

# Iron-Sulfur (Fe-S) Cluster Assembly

## THE SufBCD COMPLEX IS A NEW TYPE OF Fe-S SCAFFOLD WITH A FLAVIN REDOX COFACTOR<sup>\*[5]</sup>

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<sup>‡§¶1</sup>, Gunhild Layer<sup>‡§¶1,2</sup>, Ricardo Garcia-Serres<sup>‡§¶</sup>, Luca Signor<sup>||</sup>, Martin Clemancey<sup>‡§¶</sup>, Jean-Marc Latour<sup>‡§¶</sup>, Marc Fontecave<sup>‡§¶\*3</sup>, and Sandrine Ollagnier de Choudens<sup>‡§¶4</sup>

From the <sup>‡</sup>Commissariat à l'Energie Atomique, iRTSV/LCBM, 38054 Grenoble Cedex 09, <sup>§</sup>CNRS, UMR 5249, 38054 Grenoble, <sup>¶</sup>Université Joseph Fourier, 38054 Grenoble, the <sup>\*\*</sup>Collège de France, 11 Place Marcelin Berthelot, 75231 Paris Cedex 05, and the <sup>||</sup>Laboratoire de Spectrométrie de Masse des Protéines, Institut de Biologie Structurale, CNRS/Commissariat à l'Energie Atomique/ Université Joseph Fourier, 41 Rue J. Horowitz, 38027 Grenoble Cedex 1, France

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 SufBC<sub>2</sub>D complex, only in its reduced form (FADH<sub>2</sub>), which has the ability to reduce ferric iron. These results suggest that the SufBC<sub>2</sub>D 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 FADH<sub>2</sub>-dependent reductive mobilization of iron.

Proteins that contain an iron-sulfur (Fe-S)<sup>5</sup> 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 SufA 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

\* This work was supported by the Région Rhône-Alpes, Contract CIBLE 07 016335.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–6 and Table 1.

<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> Present address: Institut für Mikrobiologie, Technische Universität Braunschweig, D-38106 Braunschweig, Germany.

<sup>3</sup> To whom correspondence may be addressed. Tel.: 33-438789103; Fax: 33-438789124; E-mail: mfontecave@cea.fr.

<sup>4</sup> To whom correspondence may be addressed. Tel.: 33-438789115; Fax: 33-438789124; E-mail: sollagnier@cea.fr.

<sup>5</sup> The abbreviations used are: Fe-S, iron-sulfur; DTT, dithiothreitol; AcnB, aconitase B.

## Biosynthesis of Iron-Sulfur Clusters by the Suf Machinery

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<sub>2</sub>, and SufC<sub>2</sub>D<sub>2</sub> 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 *iscU*, 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 FADH<sub>2</sub>, the flavin adenine nucleotide in its reduced form, which readily reacts with oxidants such as oxygen and ferric ions. We suggest that SufBCD uses FADH<sub>2</sub> 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 aconitase B was a gift from J. R. Guest (Norwich, UK).

**Strains and Growth Conditions**—(His)<sub>6</sub>-SufE and SufAB-CDSE were produced in *E. coli* TOP10 cells (Invitrogen); (His)<sub>6</sub>-SufS, SufB, SufC, and SufD as well as AcnB 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 μg/ml ampicillin or 30 μg/ml chloramphenicol at 37 °C to an A<sub>600</sub> = 0.5 before induction with 0.2% L-arabinose (w/v) or 0.5–1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were grown at 37 °C for a further 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 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, loaded onto a butyl-Sepharose FF hydrophobic column, and eluted with a linear gradient of 1–0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. SufE-(His)<sub>6</sub>,(His)<sub>6</sub>-SufS, SufB, SufC, SufD, and AcnB 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 (Micro-mass, 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 SufBC<sub>2</sub>D 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 FADH<sub>2</sub> was calculated from the absorbance of free oxidized FAD at 450 nm ( $\epsilon = 11,300 \text{ M}^{-1}\cdot\text{cm}^{-1}$ ).

Determination of the dissociation constant for the SufBC<sub>2</sub>D-FADH<sub>2</sub> 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 SufBC<sub>2</sub>D or in a control sample without protein. After incubation, unbound flavin was separated from SufBC<sub>2</sub>D by filtration with a 100,000 molecular weight cut-off Vivaspin concentrator (Sartorius). The concentrations of SufBC<sub>2</sub>D-FADH<sub>2</sub> ([FAD<sub>bound</sub>]) and apo-SufBC<sub>2</sub>D were determined according to the calculated concentrations of unbound and total FADH<sub>2</sub> and the known amount of total apo-SufBC<sub>2</sub>D. The protein-bound FADH<sub>2</sub> as a function of unbound FADH<sub>2</sub> in solution was then plotted. The data have been fitted by a saturation hyperbola according to Equation 1,

$$[\text{FAD}_{\text{bound}}] = [\text{SufBC}_2\text{D}_{\text{tot}}] \cdot [\text{FAD}_{\text{free}}]/K_d + [\text{FAD}_{\text{free}}] \quad (\text{Eq. 1})$$

