Protein Synthesis and Degradation: Iron-Sulfur (Fe-S) Cluster Assembly: THE SufBCD COMPLEX IS A NEW TYPE OF Fe-S SCAFFOLD WITH A FLAVIN REDOX COFACTOR

Silke Wollers, Gunhild Layer, Ricardo Garcia-Serres, Luca Signor, Martin Clemanecy, Jean-Marc Latour, Marc Fontecave and Sandrine Ollagnier de Choudens

doi: 10.1074/jbc.M110.127449 originally published online May 11, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.127449

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/05/11/M110.127449.DC1.html

This article cites 48 references, 19 of which can be accessed free at http://www.jbc.org/content/285/30/23331.full.html#ref-list-1
Iron-Sulfur (Fe-S) Cluster Assembly

THE SufBCD COMPLEX IS A NEW TYPE OF Fe-S SCAFFOLD WITH A FLAVIN REDOX COFACTOR

Received for publication, March 26, 2010, and in revised form, May 10, 2010. Published, JBC Papers in Press, May 11, 2010, DOI 10.1074/jbc.M110.127449

Silke Wollers‡, Gunhild Layer‡, Ricardo Garcia-Serres†, Luca Signorini, Martin Clemaucy‡, Jean-Marc Latour§, Marc Fontecave†, and Sandrine Ollagnier de Choudens¶

Abstract

Assembly of iron-sulfur (Fe-S) clusters and maturation of Fe-S proteins in vivo require complex machineries. In Escherichia coli, under adverse stress conditions, this process is achieved by the SUF system that contains six proteins as follows: SufA, SufB, SufC, SufD, SufS, and SufE. Here, we provide a detailed characterization of the SufBCD complex whose function was so far unknown. Using biochemical and spectroscopic analyses, we demonstrate the following: (i) the complex as isolated exists mainly in a 1:2:1 (B:C:D) stoichiometry; (ii) the complex can assemble a [4Fe-4S] cluster in vitro and transfer it to target proteins; and (iii) the complex binds one molecule of flavin adenine nucleotide per SufBC2D complex, only in its reduced form (FADH2), which has the ability to reduce ferric iron. These results suggest that the SufBC2D complex functions as a novel type of scaffold protein that assembles an Fe-S cluster through the mobilization of sulfur from the SufSE cysteine desulfurase and the FADH2-dependent reductive mobilization of iron.

Proteins that contain an iron-sulfur (Fe-S)5 cluster as a prosthetic group are widely utilized in all living organisms for a great variety of cellular processes, including respiratory and photosynthetic electron transport, metabolic and biosynthetic reactions, and in the regulation of gene expression (1, 2). Fe-S clusters are not spontaneously formed in the cells. Genetic and biochemical studies have so far revealed three distinct systems responsible for Fe-S cluster biosynthesis, termed NIF, ISC, and SUF, which are encoded by the nif, isc, and suf operon, respectively (1–3). The NIF system is responsible for the maturation of nitrogenase, but it is also distributed in some anaerobic organisms lacking nitrogenase (4). The ISC machinery is found in the majority of prokaryotes and in mitochondria (5). The SUF pathway is present in cyanobacteria and in the chloroplasts of higher plants as well as in bacteria, including human pathogens such as Yersinia pestis and Mycobacterium tuberculosis (6, 7). It is generally admitted that the SUF machinery is involved in biosynthesis of Fe-S clusters during adverse stress conditions such as iron starvation and oxidative and heavy metal stresses (8–10).

The SUF machinery has been the focus of intense studies at the biochemical level, especially in Escherichia coli. The sufABCDSE operon in E. coli encodes six proteins. SufS is a cysteine desulfurase that mobilizes sulfur from free l-cysteine in the form of a protein-bound persulfide (11, 12). SufE accepts sulfur from SufS and provides it to proteins for Fe-S cluster assembly (13). In doing so, SufE acts as a sulfur transfer protein that stimulates SufS activity (14, 15). The function of SufE was more enigmatic. Some in vitro experiments had shown that SufA can bind ferric iron and transfer it to IscU during cluster assembly (16). However, other in vitro experiments had demonstrated that SufA can assemble Fe-S clusters and transfer them to apoproteins (17–19). Recently, the nature of its metal cofactor as well as its role were clarified by the characterization of the protein isolated after co-expression in E. coli with its cognate partner proteins from the suf operon, SufBCDSE (20). This study unambiguously demonstrated that SufA binds a [2Fe-2S] cluster that can be transferred to target apo-proteins (20). Consequently, SufA could be defined either as an Fe-S scaffold protein, defined as the primary site of cluster assembly, or as a carrier protein, defined as a system transferring Fe-S clusters from a scaffold to a target protein. Genetic studies supported the latter concept, and SufA was included in the family of the so-called A-type carriers (21).

The three additional components of the SUF machinery, SufB, SufC, and SufD, were shown to be essential for in vivo Fe-S biosynthesis under oxidative stress and iron limitation conditions (8, 9, 22). SufC is a soluble ATPase that exhibits striking structural similarity to the ATPase subunits of ABC transporters (23). SufB and SufD share limited sequence similarity with each other and interact with SufC to form a tight SufBCD complex (9, 14). Binding of either SufB or SufD to SufC was shown to enhance the basal ATPase activity of SufC (24, 25). Physical interaction between SufBCD and the SufSE complex results in further stimulation of the cysteine desulfurase...
activity of the SufSE complex (14, 26). Very recently, SufA was also shown to interact with SufBCD (27).

