ABC transporters, energy from ATP binding/hydrolysis acts to transport specific substances across membranes (1–5, 25). In soluble SMC proteins, the ABC ATPase utilizes ATP to recognize and bind DNA (4, 26). Despite this extreme functional diversity, these proteins share a similar architecture, consisting of two ABC ATPase domains bound to substrate/function-specific partner domains; in both types of proteins, the ABC ATPase activity drives the conformational changes in partner domains required for each function (4). Therefore, it is likely that structural changes in the SufB and SufD subunits are driven by SufC ATPase activity in the SufBCD complex and that the dynamic motion of the complex should provide important clues regarding the molecular mechanism of Fe-S cluster biogenesis.

In this study, we determined the crystal structure of the SufBCD (SufB$_2$-SufC$_2$-SufD$_1$) complex, providing the first demonstration of the quaternary configuration of the ternary complex. Biochemical experiments based on the crystal structure demonstrated that the two SufC ABC ATPase subunits form a head to tail dimer in the complex upon ATP binding, thereby inducing a structural change in the interface between the SufB and SufD subunits. These findings, together with in vivo mutational analyses, provided insights into the mechanism of Fe-S cluster assembly in the SufBCD complex.

**Experimental Procedures**

**Expression and Purification of the E. coli SufBCD Complex—**
To purify the SufBCD complex, the entire suf operon was expressed simultaneously. The plasmid pGSO164, containing the entire suf operon under the control of an arabinose-inducible promoter (22), was used to overexpress SufABCDS in the TOP10 strain of *E. coli*. The cells were grown in TB medium containing ampicillin (50 μg/ml) at 37 °C. L-Arabinose was added to 0.2% (w/v) final concentration when the cultures reached an $A_{600}$ of 0.4–0.6. After 3 h of SufABCDS expression at 37 °C, the cells were harvested by centrifugation, and the cell pellets were frozen at −80 °C. Cell pellets were lysed by sonication in 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, and 1 mM DTT. The soluble fraction was subjected to ammonium sulfate fractionation at 20% saturation. After centrifugation, the supernatant fraction was loaded onto a HiPrep Phenyl FF (low sub) 16/10 column (GE Healthcare), and the bound protein was eluted with a decreasing linear gradient of 20–0% ammonium sulfate. Fractions containing the SufBCD complex were pooled, dialyzed overnight in 50 mM Tris-HCl (pH 7.8) and 1 mM DTT,
then loaded onto a Mono Q HR 5/50 GL column (GE Healthcare), and eluted with a linear gradient of 0–1 M NaCl. The SufBCD complex was further purified by gel filtration using a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) in 50 mM Tris–HCL (pH 7.8) and 150 mM NaCl. The purified SufBCD complex was concentrated and stored at −80 °C.

Site-directed mutagenesis was performed using the pGSO164 plasmid as a template and the primers listed in Table 1. Genes were expressed in mutant cells (YT2512) in which pGSO164 plasmid served as a template and the primers listed in Table 2. Coordinates of the x-ray structure of the SufBCD complex have been deposited in the Protein Data Bank, under accession codes 5AWF and 5AWG.

The crystallographic asymmetric unit contained two SufBCD complexes, termed Complex 1 and Complex 2. Although the electron density for the SufBCD complex was mostly continuous, the densities for some regions were poorly defined: in Complex 1, SufB residues 1–33 and 80–156, SufD residues 1–7 and 422–423, SufCSufB residues 244–248, and SufCSufD residues 237–248. In Complex 2, SufB residues 1–35 and 79–157, SufD residues 1–7 and 422–423, SufCSufD residues 244–248, and SufCSufD residues 237–248. Accordingly, these residues were not included in the model.

Single-particle Electron Microscopy Reconstruction—The SufBCD complex was prepared from a peak fraction of gel filtration in 50 mM MES (pH 6.5), 150 mM NaCl, and 5 mM MgCl2. The negatively stained SufBCD complex was examined using an H9500SD transmission electron microscope (Hitachi High-Tech) operated at 200 kV at room temperature. The images were acquired on a 2k × 2k charge-coupled device camera (TVIPS) with a physical pixel size of 0.24 nm. Random conical tilt reconstruction was performed using the software package SPIDER (36). The obtained three-dimensional structure from random conical tilt was refined by using the EMAN1 software suite (37). The final reconstruction of the SufBCD complex was computed from −7,146 particles. The particle images were low pass filtered at 30 Å before refinement, and therefore the Fourier shell correlation that was calculated using eotest of EMAN1 shows higher values than 0.5 in every frequency ranges. The EM structure of the SufBCD complex has been deposited in the Electron Microscopy Data Bank under accession number EMD-3163.