**Cofactor Analysis**—Anaerobically purified SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D**—Purified SufBC<sub>2</sub>D (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(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> or <sup>57</sup>FeCl<sub>3</sub> 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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 Fridovitch (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] SufBC<sub>2</sub>D (0.3 nmol) (providing 4 eq of iron and sulfur atoms/apoAcnB) or 5-fold molar excess of Fe<sup>2+</sup> and S<sup>2-</sup> 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 SufBC<sub>2</sub>D-FADH<sub>2</sub> Complex**—SufBC<sub>2</sub>D-FADH<sub>2</sub> was incubated anaerobically with ferric citrate (10 molar excess with regard to SufBC<sub>2</sub>D) in 50 mM Tris-HCl, pH 7.5. Either the oxidation of the flavin at 450 nm or the reduction of Fe<sup>3+</sup> from ferric citrate was followed over time. Reduction and mobilization of iron were monitored after addition to the reaction mixture of the Fe<sup>2+</sup>-chelator ferrozine (30–50 molar excess/SufBC<sub>2</sub>D) via the increase of the absorbance at 562 nm corresponding to the formation of a ferrozine-Fe<sup>2+</sup> complex (ε = 27,900 M<sup>-1</sup>·cm<sup>-1</sup>). When CyaY was used as ferric iron source, 10 μM of the intermediate form of CyaY-Fe<sup>3+</sup>, containing an average of 20 iron atoms/monomer (34), was incubated with 10 μM of SufBC<sub>2</sub>D-FADH<sub>2</sub> (0.93 FADH<sub>2</sub>/complex). Ferrozine was added, and formation of the ferrozine-Fe<sup>2+</sup> 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 SufBC<sub>2</sub>D underestimates the concentration by a factor of 1.14, as determined

by the quantitative amino acid analysis of the purified SufBC<sub>2</sub>D 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 aerobically 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 SufBC<sub>2</sub>D 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/SufBC<sub>2</sub>D ratio by quantifying the SufC-containing fractions that dissociate from the SufBC<sub>2</sub>D complex. This allowed us to show that 75% of the complex exists in the SufBC<sub>2</sub>D form, the rest being in the SufBCD form derived from dissociation of one SufC molecule from the SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D. All attempts to generate such a SufBC<sub>2</sub>D complex (or a SufBCD complex) by incubating a mixture of SufB, SufC, and SufD, purified separately, failed. Only B<sub>2</sub>, B<sub>2</sub>C, and C<sub>2</sub>D<sub>2</sub> combinations were obtained emphasizing the absolute necessity to use the as-isolated complex for further characterizations.

**SufBC<sub>2</sub>D Complex Binds a [4Fe-4S] Cluster after Reconstitution**—Chahal *et al.* (27) proposed recently that the SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D complex. The SufBC<sub>2</sub>D complex, reconstituted with iron and sulfur as described under “Experimental Procedures,” was indeed shown to contain 3.5 irons/complex 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]<sup>2+</sup> cluster with only one broad band at around 420 nm (Fig. 1). As shown for SufB (26), the cluster of SufBC<sub>2</sub>D is sensitive to oxygen (*t*<sub>1/2</sub>, 10 min) (supplemental Fig. 2). To accurately determine the nature of its Fe-S cluster, SufBC<sub>2</sub>D was reconstituted anaerobically with <sup>57</sup>Fe 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<sub>Q</sub> = 3.50 mm/s) consistent with high spin Fe<sup>2+</sup>. This species was assigned to non-specifically bound ferrous ions. The Mössbauer spectrum of the reconstituted SufBC<sub>2</sub>D-[<sup>57</sup>Fe-S] complex (Fig. 2b), containing

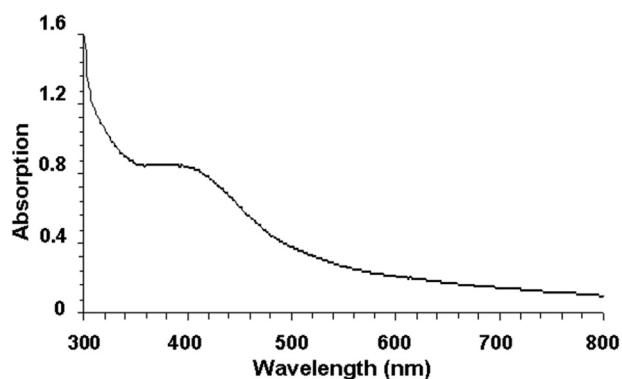


FIGURE 1. UV-visible spectrum of reconstituted SufBC<sub>2</sub>D-[Fe-S] complex. SufBC<sub>2</sub>D-[Fe-S] complex (50  $\mu$ M, 3.8 iron and 5 sulfur/complex) in 50 mM Tris-HCl, pH 8.