Despite the progress in elucidating some of its biochemical properties, including three-dimensional crystal structures of SufC, SufD, and SufC2D2 proteins (23, 28, 29), our understanding of the role of the SufBCD complex and the molecular mechanism by which it functions remains elusive. Genetic studies have recently shown that the simultaneous inactivation of iscll, encoding the scaffold protein IscU of the ISC system, and sufBCD in E. coli is lethal and that none of the A-type carriers (IscA, SufA, and ErpA) is able to promote maturation of Fe-S proteins, thus supporting the hypothesis of SufBCD functioning as a scaffold protein (21). This is in agreement with our finding that the SufB protein is a [4Fe-4S] protein (26), and a recent report by Chahal et al. (27) showing that SufBCD binds an Fe-S cluster that can be transferred to apo-SufA, whereas SufA is unable to transfer its cluster to SufBCD. These results clarify the SufBCD–SufA duality, with SufBCD being the scaffold protein, and SufA is a cluster carrier protein with an unidirectional Fe-S cluster transfer from SufBCD to SufA. More interestingly, we propose in this paper that the SufBCD complex is a novel type of scaffold protein on the basis of the unexpected observation that the anaerobically purified SufBCD complex contains 1 eq of FADH2, the flavin adenine nucleotide in its reduced form, which readily reacts with oxidants such as oxygen and ferric ions. We suggest that SufBCD uses FADH2 as a redox cofactor for mobilizing iron during assembly of its own cluster.

**Experimental Procedures**

**Materials and Plasmids**—Ferric-dicitrate was made by mixing a 2-fold excess of citric acid with ferrous ammonium sulfate in water. During neutralization with NaOH, iron oxidizes, and the solution turns green-brown. Plasmid pGSO164 containing the entire suf operon from *E. coli* was used to express and purify the SufBCD complex (14). Plasmids pET-Shis, pET-Ehis encoding the His-tagged SufS and SufE, as well as pET3aSufB, pET3aSufC, and pET3aSufD encoding SufB, SufC, and SufD proteins were obtained as described previously (13, 19, 26). Plasmid pG5783 encoding aconitate B was a gift from J. R. Guest (Norwich, UK).

**Strains and Growth Conditions**—(His)_6–SufE and SufABCDSE were produced in *E. coli* TOP10 cells (Invitrogen); (His)_6–SufS, SufB, SufC, and SufD as well as AcmB were produced in *E. coli* BL21(DE3) pLysS cells (Invitrogen) as described previously (14, 19, 26, 30). Cells were grown in LB medium in the presence of 100 mg/ml ampicillin or 30 mg/ml chloramphenicol at 37 °C to an *A*OD600 of 0.5 before induction with 0.2% 1-arabinose (w/v) or 0.5–1 mM isopropyl β-D-galactopyranoside. Cells were grown at 37 °C for 5 h with the exception of SufB (26).

**Protein Purification**—For SufBCD, cell lysis was achieved by three freeze/thaw cycles in the presence of 0.7 mg/ml lysozyme followed by centrifugation at 45,000 × g for 90 min. The obtained supernatant was cleared of DNA with 2% (w/v) streptomycin sulfate and loaded onto a Q-Sepharose FF anion exchange column (GE Healthcare). SufBCD was eluted with a linear gradient of 0–1 M NaCl. Fractions containing SufBCD were pooled, diluted 1:2 with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM (NH4)_2SO4, loaded onto a butyl-Sepharose FF hydrophobic column, and eluted with a linear gradient of 1–0 M (NH4)_2SO4. SufE-(His)_6, SufS, SufB, SufC, SufD, and AcmB were purified as described previously (15, 19, 26, 30).

**Mass Spectrometry**—Noncovalent mass spectrometry measurements were performed on a Q-TOF micro mass spectrometer equipped with a Z-spray electrospray ion source (Micromass, Manchester, UK). Mass spectra were acquired with a needle voltage of 3 kV, sample cone of 30 V, extraction cone of 0.1, source temperature of 80 °C, and desolvation temperature of 150 °C. Backing Pirani pressure was set at 7.3 mbar. The SufBCD sample was infused continuously at a 10 μl/min flow rate in 50 mM ammonium acetate buffer, pH 6.8. Data were recorded in the positive ion mode in the 1800–7000 m/z range with a 1-s scan time and processed with MassLinx 4.0 software (Waters). A 1 mg/ml CsI solution in isopropyl alcohol/water (1:1, v/v) was used to calibrate the instrument.

**Mössbauer Spectroscopy**—Mössbauer spectra were recorded at 4.2 K, either on a low field Mössbauer spectrometer equipped with a Janis SVT-400 cryostat or on a strong field Mössbauer spectrometer equipped with an Oxford Instruments Spectromag 4000 cryostat containing an 8 tesla split pair superconducting magnet. Both spectrometers were operated in a constant acceleration mode in transmission geometry. The isomer shifts are referenced against that of a metallic iron foil at room temperature. Analysis of the data was performed with the program WMOSS (WEB Research).

**Reconstitution of Suf Proteins with Flavin and Binding Measurements**—The proteins SufB, SufC, SufD, and SufBCD were incubated in 50 mM Tris-HCl, pH 7.5, under anaerobic conditions with a 5 molar excess of FAD. Photo-induced reduction of the flavin was achieved by irradiation with a commercial slide projector placed at a distance of 3 cm in the presence of 5–10 mM DTT (31). The resulting colorless solution was desalted via a NAP-25 (GE Healthcare) column to remove unbound flavin. After aerobic heat denaturation of the protein, the concentration of protein-bound FADH2 was calculated from the absorbance of free oxidized FAD at 450 nm (ε = 11,300 M⁻¹ cm⁻¹).