Solution Scattering Data Collection and Analysis—The SufBCD complex (2–18 mg/ml) for small angle x-ray scattering (SAXS) experiments was prepared in 50 mM Tris–HCL (pH 7.8) and 150 mM NaCl. SAXS experiments were performed at room temperature on a Rigaku BioSAXS-1000, using CuKα radiation from the Rigaku FR-X rotating anode x-ray generator. The scattering vector range was set from q_{min} = 0.009 Å^{-1} to q_{max} = 0.69 Å^{-1} (q = 4πsinθ/λ). Protein samples were placed in a quartz capillary with a diameter of 1.0 mm using an exposure time of 15 min/frame. The final scattering curve was radially averaged from eight frames with the program SAXS Lab (Rigaku). Subsequent data were analyzed by the ATSAS program package (38). Data quality was assessed on the basis of the linearity of Guinier plots. Scattering profile simulations from the crystal structure were carried out using CRYSOL (39). Ab initio models were generated using DAMMIF (40). 10 individual reconstructions were aligned and averaged, and the most typical model was generated using DAMAVER (41). The crystal structure was fitted to the dummy model by manually. The SAXS data at 8 mg/ml measurement were used for Fig. 4. Data collection and structural parameters are summarized in Table 3.

In Vivo Complementation Assay with Mutated SufC—Site-directed mutations were generated in plasmid pBBR-sufC and introduced into E. coli mutant strain UT109 (16) harboring two plasmids, pUMV22 and pRK-sufAB-DSE (ΔsufCp). UT109
Structure of SufBCD Complex, a Novel Type of ABC Protein

TABLE 2
Data collection, phasing, and refinement statistics for x-ray crystallography
The values in parentheses correspond to the highest resolution shell.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Native</th>
<th>Hg derivative 1</th>
<th>Hg derivative 2</th>
<th>Pt derivative</th>
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<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
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<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>a, b, c (Å)</td>
<td>119.5, 139.6, 124.7</td>
<td>119.8, 139.4, 124.4</td>
<td>120.2, 140.0, 124.6</td>
<td>119.8, 140.4, 124.5</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 113.1, 90.0</td>
<td>90.0, 113.6, 90.0</td>
<td>90.0, 113.5, 90.0</td>
<td>90.0, 113.1, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>2.95 (3.06–2.95)</td>
<td>4.30 (4.45–4.30)</td>
<td>4.50 (4.66–4.50)</td>
<td>3.45 (3.57–3.45)</td>
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<td>Rmerge (%)</td>
<td>6.0 (37.1)</td>
<td>12.7 (29.5)</td>
<td>14.4 (29.8)</td>
<td>10.7 (34.1)</td>
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<td>Complete (%)</td>
<td>98.5 (98.1)</td>
<td>99.3 (99.9)</td>
<td>92.8 (94.5)</td>
<td>99.1 (99.7)</td>
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<td>Redundancy (%)</td>
<td>3.8 (3.6)</td>
<td>5.6 (5.6)</td>
<td>6.0 (5.9)</td>
<td>5.6 (5.6)</td>
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<table>
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<tr>
<th>Refinement</th>
<th>P2₁</th>
<th>P2₁</th>
<th>P2₁</th>
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<tr>
<td>Resolution (Å)</td>
<td>41.1–2.95</td>
<td>43.94–4.30</td>
<td>43.94–4.30</td>
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<td>50,117</td>
<td>50,117</td>
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<td>29.5/34.0</td>
<td>29.5/34.0</td>
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<tr>
<td>No. atoms</td>
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<td>18,804</td>
<td>18,804</td>
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<td>Protein (Å²)</td>
<td>83.6</td>
<td>110.3</td>
<td>110.3</td>
<td>110.3</td>
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<tr>
<td>Ion (Å)</td>
<td>–</td>
<td>148.8</td>
<td>148.8</td>
<td>148.8</td>
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<tr>
<td>r.m.s.d.</td>
<td>0.006</td>
<td>0.007</td>
<td>0.007</td>
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<tr>
<td>Bond lengths (Å)</td>
<td>0.1241</td>
<td>1.672</td>
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<td>Ramachandran plot</td>
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<tr>
<td>Most favored (%)</td>
<td>92.3</td>
<td>86.5</td>
<td>86.5</td>
<td>86.5</td>
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<tr>
<td>Additionally allowed (%)</td>
<td>6.9</td>
<td>12.9</td>
<td>12.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Generously allowed (%)</td>
<td>0.8</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

a Hg, methylmercury(II) acetate.
b Hg, methylmercury(II) chloride.
c Pt, potassium tetranitro platinate(II).