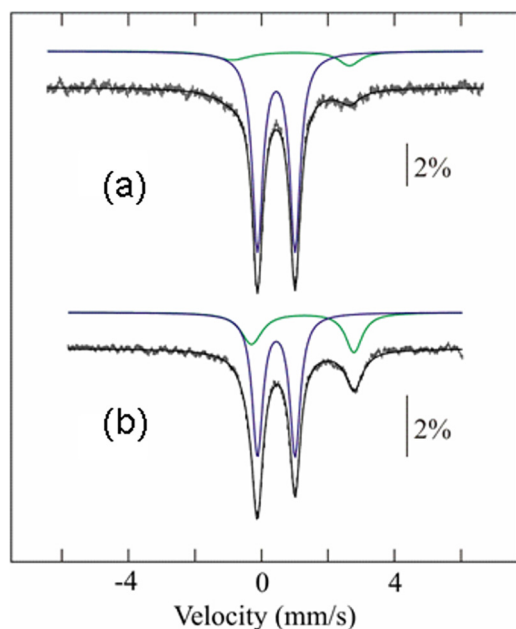


FIGURE 2. Mössbauer spectra of SufB-[<sup>57</sup>Fe-S] and SufBC<sub>2</sub>D-[<sup>57</sup>Fe-S]. *a*, SufB (125  $\mu$ M, 3.8 iron and 4.9 sulfur/monomer) and *b*, SufBC<sub>2</sub>D complex (210  $\mu$ M, 3.1 iron and 5 sulfur/complex) in 50 mM Tris-HCl, pH 7.5. Spectra were recorded at 4.2 K in a magnetic field of 50 milliteslas applied perpendicular to the  $\gamma$  beam. The black solid lines are composite quadrupole doublet simulations with the parameters cited in the text. The colored solid lines are contributions from [4Fe-4S]<sup>2+</sup> clusters (blue) and nonspecifically bound Fe<sup>2+</sup> (green).

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]<sup>2+</sup> cluster in SufB, and a minor doublet (30% of total iron) ( $\delta = 1.24$  mm/s and  $\Delta E_Q = 3.08$  mm/s) also assigned to nonspecifically bound Fe<sup>2+</sup>. 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 SufBC<sub>2</sub>D (supplemental Fig. 3). The quantification of bound iron and sulfur together with these spectroscopic analyses demonstrate clearly that the SufBC<sub>2</sub>D complex assembles a [4Fe-4S]<sup>2+</sup> cluster during *in vitro* reconstitution, as does SufB, and thus suggest that this cluster is located on the SufB component within the SufBC<sub>2</sub>D complex.

**Anaerobically Purified SufBC<sub>2</sub>D Contains a Protein-bound Flavin Adenine Dinucleotide (FADH<sub>2</sub>)**—Recently, we were able to isolate the SufA protein with its Fe-S cluster by strict adher-

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 SufBC<sub>2</sub>D complex could be isolated with its metal cofactor when purified under similar conditions. After expression of the *suf* operon using the pGSO164 plasmid, SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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. 3*a*). 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 SufBC<sub>2</sub>D (data not shown). The same supernatant was analyzed spectrophotometrically. Using an extinction coefficient of 11.3  $\text{mm}^{-1}\cdot\text{cm}^{-1}$  for free FAD (35), we calculated a ratio of about 0.8 mol of FAD per mol of SufBC<sub>2</sub>D. From these analyses, it can be concluded that the anaerobically purified SufBC<sub>2</sub>D complex binds 1 eq of visible light-transparent FADH<sub>2</sub>, 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 SufBC<sub>2</sub>D 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. 3*b*, 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. 3*b*, 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 an  $S = 1/2$  signal centered at  $g = 2.003(7)$  (supplemental Fig. 4). Interestingly, the air-dependent oxidation of the reduced flavin in SufBC<sub>2</sub>D is slower ( $t_{1/2} = 4.5$  min) than that of free reduced flavin in solution ( $t_{1/2} = 0.5$  min), as shown in Fig. 3*c*. This is consistent with the flavin being protein-bound and thus partially protected from exogenous reactants.