Determination of the dissociation constant for the SufBCD–FADH2 complex was performed by an ultrafiltration assay (32). 0–200 μM free reduced flavin were anaerobically co-incubated for 60 min at 18 °C either with 20 μM SufBCD or in a control sample without protein. After incubation, unbound flavin was separated from SufBCD by filtration with a 100,000 molecular weight cut-off Vivaspin concentrator (Sartorius). The concentrations of SufBCD–FADH2 ([FADbound]) and apo-SufBCD were determined according to the calculated concentrations of unbound and total FADH2 and the known amount of total apo-SufBCD. The protein-bound FADH2 as a function of unbound FADH2 in solution was then plotted. The data have been fitted by a saturation hyperbola according to Equation 1,

\[
[FAD_{bound}] = [SufBCD_{tot}] \times [FAD_{free}] \times K_d \div [FAD_{free}]
\]

(Eq. 1)
Cofactor Analysis—Anaerobically purified SufBC₂D was boiled for 10 min, chilled on ice, and microcentrifuged for 10 min to precipitate the protein. The supernatant was analyzed by thin layer chromatography on Silica Gel 60 F254 (Merck) with a butan-1-ol/acetic acid/water (12:3:5 by volume) development system. Pure FMN and FAD were run as flavin standards.

Iron-Sulfur Cluster Reconstitution on SufBC₂D—Purified SufBC₂D (135 μM) was incubated with catalytic amounts (1.5 μM) of SufS and SufE, an excess (2 mM) of l-cysteine, and a 5-fold excess (810 μM) of Fe(NH₄)₂(SO₄)₂ or 5⁷FeCl₃ in the presence of 5 mM DTT at 18 °C under anaerobic conditions. After 4 h of incubation, EDTA (135 μM) was added, and after 15 min, the mixture was desalted using a Nap-25 column (GE Healthcare). UV-visible spectrum of reconstituted SufBC₂D was recorded on a Cary 1 Bio (Varian) spectrophotometer. The iron and sulfur content of the complex was determined as described previously (26).

Iron-Sulfur Cluster Transfer Reactions—All Fe-S transfer experiments were performed anaerobically at 18 °C. Aconitase B in its apo-form (0.2 nmol) was incubated in 50 mM Tris-HCl, pH 7.6, containing 5 mM DTT with either a 1.5-fold molar excess of the SufBC₂D complex (0.3 nmol) to provide sufficient equivalents of iron and sulfide to build a [4Fe-4S]/AcnB or 5 molar excess of iron and sulfide. Aconitase activity was assayed after 5 min of incubation in 100 μl by monitoring the formation of NADPH via the increase of absorbance at 340 nm as described by Gardner and Fridovich (33). For the experiment in the absence of DTT during the FeS transfer, apo-aconitase B was first pretreated with 5 mM DTT for 30 min, before desalting the protein solution via a MicroBiospin column (Bio-Rad). The resulting protein (0.2 nmol) was tested for aconitase activity as described above. For the Fe-S transfer experiment in the presence of the iron chelator bathophenanthroline disulfate, apo-aconitase B (0.2 nmol) was incubated anaerobically with either [4Fe-4S] SufBC₂D (0.3 nmol) (providing 4 eq of iron and sulfide atoms/apoAcnB) or 5-fold molar excess of Fe²⁺ and S²⁻ in 50 mM Tris-HCl, pH 7.6, 5 mM DTT with increased amounts of bathophenanthroline, and the aconitase activity was measured after 15 min of incubation as described above.

Ferric Reduction by the SufBC₂D-FADH₂ Complex—SufBC₂D-FADH₂ was incubated anaerobically with ferric citrate (10 mM excess with regard to SufBC₂D) in 50 mM Tris-HCl, pH 7.5. Either the reduction of the flavin at 450 nm or the reduction of Fe³⁺ from ferric citrate was followed over time. Reduction and mobilization of iron were monitored after addition to the reaction mixture of the Fe²⁺-chelator ferrozine (30–50 μM excess/SufBC₂D) via the increase of the absorbance at 562 nm corresponding to the formation of a ferrozine-Fe³⁺ complex (ε = 27,900 M⁻¹·cm⁻¹). When CyaY was used as ferric iron source, 10 μM of the intermediate form of CyaY-Fe³⁺, containing an average of 20 iron atoms/monomer (34), was incubated with 10 μM of SufBC₂D-FADH₂ (0.93 FADH₂/complex). Ferrozine was added, and formation of the ferrozine-Fe³⁺ complex was followed at 562 nm.

Determination of Protein Concentration—Protein concentrations were measured by the method of Bradford using bovine serum albumin as a standard that in the case of SufBC₂D underestimates the concentration by a factor of 1.14, as determined by the quantitative amino acid analysis of the purified SufBC₂D complex.