TABLE 3
Data collection and structural parameters for SAXS analysis

<table>
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<tr>
<th>Data collection parameters</th>
<th>Value</th>
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<tr>
<td>Instrument</td>
<td>Rigaku BioSAXS-1000</td>
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<tr>
<td>X-ray source</td>
<td>Rigaku FR-X</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.54</td>
</tr>
<tr>
<td>q range (Å⁻¹)</td>
<td>0.009–0.69</td>
</tr>
<tr>
<td>Exposure time (min)</td>
<td>15</td>
</tr>
<tr>
<td>Concentration range (mg/ml)</td>
<td>2–18</td>
</tr>
<tr>
<td>Temperature</td>
<td>Room temperature</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Structural parameters</th>
<th>Value</th>
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<tr>
<td>I(Io) from P(r) (Å)</td>
<td>0.225 ± 0.001</td>
</tr>
<tr>
<td>R, from P(r) (Å)</td>
<td>40.8 ± 0.6</td>
</tr>
<tr>
<td>I(Io) from Guiner (Å)</td>
<td>0.222 ± 0.001</td>
</tr>
<tr>
<td>R, from Guiner (Å)</td>
<td>39.9 ± 0.1</td>
</tr>
<tr>
<td>Dmax (Å)</td>
<td>138.5</td>
</tr>
<tr>
<td>x² of DAMMIF models</td>
<td>1.22</td>
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</table>

<table>
<thead>
<tr>
<th>Molecular mass determination</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Mf, from I(Io) (kDa)</td>
<td>150.2</td>
</tr>
<tr>
<td>Mf, from sequence (kDa)</td>
<td>156.7</td>
</tr>
</tbody>
</table>

a Reported for 8 mg/ml measurement.

contains deletions of the chromosomal suf (ΔsufABCΔDSE) and isc (ΔiscUA-hscBA) operons. Normally, deletion of both pathways is lethal because of the lack of the biosynthetic apparatus for Fe-S clusters (10). However, plasmid pUMV22, which harbors genes for mevalonate kinase, phosphomevalonate kinase, and diposphomevalonate decarboxylase cloned from Streptomyces sp., allows UT109 to grow in the presence of d-mevalonate because the essential Fe-S enzymes IspG and IspH involved in the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis can be bypassed by the foreign mevalonate pathway. Upon shift to the absence of mevalonate, the cells are unable to grow without introduction of a functional sufC gene (in this case from pBBR-sufC) to complete the partial Suf system provided by pRK-sufAB-DSE (ΔsufCp).

For the construction of plasmid pRK-sufAB-DSE (ΔsufCp), the sufAB fragment was amplified using primers SufAF and SufB-Rsc3, and sufDSE was amplified using primers SufD-Fsc5 and SufER (Table 1). Because the coding region of sufC contains the promoter elements for sufDSE, an artificial promoter sequence was added to the upstream region of sufD in the SufD-Fsc5 primer. After digestion with restriction enzymes, the two PCR fragments were cloned simultaneously into the Xhol/Nhel sites of pRKNSE (42). The expression plasmid pBBR-sufC was constructed by transferring the Xbal-Sacl fragment carrying the ribosome-binding sequence and the SufC coding region from the pET-21a (+) derivative (20) to the pBBR1MCS-4 plasmid (43), in which expression was driven by the lac promoter. Mutagenesis of SufC was performed using the pBBR-sufC plasmid as a template, and the primers are listed in Table 1.

ATP Hydrolysis Measurement—ATP hydrolysis rates were determined by a linked enzyme assay that coupled the formation of ADP to the oxidation of NADH, as described previously (44).

Disulfide Cross-linking Experiment—The purified mutant complexes (1 mg/ml) were incubated at room temperature for 30 min in the presence of 5 mM ATP, 5 mM MgCl₂, and 0.05 mM CuSO₄, the resultant products were analyzed by Western blot of native PAGE (7.5% gel) and nonreducing SDS-PAGE (12.5% gel) using antibodies against SufB, SufC, and SufD.

Fluorescence Labeling Experiment—For assays using 1-anilinonaphthalene-8-sulfonate (ANS), the purified mutant complexes (1 mg/ml) were mixed with 50 μM ATP, 50 μM MgCl₂, 5 μM CuSO₄, and 30 μM ANS, and time-dependent changes in fluorescence at room temperature were measured for 30 min.

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For the N-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM) assays, the purified mutant complexes (1 mg/ml) were incubated at room temperature for 30 min in the presence of 50 μM ATP, 50 μM MgCl₂, 5 μM CuSO₄, and 10 μM DACM, and then their fluorescence was measured. All fluorescence spectra were recorded on a FP-8200 fluorescence spectrometer (JASCO).

In Vivo Iron-Sulfur Cluster Formation Analysis—Site-directed mutagenesis was performed using the pGSO164 plasmid as a template and the primers listed in Table 1, and the genes were expressed in YT2512 (10). The cells were grown in LB medium containing ampicillin (50 μg/ml) and ferric ammonium citrate (0.1 mg/ml) at 37 °C. L-Arabinose was added to 0.2% (w/v) final concentration when the cultures obtained an A₆₀₀ of 0.4–0.6. After 3 h of expression of SufABCDSE at 37 °C, the cells were harvested by centrifugation. UV-visible absorption spectra were recorded at room temperature on a V-630 spectrophotometer (JASCO).

Protein Sequences—The multiple sequence alignments in Figs. 1 and 6 were performed using Clustal Omega (45), and the figures were prepared with NJplot (46) and ESPript (47). EcoGene (48) accession numbers for the proteins from E. coli K-12 aligned in Figs. 1 and 6 are as follows: SufC, EG13964; UgpC, EG11048; MalK, EG10558; PotA, EG10749; PotG, EG11630; ThiQ, EG11572; TauB, EG13299; SsuB, EG12358; ProV, EG10771; GltL, EG12663; YhdZ, EG12837; GlnQ, EG10389; HisP, EG10452; ArtP, EG11624; CysA, EG10183; PstB, EG10783; FetA, EG13259; FepC, EG10295; FecE, EG10290; FhuC, EG10304; BtuD, EG10128; CcmA, EG12059; DppD, EG12627; SapD, EG12304; DdpD, EG13787; DppF, EG12628; UvrA, EG11061; SbcC, EG10927; MutS, EG10625; RecN, EG10831; MukB, EG10618; and RecF, EG10828.

Results—Structure of the SufBCD Complex—We determined the first crystal structure of the SufBCD complex from E. coli at 2.95 Å resolution (Fig. 2, A and B) by the multiple isomorphous replacement method coupled with anomalous scattering phasing from Hg/Pt derivatives. The SufBCD complex consists of one SufB subunit, two SufC subunits, and one SufD subunit with a stoichiometry of 1:2:1, consistent with previous biochemical experiments (14, 20). Each of the SufC subunits is bound to a subunit of the SufB-SufD protomers and is accordingly termed SufCSufB and SufCSufD. This overall configuration is common among ABC proteins, in which two ABC ATPase subunits bind to function-specific subunits with their ATP-binding motifs facing each other. The two bound SufC subunits, however, are spatially separated, in contrast to the analogous domains/subunits of canonical ABC transporters; the distance between the SufC subunits within the SufBCD complex is more than 40 Å (Fig. 2B). The asymmetric unit contains two complexes with almost identical structures; superposition
of them showed some mobility of two SufC subunits that shifted < 1 Å toward each other. The r.m.s.d. is less than 0.71 Å for the main chain Cᵦ atoms.

The structure of the SufBCD complex was further examined by three-dimensional reconstruction imaging based on negative stain electron microscopy (Fig. 3). The structures obtained by both methods agreed closely, confirming the quaternary structure of the SufBCD complex. In addition, the crystal structure was consistent with SAXS data from the as-isolated SufBCD complex in solution (Fig. 4), indicating that the configuration of the SufBCD complex in the crystalline state was not affected by crystal packing.

The structures of SufB and SufD are similar and share a common domain organization: an N-terminal helical domain, a core domain consisting of a right-handed parallel β-helix, and a C-terminal helical domain that contains the SufC binding site (Fig. 2A). The β-helix in the core domain of SufB is partly composed of shorter strands than the corresponding domain of SufD, whereas the C-terminal helical domain and the mode of SufC binding are strikingly similar between SufB and SufD. Intriguingly, the mode of binding between the SufC and SufB/SufD subunits is conserved in ABC transporters, an interaction termed the “transmission interface” (49) (discussed below). The heterodimer interface of SufB-SufD protomers consists primarily of 25 hydrogen bonds that form two anti-parallel β-sheets. Although the structure of the SufD subunit in the SufBCD complex was almost identical to that of the previously reported SufD homodimer crystallized alone (50), some structural difference was observed around the interaction site with SufC. The SufD monomers are superimposable, with an r.m.s.d. of 0.59 Å between Cᵦ atoms. Previous biochemical study has suggested that the SufBCD complex binds a flavin as redox cofactor and proposed four motifs involved in FAD binding (24). One proposed motif is located in the disordered region and could not be observed. The remaining motifs were spatially separated in the SufBCD complex.
Structure of SufBCD Complex, a Novel Type of ABC Protein