RESULTS

SufBCD Complex Exists Mainly in a 1:2:1 Stoichiometry—The stable SufBCD complex used in this study was isolated directly from an E. coli strain expressing the whole suf operon and purified anaerobically as described previously (26). After aerobic purification, the SufBCD complex does not contain any cofactor and, as a consequence, exists in the apo-form. To determine the stoichiometry of the native complex, with an estimated purity of >95% (supplemental Fig. 1a), we analyzed it by electrospray ionization-mass spectrometry. The mass spectrum of the purified complex displayed three main species (1–3) giving the following experimental molecular masses: 1) 27,583.1 ± 3.2 Da, which corresponds to a SufC monomer; 2) 128,979.4 ± 4.2 Da, which is in accordance with a SufBCD complex; and 3) 156,644.7 ± 12.6 Da, which is consistent with the mass of a SufBC₂D complex (supplemental Fig. 1, b and c). No other combinations match these mass values. Because the mass spectrometry is not a quantitative method, we used size exclusion chromatography to determine the SufBCD/SufBC₂D ratio by quantifying the SufC-containing fractions that dissociate from the SufBCD complex. This allowed us to show that 75% of the complex exists in the SufBC₂D form, the rest being in the SufBCD form derived from dissociation of one SufC molecule from the SufBC₂D complex (supplemental Fig. 1d). These data support an organization of the native complex predominantly with a 1:2:1 (B:C:D) stoichiometry, as suggested previously (24, 29), and a propensity of that complex to lose one SufC component. From now on, this complex will be named SufBCD₃. All attempts to generate such a SufBCD₃ complex (or a SufBCD complex) by incubating a mixture of SufB, SufC, and SufD, purified separately, failed. Only B₂,B₂C, and C₂D₂ combinations were obtained emphasizing the absolute necessity to use the as-isolated complex for further characterizations.

SufBC₂D Complex Binds a [4Fe-4S]²⁺ Cluster after Reconstitution—Chahal et al. (27) proposed recently that the SufBC₂D complex contains an Fe-S cluster similar to that of SufB characterized by us (26). Here, we present the first detailed characterization of the Fe-S cluster bound to the SufBC₂D complex. The SufBC₂D complex, reconstituted with iron and sulfur as described under “Experimental Procedures,” was indeed shown to contain 3.5 iron/sulfur and generally slightly larger amounts of sulfur (5 sulfur/complex), as observed in the case of SufB alone (26). It displayed the characteristic UV-visible spectrum of a [4Fe-4S]²⁺ cluster with only one broad band at around 420 nm (Fig. 1). As shown for SufB (26), the cluster of SufBC₂D is sensitive to oxygen (t₁/₂, 10 min) (supplemental Fig. 2). To accurately determine the nature of its Fe-S cluster, SufBC₂D was reconstituted anaerobically with ⁵⁷Fe under the same conditions as described above and characterized by Mössbauer spectroscopy. A minority species accounting for 14% of total iron is detected as a broad asymmetric quadrupole doublet with parameters (δ = 0.89 mm/s and ΔE Q = 3.50 mm/s) consistent with high spin Fe²⁺. This species was assigned to nonspecifically bound ferrous ions. The Mössbauer spectrum of the reconstituted SufBC₂D-[⁵⁷Fe-S] complex (Fig. 2b), containing
Biosynthesis of Iron-Sulfur Clusters by the Suf Machinery


3.1 iron and 5 sulfur/complex, could be fitted with a major quadrupole doublet (70% of total iron), whose parameters are identical to those of the [4Fe-4S]2+ cluster in SufB, and a minor doublet (30% of total iron) (Δ = 1.24 mm/s and ΔE_Q = 3.08 mm/s) also assigned to nonspecifically bound Fe2+. It is worth noting that the presence of ATP during the reconstitution process did not change either the type or the amount of cluster present in SufBC2D (supplemental Fig. 3). The quantification of bound iron and sulfur together with these spectroscopic analyses demonstrate clearly that the SufBC2D complex assembles a [4Fe-4S]2+ cluster during in vitro reconstitution, as does SufB, and thus suggest that this cluster is located on the SufB component within the SufBC2D complex.

Anaerobically Purified SufBC2D Contains a Protein-bound Flavin Adenine Dinucleotide (FADH2)—To analyze the flavin-binding properties of apo-SufBC2D, the complex was co-incubated with a 5 mol excess of FAD either under aerobic conditions or anaerobically in the presence of an excess of DTT while irradiating to obtain FADH2. After separation of unbound flavin on a NAP-25 column, the reconstituted SufBC2D protein was assayed for its flavin content as described under “Experimental Procedures.” Between 0.8 and 1 mol of ence to anaerobiosis during extraction of soluble proteins and protein purification from an E. coli strain expressing the whole suf operon (20). We wondered also whether the SufBC2D complex could be isolated with its metal cofactor when purified under similar conditions. After expression of the suf operon using the pGSO164 plasmid, SufBC2D was thus purified anaerobically. The protein solution exhibited a very pale yellow color, suggesting that either only very little or no Fe-S cluster was associated with the protein or that the cluster was in a reduced state. However, analysis of the iron and sulfide contents revealed only very little protein-bound iron and sulfide (0.1 iron and 0.2 sulfur/complex). This showed that under the chosen growth conditions, intracellular SufBC2D is mainly cluster-free, in contrast to SufA, although we cannot exclude that the cluster is so labile that it was lost during purification steps.

When a fraction of the pure complex was exposed to air, surprisingly the solution became bright yellow. The resulting UV-visible spectrum displayed absorption maxima, at 370 and 450 nm, which are characteristic for the presence of an oxidized flavin (Fig. 3a). Analysis of the flavin species was done by thin layer chromatography of the supernatant, after heat denaturation of the protein, and unambiguously established the exclusive presence of flavin adenine dinucleotide in SufBC2D (data not shown). The same supernatant was analyzed spectrophotometrically. Using an extinction coefficient of 11.3 mM⁻¹·cm⁻¹ for free FAD (35), we calculated a ratio of about 0.8 mol of FAD per mol of SufBC2D. From these analyses, it can be concluded that the anaerobically purified SufBC2D complex binds 1 eq of visible light-transparent FADH2, the reduced form of FAD, which converts to FAD, the yellow oxidized form, during in vitro exposure to oxygen.