The SufC subunit has two domains, as observed in the members of the ABC ATPase family: a catalytic α/β domain that contains the nucleotide-binding Walker A and Walker B motifs, and a helical domain specific to ABC ATPases containing an ABC signature motif (Fig. 5). The two domains are connected by a Q-loop that contains a strictly conserved glutamine residue (Fig. 6). SufC is almost identical to other typical ABC proteins. These structural changes rearrange the local structure of SufC to be suitable for ATP binding and hydrolysis; consequently, the local structure of SufC more closely resembles that of active ABC ATPases. These findings are consistent with recent kinetic experiments showing that the ATPase activity of SufC is enhanced by complex formation with SufB/SufD (19).

Key ABC ATPase Motifs of SufC Are Critical for Suf Function—Superposing the structure of E. coli SufC on the typical ABC ATPase, E. coli HlyB of the α-hemolysin export protein (53), reveals very similar overall topologies (Fig. 5). SufC contains highly conserved sets of amino acid residues including an ABC signature motif, Q-loop, D-loop, and H-motif in addition to the Walker A and Walker B motifs, all of which are characteristic of ABC ATPases (Fig. 6). We focused on three strictly conserved amino acid residues considered to be essential for ATP hydrolysis in the ABC ATPases (52–54): the Lys residue in the Walker A motif (corresponding to the Lys-40 in SufC), the Glu residue immediately following the Walker B motif (the Glu-171 in SufC), and the His residue in the H-motif (the His-203 in SufC). In vitro measurements of ATPase activity clearly demonstrated that SufBCD complexes containing mutated SufC proteins (K40R, E171Q, and H203A) almost completely lacked activity (Fig. 8). These mutations did not impair the structural stability of SufC or its interaction with partner proteins (Fig. 9). These results prove that as in the canonical ABC ATPase, the residues of the ABC sequence motifs are responsible for ATPase activity in SufC.

To determine whether the ABC ATPase activity of SufC is necessary for the Fe–S cluster biogenesis, we assessed its in vivo function using a recently established method4 in the E. coli Δisc mutant strain UT109 (16). The site-directed mutants of SufC, K40R, E171Q, and H203A were not able to complement mutant cells, indicating that these residues are indispensable for in vivo Fe–S cluster biosynthesis (Fig. 10A). Our results are in good agreement with previous experiments regarding the SufC K40R mutant (18). Thus, SufC can behave as an ABC-type ATPase, and the activity is indispensable for in vivo Fe–S cluster assembly.

SufC Forms the Transient Head to Tail Dimer—The structure of the SufB1-SufC2-SufD1 complex revealed the configuration of each subunit: the SufC subunit of the ABC ATPase binds to the C-terminal helical domains of the SufB/SufD subunits, and the two SufCs are oriented face to face. According to the current consensus model, ABC ATPases form a transient head to tail dimer in which two nucleotides are sandwiched at the dimer interface between the Walker motifs of one subunit and the ABC signature motif of the other subunit (Fig. 11A). Based on this concept, we generated a putative dimer model of SufC by superimposing the structure of SufC onto the dimeric form of the ATP-bound HlyB (H662A) ABC ATPase (53). The resulting model showed that the local structural changes in SufC (mentioned above) enable an ideal association for the head to tail dimer without steric hindrances (Fig. 11B). Despite the favorable modeling results, SufC and SufC subunits are spatially separated in the SufBCD complex, with their ATP-binding motifs facing one another (Fig. 2B); they would have to move ~20 Å closer to each other to form the head to tail dimer, a distance that is unusually long compared with other structurally characterized ABC proteins.

We conducted disulfide cross-linking experiments to determine whether the separated SufC subunits could transiently associate with each other in the SufBCD complex. In the putative dimer model, the Cα atoms of Tyr-86 in each SufC subunit are in close proximity (<5.8 Å) (Fig. 11B). Hence, we replaced Tyr-86 with a cysteine to allow for covalent trapping of the transient SufC dimer via disulfide bond formation between the subunits. To simplify analysis, we also replaced the sole native cysteine residue on SufC, Cys-167, with an alanine. These mutations did not affect the function of the SufBCD complex (Fig. 10B). After the mutated complex (SufC-186C/C167A) was incubated in the presence of ATP/Mg2+ and an oxidant (CuSO4) to enhance disulfide-bond formation, disul-
fide-bond formation was assessed by native PAGE analysis. The results revealed an additional band on the gel that migrated more quickly than the as-isolated SufBCD complex (Fig. 11C).

No such band was observed when a reducing agent (DTT) was incubated with the sample (data not shown), indicating that disulfide bond gives the new band. Because Western blot analyses using antibodies against SufB, SufC, and SufD revealed all of the corresponding signals (Fig. 11C), we conclude that the novel band represents a conformationally distinct form of the SufBCD complex. In addition, nonreducing SDS-PAGE/Western blot analyses using an antibody against SufC also revealed an additional band whose molecular size was consistent with a molecule 2-fold larger than SufC (Fig. 11D). These findings strongly support the idea that SufC can form a transient dimer, even within the SufBCD complex, in the presence of ATP/Mg\(^{2+}\). The mobility shift on native PAGE demonstrates that a structural change occurs in the SufBCD complex upon SufC dimerization.

Gross Structural Change of SufB-SufD Protomers upon SufC Dimerization—We detected the conformational change of the SufBCD complex, initiated by SufC dimerization, in fluorescent labeling experiments using ANS. ANS, which is poorly fluores-
cent in an aqueous environment, is highly fluorescent upon binding to hydrophobic regions on protein surfaces (55). To determine whether SufC dimerization induces the exposure of hydrophobic regions in SufB-SufD protomers, we compared the fluorescence of the native and cross-linked complexes (described above). After adding ATP/Mg\(^{2+}\)/H\(_{11001}\) and an oxidant to the purified mutant SufBCD complex (SufC-Y86C/C167A), we added ANS to the mixture and immediately measured its fluorescence. The results revealed a remarkable increase in fluorescence intensity, depending on the incubation time (Fig. 12A), indicating that a gross structural change of the SufBCD complex accompanied SufC dimerization (cross-link formation). No such fluorescence increase was observed when ATP/Mg\(^{2+}\)/H\(_{11001}\) or oxidant was omitted from the reaction mixtures or in a control experiment using the single-mutant SufBCD complex where the dimer is not covalently stabilized (SufC-C167A) (Fig. 12A). Therefore, this conformational change was surely elicited by SufC dimerization.

Next, we used another fluorescent reagent to determine whether the interface between SufB and SufD protomers is exposed. To this end, we used the fluorescent thiol reagent DACM, which has a high quantum yield when it reacts with the free cysteine residues on the protein surface (56). The SufBCD complex has a large number of cysteine residues (13 cysteines in SufB, 3 cysteines in SufD, and 1 cysteine in SufC), most of which are buried inside the molecule. We focused on Cys-405 of SufB, which is located at the heterodimer interface between the SufB and SufD protomers (Fig. 13A). This cysteine residue, which is strictly conserved among SufB homologues, is a potential Fe-S cluster assembly site (20). To ascertain whether the Cys-405 could be exposed and detected by DACM, we replaced Cys-405 of SufB with an alanine, in combination with the SufC-Y86C/C167A mutation. Each mutant complex (Y86C/C167A/C405A and Y86C/C167A) was incubated under cross-linked conditions (in the presence of ATP/Mg\(^{2+}\) and an oxidant) and further incubated after addition of DACM. Fluorescence intensity increased following formation of the SufC cross-linked dimer in the complex, indicating that several cysteine residues in the
complex were exposed, whereas a significant decrease in fluorescence intensity was observed upon introduction of the SufB C405A mutation (Fig. 12B). Control experiments, in which the incubation was performed under non-cross-linked conditions (i.e. in the absence of ATP/Mg²⁺/H₁₁₀₀₁ and oxidant), exhibited no difference between mutant complexes. These results demonstrated that SufC dimer formation leads to exposure of the heterodimer interface of the SufB-SufD protomers (at least of Cys-405 of SufB, which is otherwise buried inside the dimer interface). Notably, the invariant residue His-360 of SufD, another candidate for the cluster coordination residue (20), is located close to the Cys-405 of SufB (Fig. 13A), strongly implying that His-360 of SufD could also be exposed by the conformational change.