Further evidence for the presence of a reduced flavin in SufBC2D came from the observation of the semi-reduced state of FAD during careful spectroscopic monitoring of the air-dependent oxidation reaction. As shown in Fig. 3b, during the very first minutes of the reaction, we could observe the transient appearance of an absorption band at 590 nm and a shoulder at around 640 nm with a maximal absorption after about 7 min. These absorption bands, which then disappeared upon further incubation, are indeed characteristic for a neutral semiquinone species (Fig. 3b, inset). Confirmation of the formation of such a radical species was obtained from the EPR spectrum of the solution, after about 10 min incubation, which displayed absorption maxima, at 370 and 450 nm, which are characteristic for the presence of an oxidized flavin (Fig. 3c). This is consistent with the flavin being protein-bound and thus partially protected from exogenous reagents.
Biosynthesis of Iron-Sulfur Clusters by the Suf Machinery

FAD per mol of SufBC₂D could be reproducibly determined in the case of the anaerobic reaction mixture containing FADH₂ and SufBC₂D, whereas no protein-bound flavin could be detected in the case of the aerobic FAD/SufBC₂D incubation mixture clearly showing that only FADH₂ binds to the protein complex. Accordingly, when the FADH₂-containing protein was exposed to air and then desalted on a NAP-25 column, flavin could not be detected anymore on SufBC₂D. During incubation of SufBC₂D with FMNH₂ or reduced riboflavin instead of FADH₂, less flavin remained protein-bound after the desalting step (0.4 and 0.1 mol/mol of SufBC₂D, respectively). Again, no binding of oxidized FMN or riboflavin could be observed after co-incubation under aerobic conditions. We also investigated the ability of the SufBC₂D complex, in its iron-sulfur cluster form ([4Fe-4S]), to bind the reduced flavin. The presence of the cluster or addition of ATP had no influence on the flavin content, which was determined to be approximately one reduced flavin per complex (supplemental Table 1). The fact that the reduced flavin and the cluster under its +2 oxidation state co-existed in the same complex shows that no electron transfer between the two species could occur.

To determine which protein subunits of the SufBC₂D complex are involved in the binding of reduced flavin, we repeated the same experiment as above with single proteins SufB, SufC, and SufD and also with some combinations of the three proteins. After treatment with a 5 molar excess of FAD under anaerobic conditions and irradiation, only SufB alone or SufB in the presence of SufC was able to bind FADH₂, albeit to a lesser extent (0.1–0.3 mol of FADH₂ per mol of protein, respectively). SufC alone and SufD were shown not to bind the flavin. Thus, only the whole SufBC₂D complex could bind 1 mol of flavin per mol of complex demonstrating that the association of the three proteins is required for full binding of FADH₂.

To determine the dissociation constant ($K_d$) for the binding of FADH₂ to SufBC₂D, an ultrafiltration assay was used as described under “Experimental Procedures.” The flavin-free apo-SufBC₂D complex (20 μM) was co-incubated at 18 °C anaerobically with different concentrations of FADH₂ (0–200 μM) obtained by photo-induced reduction of FAD. After co-incubation, the samples were transferred to a 100,000 molecular weight cutoff concentrator, and unbound FADH₂ was separated from SufBC₂D by ultrafiltration. As a control, the same experiment was performed in the absence of apo-SufBC₂D. The flavin content of the flow-through fraction of the samples incubated in the presence of apo-SufBC₂D was determined as described under “Experimental Procedures.” The amount of complex (SufBC₂D-FADH₂) and the concentrations of free SufBC₂D were determined according to the calculated concentrations of free and total FADH₂ and the known amount of total apo-SufBC₂D. Under these conditions, a dissociation constant of 12 μM was determined (Fig. 4).

Bound Flavin Is Not Required for SufBC₂D [4Fe-4S] Cluster Transfer—Aconitase B (AcnB), an enzyme containing a [4Fe-4S] cluster in its active form, was used as a target for Fe-S transfer experiments. Both forms of holo-SufBC₂D ([Fe-S] and [Fe-S] + FADH₂) were used as a potential source of clusters. In a typical experiment, an excess of holo-SufBC₂D (0.3 nmol) was co-incubated anaerobically with apo-aconitase B (0.2 nmol) to provide a sufficient amount of Fe-S cluster to build a [4Fe-4S] cluster in AcnB. After 5 and 20 min of reaction, AcnB activity was monitored as described under “Experimental Procedures.” As shown in Fig. 5a, AcnB is fully active after 5 min of reaction, and no significant differences could be observed between the two forms of holo-SufBC₂D used as the Fe-S source. A similar activation of aconitase could be achieved when apo-AcnB was incubated with a 5-fold molar excess of iron and sulfide but only in the presence of DTT in the reaction mixture (Fig. 5a). Indeed, very little activity in the control was detected in the absence of DTT (Fig. 5b). On the contrary, AcnB can be matured in a time-dependent manner by both SufBC₂D-[Fe-S] and SufBC₂D-[Fe-S] + FADH₂ even in the absence of DTT (Fig. 5b). Thus, these data show for the first time a cluster transfer from SufBC₂D to a target protein different from SufA and also exclude a role of the reduced flavin in this process. Finally, the experiment shown in Fig. 5c nicely differentiates the SufBC₂D-dependent and the chemical aconitase activation. Indeed, addition of increasing concentrations of a strong iron chelator,
bathophenanthroline, to the standard reaction mixture had very little effect on the Fe-S cluster transfer from SufBC2D to AcnB, although it completely inhibited the chemical reconstitution of the aconitase. These data, the lack of requirement for DTT and inhibition by a chelator, thus show that cluster transfer from SufBC2D to aconitase is a concerted process that does not involve intermediate disassembly of the cluster, release of iron and sulfur in solution, and then reassembly in the target protein.