In Vivo Iron-Sulfur Cluster Formation—During the course of phase determination using the heavy atoms, we noticed that two clear electron densities derived from Hg²⁺/H₁₁₀₀₁ ion appeared inside the heterodimer interface between the SufB and SufD protomers (Fig. 13A): one Hg²⁺ ion bound to Cys-405 in SufB and the other bound to Cys-358 in SufD. One coordinated cysteine residue from SufB was the invariant Cys-405, which is presumably one of the residues composing the assembly site for the nascent Fe-S cluster (20). Interestingly, Cys-358 in SufD is located adjacent to His-360 of SufD, another candidate for cluster binding. This observation raises the possibility that Hg²⁺ ion binds to the authentic iron-binding site involved in Fe-S cluster assembly. Hence, we speculated that cluster formation ability could be evaluated based on the color of the harvested cells prior to exposure of the nascent Fe-S cluster to air by disruption. Harvested cells expressing the wild-type SufBCD complex had a blackish-green color (Fig. 13C), quite similar to that of the partially purified SufBCD complex (Fig. 13B, inset). By contrast, control cells harboring only a vector plasmid were an unremarkable white (Fig. 13C). We thus reasoned that the color of the cells reflects in vivo cluster formation ability, at least for the SufBCD complex, even though the cells included other Fe-S proteins.

As expected, both SufB C405A and SufD H360A mutants had white cells, indicating that these residues are indispensable for cluster assembly, whereas Cys-358 of SufD, the other binding site for Hg²⁺ ions, was not involved in cluster formation (Fig. 13C). These results suggest that Cys-405 of SufB and His-360 of SufD could serve as the in vivo cluster binding sites. Furthermore, mutants in residues essential for SufC ATP hydrolysis (K40R, E171Q, and H203A; described above) also had white cells (Fig. 13C). In combination with the findings described above, SufC dimerization and conformational changes are indispensable for nascent Fe-S cluster formation (discussed below).
The wild-type and mutant SufBCD proteins were present at equal levels in the cells as confirmed by immunoblot analyses (Fig. 13D).

Discussion

Structural Features—In this study, we determined the crystal structure of the SufBCD complex for the first time, revealing its unusual architecture. The core domains of the SufB-SufD protomers consisted of many long strands (8–13 residues in each strand) arranged in a helical architecture, a so-called /H9252\-helix. Indeed, the SufD fold (and probably the SufB fold as well) has been categorized as a novel folding superfamily (superhelix turns made of two very long strands each) in the SCOP2 classification database. Moreover, the respective /H9252\-helix core domains in SufB and SufD are associated by anti-parallel /H9252\-strands to form a novel heterodimeric structure. In the ABC transporters in general, the transmembrane domains consist of a total of 12 helices with 6 helices per monomer, but they have structural diversity appropriate for their respective substrates and functions. In SMC proteins, the function-specific domain consists of a long coiled-coil arm that forms a V-shaped dimeric molecule by interacting with the hinge region for DNA binding. Therefore, the novel /H9252\-helix architecture of the SufB-SufD protomers appears to be specialized for the Fe-S cluster biogenesis systems.

Despite the structural variations in their substrate- and function-specific domains or subunits, ABC proteins share a common mechanism for transmitting the driving force for their transport, as evidenced by the Arrhenius plots in Figure 12B.
respective functions. Recent extensive structural analyses in ABC transporters revealed that the so-called “transmission interface” transmits the dynamic motion of the ABC ATPase to the transmembrane domain during ATP binding and hydrolysis (49), where the Q-loop in the ABC ATPase domain associates with the two short helix-turn-helix motifs in the transmembrane domains. Structural motifs involved in the interaction between SufC and SufB/SufD bear striking similarity to the corresponding configurations in other structurally characterized ABC proteins (Fig. 14). From a structural standpoint, the SufBCD complex also shares the mode of the transmission of the driving force with other ABC proteins: the ATPase activity of SufC drives the conformational change of SufB-SufD protomers for Fe-S cluster biogenesis.

Insight into Iron-Sulfur Cluster Biogenesis—In vitro biochemical experiments and in vivo functional analyses based on the crystal structure of the SufBCD complex provided unprecedented insights into the molecular mechanism of Fe-S cluster biogenesis. The main findings are summarized as follows: 1) SufC of ABC-type ATPase forms a transient head to tail dimer within the SufBCD complex during the catalytic step of ATP binding and hydrolysis; 2) SufC dimerization drives gross structural changes of the SufB-SufD protomers, leading to the exposure of Cys-405 of SufB (and probably also His-360 of SufD) inside the heterodimer interface; 3) the conformational changes are directly related to nascent Fe-S cluster formation on the SufBCD complex; and 4) Cys-405 of SufB and His-360 of SufD are most likely to work in concert, possibly serving as the site of in vivo cluster synthesis.