Reduction of Ferric Complexes by the SufBC2D-FADH2 Complex—Reduced flavins are excellent ferric iron-reducing agents (36, 37). We thus investigated the potential of the reduced flavin of the SufBC2D complex for reduction of ferric complexes. This was tested using ferric citrate, a small iron complex (38), and CyaY, the bacterial frataxin homologue (34, 39), as electron acceptors. SufBC2D-FADH2 complex (30 μM, 1 FADH2/complex) was incubated anaerobically with ferric citrate (300 μM), and electron transfer from FADH2 to ferric citrate was monitored by UV-visible spectroscopy from the increase of the absorbance at 450 nm, reflecting formation of oxidized flavin. As a control experiment, SufBC2D-FADH2 complex was incubated with buffer instead of ferric citrate. A time-dependent (t1/2 = 14 min) oxidation of the flavin was observed in the reaction mixture containing the ferric citrate (Fig. 6a), whereas no oxidation of the flavin occurred in the control experiment (Fig. 6a, inset). From the absorption at 450 nm, we could calculate, at the end of the reaction (~60 min) (Fig. 6a), that 26 μM of the flavin was oxidized (90% yield). We also monitored the reduction of ferric iron by the FADH2 cofactor using ferrozine in excess as an effective Fe2+ chelator and an Fe2+ probe. The chelator mobilizes ferrous ions from the iron source and forms a complex with a maximal absorption at 562 nm. Under these conditions and using 10 μM of SufBC2D-FADH2 complex containing 0.74 FADH2/complex, we observed a time-dependent formation of the ferrozine-Fe2+ complex (t1/2 = ~5 min) indicating reduction of ferric citrate by FADH2 and mobilization of Fe2+ by ferrozine (Fig. 6b). At the end of the reaction, about 14 μM of ferrozine-Fe2+ complex...
were formed corresponding to 95% of reducing equivalents present on the SufBC2D complex under the FADH$_2$ form (14.8 μM). Addition of ATP/MgCl$_2$ had neither an effect on the rate nor on the yield of the reaction (Fig. 6b). For comparison, free FADH$_2$ (30 μM) reacted with ferric citrate (300 μM) at a faster rate ($t_{1/2}$ < 1 min), further confirming that the flavin bound to the protein complex is partially protected from oxidants in solution.

We also used the CyaY protein as a ferric iron source. The SufBC$_2$D-FADH$_2$ complex (9.3 μM FADH$_2$) was incubated anaerobically with 10 μM of the CyaY-Fe$^{3+}$. The CyaY-Fe$^{3+}$ protein that we used contained about 20 iron/monomer protein (34). The reduction of Fe$^{3+}$ from CyaY to Fe$^{2+}$ and its release was monitored by UV-visible spectroscopy using 1 mM ferrozine. We also observed a time-dependent formation of the ferrozine-Fe$^{2+}$ complex, as shown by the increase of the absorption at 562 nm (Fig. 6c) and the concomitant oxidation of the flavin (increase of the absorption band at 450 nm) indicating reduction of CyaY-Fe$^{3+}$ by the reduced FADH$_2$ and mobilization of Fe$^{3+}$ by ferrozine. The reaction is slower than with ferric citrate ($t_{1/2} = 35$ min). For a time of 70 min, beyond which there was no significant change in the absorption, we could measure the formation of 19 μM Fe$^{2+}$-ferrozine complex. This matches the amount of reducing equivalents available within the complex under the form of the reduced flavin FADH$_2$. No reduction of CyaY-Fe$^{3+}$ could be observed in a reaction mixture containing apo-SufBC$_2$D, CyaY-Fe$^{3+}$, and ferrozine (supplemental Fig. 5). Cytochrome c was also shown to be an oxidant of the protein-bound reduced flavin (FADH$_2$) (supplemental Fig. 6).

**DISCUSSION**

The results presented here provide a thorough biochemical characterization of the SufBC$_2$D complex that forms a strong basis for a better understanding of its function during Fe-S cluster biosynthesis. All experiments have been carried out on a complex isolated from growing E. coli cells expressing the whole suf operon. This complex exists in solution mainly as SufBC$_2$D, and we propose that this is the functional form, but we cannot exclude that the SufBCD form is also functional. It is quite remarkable that such a complex with a 1:2:1 (B:C:D) stoichiometry cannot be prepared by the incubation of the three isolated proteins in vitro. From our work and previous studies, it seems that SufB and SufD are prone to dimerize, forming rather stable species, whereas SufC is monomeric in solution (24). The three-dimensional structures of SufC and the SufD$_2$ dimerization and how they are strained to make a SufBD heterodimer (23, 28, 40). Clearly, formation of these dimers (SufB$_2$ and SufD$_2$) under in vitro conditions makes it difficult for SufB, SufC, and SufD to assemble as a SufBC$_2$D complex. Furthermore, it seems that SufB and SufD compete for SufC binding because some SufB$_2$C and SufC$_2$D$_2$ can be formed when the three proteins are mixed (our data and see Ref. 29). In the absence of SufD, a mixture of SufB and SufC has been shown to generate a SufB$_2$C$_2$ complex (24). It is still not understood how SufB and SufD in the cytosol are prevented from dimerization (23, 28, 40). Clearly, formation of these dimers (SufB$_2$ and SufD$_2$) under in vitro conditions makes it difficult for SufB, SufC, and SufD to assemble as a SufBC$_2$D complex. Furthermore, it seems that SufB and SufD compete for SufC binding because some SufB$_2$C and SufC$_2$D$_2$ can be formed when the three proteins are mixed (our data and see Ref. 29). In the absence of SufD, a mixture of SufB and SufC has been shown to generate a SufB$_2$C$_2$ complex (24). It is still not understood how SufB and SufD in the cytosol are prevented from dimerization and how they are strained to make a SufBD heterodimer to which monomers of SufC would bind to generate the SufBC$_2$D complex (Fig. 7).