Based on these findings, we propose a mechanism for Fe-S cluster biogenesis for the SufBCD complex (Fig. 15). In the resting state, the SufC ABC-type ATPase in the complex is ready for ATP binding, and the nascent cluster assembly site at the SufB and SufD interface is buried inside the complex. When SufC forms the head to tail dimer upon ATP binding, its dynamic motion is transmitted to the SufB-SufD protomers of the function-specific subunits via the transmission interface. Consequently, the invariant residues involved in Fe-S cluster assembly, Cys-405 in SufB and His-360 in SufD, are exposed to the surface to construct and transfer the nascent Fe-S cluster.

New Perspective on the Suf System—The Suf machinery is thought to represent the ancestral system for Fe-S cluster biogenesis in all kingdoms of life (16). Genes homologous to SufB and SufC are present in a wide range of bacteria, Archaea, and plastids, suggesting that the Suf system is almost ubiquitous in nature. In this study, we revealed that SufB and SufD share novel structural features. Therefore, the dynamic motion of the SufB$_2$-SufC$_2$-SufD$_1$ complex, experimentally demonstrated here, is universally applicable to all Suf systems, even the SufB$_2$-SufC$_2$ complex in the Archaeal Suf system.

FIGURE 13. In vivo Fe-S cluster formation on the SufBCD complex. A, anomalous difference map for Hg$^{2+}$ ion (contoured at 6.0 σ) in the Hg derivative of the SufBCD complex. A square highlights the binding site of Hg$^{2+}$, of which the right panel shows a close-up view. Two Hg$^{2+}$ ions bound to Cys-405 in SufB and Cys-358 in SufD are adjacent to His-360. These residues are depicted with a stick model, and Hg$^{2+}$ ions are shown as orange balls. B, UV-visible absorption spectrum of the SufBCD complex at an early purification stage. The inset shows the sample solution and SDS-PAGE analysis of the partially purified SufBCD complex used in this measurement. C, colors of the harvested host cells overproducing the SufBCD complex. D, comparison of the expression level of Suf/SufC/SufD among the cells harboring the wild-type plasmid or the various mutant plasmids by immunoblot analyses using antibodies against SufB, SufC, and SufD. cont., control.
**Structural and mechanistic understanding of Suf systems may enable the development of new antibiotics that target the SufBCD complex. Indeed, a recent study demonstrated that the Suf system of malaria parasites is essential for survival and plays a fundamental role in maintaining the apicoplast organelle (58). In eukaryotes, including mammalian cells, the ISC system (59) and its dependent CIA system (60) are responsible for nascent Fe-S cluster biogenesis.**

![Figure 14. Comparison of the transmission interface in the SufBCD complex and ABC proteins from E. coli. A–D, close-up views of the interface between the substrate/function-specific subunit and the ABC ATPase subunit in the resting (inward-facing) state of MalFGK maltose transporter (Protein Data Bank code 3FH6) (A), the inward-facing state of the MetNI methionine transporter (Protein Data Bank code 3DHW) (B), and the resting state of the SufBCD complex at the SufB-SufC interface (C) and the SufD-SufC interface (D). Each subunit in the complex is depicted in a different color. Substrate/function-specific subunit is displayed only for the two short helix-turn-helix involved in the interaction. Color coding for the conserved motifs in ABC ATPases is the same as in Fig. 5.](image)

![Figure 15. Proposed mechanism of Fe-S cluster biogenesis for the SufBCD complex. Biogenesis cycle starts (at left) in the resting state in which SufC is ready for ATP binding. Upon binding of ATP, SufC forms a head to tail dimer. Consequently, the Fe-S cluster binding site between the SufB and SufD interface is exposed to the surface. Nascent Fe-S cluster is built/transfered and ATP is hydrolyzed, restoring the SufBCD complex to its resting state.](image)
tein of the ISC machinery, IscU, has a completely different sequence and tertiary/quaternary structure than the SufBCD complex (61). Therefore, the Suf system, especially the SufB-SufD protoners with its characteristic β-helix fold and dynamic motion, is an eligible target for drug design with minimal risk of harm to the human body.

Author Contributions—F. W. O., K. F., Y. T., and K. W. conceived the project; G. K., K. F., Y. T., and K. W. designed and conducted the experiments; K. H. performed protein purification and crystallization and determined the crystal structure; K. H., E. Y., and N. T. carried out functional analyses and genetic studies; S. K. and K. I. performed single-particle EM reconstructions; K. H. and T. M. performed SAXS experiments; K. H., K. I., T. M., F. W. O., K. F., Y. T., and K. W. wrote the manuscript.

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References

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