The SufC$_2$D$_2$ complex has been obtained and the three-dimensional structure has been solved (29). In that structure, each SufC subunit is bound to the C-terminal helical domain of...
the SufD homodimer. Because the structures of SufB and SufD were predicted to share a striking similarity, especially in the regions corresponding to the β-helix core domain and the C-terminal helical domain, which are involved in the inter-subunit interactions, the SufBC2D complex is likely to share a common architecture with the SufC2D2 complex with SufB interacting with both SufD and SufC (Fig. 7).

The second property of the SufBC2D complex resides in its ability to bind, during reaction with iron and the SufSE cysteine desulfurase system, an Fe-S cluster, for the first time well characterized by Mössbauer spectroscopy. The Mössbauer parameters unambiguously demonstrate that it is a [4Fe-4S]2+ cluster identical to the one found in the reconstituted SufB protein, so it is also tempting to suggest that in the SufBC2D complex the cluster is exclusively chelated by cysteines of SufB. We exclude that the cluster has ligands from both SufB and another protein of the complex (SufC and/or SufD) because of the following: (i) neither SufC nor SufD contains cysteine residues that are conserved, and (ii) the single SufB protein is able to bind a [4Fe-4S] with similar properties to those of the [4Fe-4S] within the SufBC2D complex.

Even though the cluster in SufB or in SufBC2D is stable under strict anaerobiosis, degradation and loss of iron and sulfide upon exposure to oxygen have been observed. Furthermore, very little iron could be detected in the anaerobically as-isolated form of SufBC2D pointing to a significant lability of that cluster. This feature is more in line with a function as a cluster scaffold protein rather than with an electron-transfer role. Scaffold proteins have been defined as proteins displaying the following: (i) a site for primary assembly of an Fe-S cluster; (ii) an ability to mobilize iron and sulfur atoms for synthesis of the cluster; and (iii) an ability to transfer its rather unstable cluster to an apoprotein for maturation. The SufBC2D complex indeed displays all these properties, and in particular we demonstrated that its [4Fe-4S] cluster could be transferred to the apo-form of aconitase, used as a model target, and converted into a catalytically functional form. Chahal et al. (27) showed that clusters could be transferred from the SufBC2D complex to SufA but not from SufA to SufBC2D. SufBC2D is thus the Fe-S cluster scaffold of the SUF machinery, playing the same role as IscU in the ISC system, and SufA is an Fe-S cluster carrier protein, shuttling clusters to cluster acceptor proteins. These clusters could be provided under normal growth conditions by IscU and under stress conditions by SufBC2D as recently suggested by Barras and co-workers (21) from genetic studies. The fact that the simultaneous inactivation of iscU and sufBCD is lethal is fully consistent with the SufBC2D complex being the scaffold within the SUF system. This function would explain why under normal growth conditions SufBC2D contains no or little cluster because it is designed to bind it only transiently.

Finally, a major discovery is the finding that SufBC2D is a flavin-binding protein. Characterization of the flavin-protein interaction clearly established that the SufBC2D complex could bind 1 eq of reduced flavin adenine dinucleotide, FADH₂. That the flavin is protein-bound is consistent with its relative resistance to exogenous electron acceptors (oxygen, ferric iron), as compared with free flavin. The presence of a SufBC2D-FADH₂ complex within cells is very likely because the anaerobically as-isolated protein contains FADH₂ exclusively even though SufBC2D has the potential to bind reduced FMN and riboflavin.
to some extent *in vitro*. The site of flavin binding within the SufBC2D complex is suggested to reside on SufB because the SufB protein in the absence of SufC and SufD binds a small amount of FADH₂ *in vitro* contrary to SufC and SufD. The *E. coli* SufB sequence contains several signatures that are characteristic of a flavin-binding motif found in the p-cresol-methylhydroxylase family (Fig. 8) (41). The three following conserved sequence motifs of this family are present in SufB: the GXXL motif, which interacts with the adenine ring; the P(X)₆G(A)NX motif, which forms a loop that binds to the adenine ring and compensates for the negative charge of the FAD molecule; the R(X)ₓEXXXYXXXGXₘₙY motif, whose central part is located near the adenine ring, in close proximity to the two first sequence motifs. Furthermore, a conserved arginine residue at the C-terminal part has been described to be within hydrogen bonding distance of both the O₂ of the isoalloxazine ring and O₃ of the ribitol, to provide charge compensation. Site-directed mutagenesis of some residues of these sequence motifs involved in FAD binding to SuFb are under investigation in our laboratory. On the other hand, whereas these motifs are also present in some other SuFb proteins, e.g. from *Salmonella* and *Yersinia*, they are not conserved among all SuFb proteins. In the case of SuFb proteins lacking these motifs, the question is whether they are able to bind FADH₂.

It is worth noting that although SuFb alone is able to bind small amounts of flavin, only the whole SufBC2D complex binds 1 eq of flavin showing that optimal interaction of the flavin with SuFb is only obtained when SuFb binds to SuFD and SuFC, probably as a consequence of conformational adaptations.

One of the remarkable properties of SuFbCD is that it does not bind the oxidized FAD form. This has been shown both from binding experiments and from the observation of a rapid loss of the flavin in solution upon exposure of the SufBC2D-FADH₂ complex to oxygen. In that respect, the system should be defined as a flavin-binding protein, which uses the reduced flavin as a substrate, rather than a flavoprotein, which uses it as a prosthetic group. It strikingly resembles the oxygenase component of the flavin-dependent two-component monooxygenases. These proteins belong to a growing family of bacterial enzymes that are involved in oxidation reactions in a huge number of metabolic and biosynthetic pathways (42, 43). They are made up of two components. One is a flavin reductase that binds a free oxidized flavin and catalyzes its reduction by reduced pyridine nucleotides. The reduced flavin is released and then efficiently and rapidly fixed by a second protein, the oxygenase component. There it reacts with oxygen to generate a flavin hydroperoxide species that is used for oxygen transfer to and oxidation of a specific substrate (42). The resulting oxidized flavin, for which the enzyme has low affinity, is then lost in solution and recovered by the flavin reductase for a second cycle. Thus, the SufBC2D complex and the oxygenase component of this class of enzymes have in common a selective affinity for reduced flavins as substrates. Nevertheless, this analogy does not help much because it is so far excluded that SufBC2D plays a role in oxygen activation and oxidation of a substrate. However, it may suggest that SuFbCD is coupled to a specific or nonspecific flavin reductase as a source of reduced
flavins in a novel two-component system and uses reduced flavin to reduce a specific substrate. Thus, what could be the function of the reducing power present in SufBC2D? Our results exclude a role of the flavin in the transfer of the clusters from SufBC2D to an apoprotein such as aconitase. In the process of Fe-S cluster synthesis, electrons are required particularly for iron reduction and mobilization of ferrous ions from ferric iron sources. In the case of the ISC system, the only redox protein is the product of the fdx gene, the [2Fe-2S] ferredoxin. It is generally proposed that indeed Fdx has a redox function during ISC-dependent Fe-S cluster assembly, but this still requires more experimental evidence. An effect of Fdx has only been observed during conversion of the [2Fe-2S] cluster of IscU into a [4Fe-4S] cluster, which requires iron reduction (44). Obviously, there is a huge literature, including from our laboratory, illustrating the potential of reduced flavins for ferric reduction and for mobilization of iron from ferritins, ferrisiderophores, and ferric citrate (38, 45). It has been demonstrated in E. coli that reduced flavins efficiently promote oxidative DNA damage, including DNA strand breaks, by delivering electrons to free iron and allowing production of hydroxyl radicals (46). In 1994, we emphasized such a reactivity with a paper entitled “Flavin Reductases or Ferric Reductases?” (37). The data reported here demonstrate clearly that the reduced FADH2 cofactor of SufBC2D is accessible and reactive enough to give its electrons not only to small iron complexes such as ferric citrate but also to iron-binding proteins such as CyaY, the bacterial frataxin, an important component of the Fe-S cluster assembly machinery as it represents one of the potential sources of iron (34). We previously showed that reducing agents such as cysteine ($E = -250 \text{ mV}$ at pH 7.4) could reduce CyaY ferric iron, and this provides a mechanism to mobilize iron from CyaY because the protein binds Fe$^{2+}$ only very weakly (34). Reduced flavins display comparably low redox potentials, and thus, even though we have not measured that of the SufBC2D-FADH2 complex, it is not surprising from a thermodynamic point of view that this complex is able to reduce CyaY-bound iron. Thus, we propose that the SufBC2D complex is a different version of the flavin-dependent two-component systems in which the flavin serves to reduce ferric iron from a so far unknown iron source rather than oxygen and, furthermore, that SufBC2D is a novel type of cluster scaffold protein, integrating a scaffold and a redox function. The novel observations reported here lead us to suggest a mechanism for Fe-S cluster assembly by the SUF system (Fig. 9). In a first step, the SufBC2D complex binds 1 eq of FADH2. This reduced flavin is produced via the action of a NAD(P)H: flavin oxidoreductase or flavin reductase. Then the components of the cysteine-desulfurase, SufS and SufE, transiently bind to the SufBC2D-FADH2 complex. The whole complex reacts with cysteine to generate persulfides on SufB (26) through trans-persulfuration reactions from SufS to SufE and then to SufB, as previously shown, and SufSE is released. At the same time, the FADH2 cofactor reduces ferric iron from CyaY, and the resulting ferrous ions are chelated by SufB where they react with persulfides to generate the [4Fe-4S] cluster. During this reaction, the flavin is oxidized and released in solution where it can be recycled by a flavin reductase. Thereafter, the holo-form of SufBC2D can transfer its cluster to an apoprotein either directly or via SufA (Fig. 9). We conclude by suggesting that there is an advantage of using a flavin-dependent system for reduction reactions under oxidative stress and iron limitation conditions, under which the SUF machinery operates, as compared with an iron-sulfur-electron transfer enzyme, such as the ferredoxin, involved in the ISC machinery. Indeed, under such deleterious conditions, an Fe-S enzyme would be degraded and be unable to fulfill its function. Flavins, in contrast, are not sensitive to reactive oxygen species and obviously not to a lack of iron. It is well established that the synthesis of nonessential iron-requiring proteins is decreased (47) and that flavodoxins substitute for ferredoxins under iron-limited growth conditions in a number of microorganisms (48). Here, we have an additional example of a shift from ferredoxin to a flavin-dependent enzyme, associated with the shift from ISC to SUF, when the growth conditions become too adverse.

REFERENCES

Biosynthesis of Iron-Sulfur Clusters by the Suf Machinery