**Flavin-binding Properties of SufBC<sub>2</sub>D**—To analyze the flavin-binding properties of apo-SufBC<sub>2</sub>D, the complex was co-incubated with a 5 molar excess of FAD either under aerobic conditions or anaerobically in the presence of an excess of DTT while irradiating to obtain FADH<sub>2</sub>. After separation of unbound flavin on a NAP-25 column, the reconstituted SufBC<sub>2</sub>D protein was assayed for its flavin content as described under “Experimental Procedures.” Between 0.8 and 1 mol of

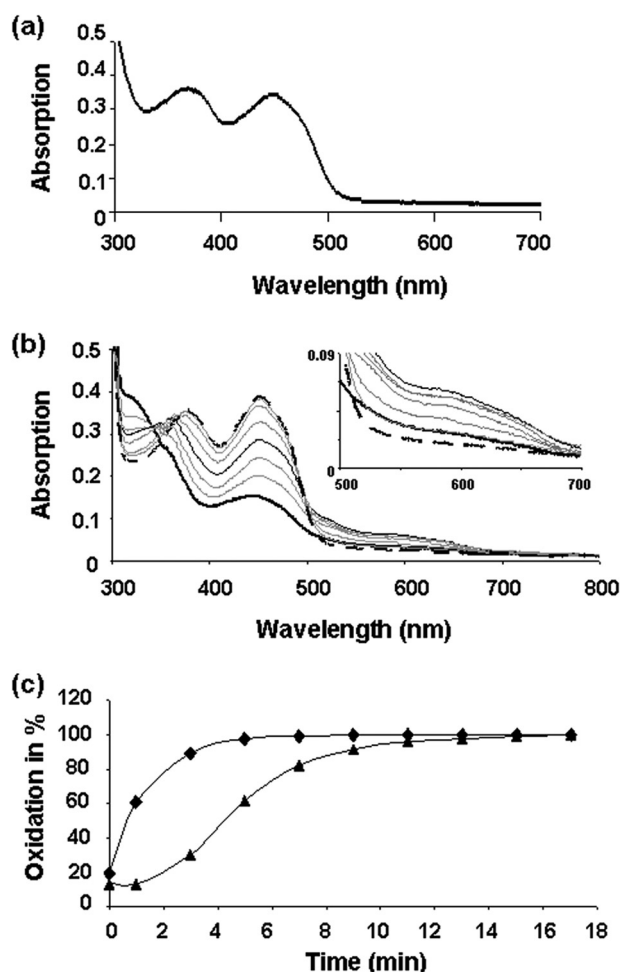


FIGURE 3. **Properties of SufBC<sub>2</sub>D-FADH<sub>2</sub>.** (a) UV-visible spectra of anaerobically purified SufBC<sub>2</sub>D complex after exposure to air; (b) kinetic oxidation of SufBC<sub>2</sub>D complex ( $t = 1$  min (bold black line) and  $t = 3, 5,$  and  $7$  min (black line);  $t = 10, 15, 20,$  and  $30$  min (dashed bold black line)) showing formation of the neutral semiquinone species (FADH<sup>•</sup>) at 590 nm (maximum formation at 7 min (black line)). Inset, enlargement of the 500–700 nm region. c, comparison of the air oxidation kinetic of the flavin within the SufBC<sub>2</sub>D complex (21  $\mu$ M flavin) (■) with free reduced flavin in solution (16  $\mu$ M) (▲).

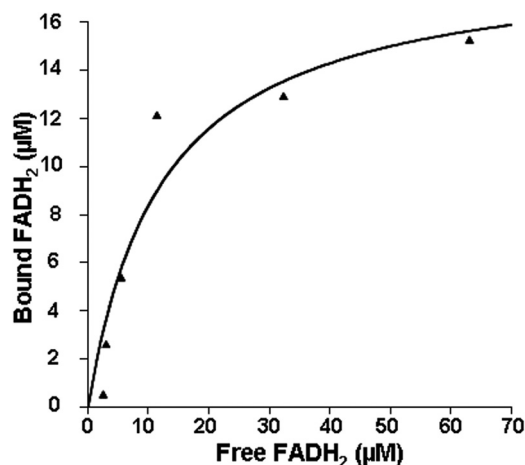
FAD per mol of SufBC<sub>2</sub>D could be reproducibly determined in the case of the anaerobic reaction mixture containing FADH<sub>2</sub> and SufBC<sub>2</sub>D, whereas no protein-bound flavin could be detected in the case of the aerobic FAD/SufBC<sub>2</sub>D incubation mixture clearly showing that only FADH<sub>2</sub> binds to the protein complex. Accordingly, when the FADH<sub>2</sub>-containing protein was exposed to air and then desalted on a NAP-25 column, flavin could not be detected anymore on SufBC<sub>2</sub>D. During incubation of SufBC<sub>2</sub>D with FMNH<sub>2</sub> or reduced riboflavin instead of FADH<sub>2</sub>, less flavin remained protein-bound after the desalting step (0.4 and 0.1 mol/mol of SufBC<sub>2</sub>D, 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 SufBC<sub>2</sub>D 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 oxida-

tion 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 SufBC<sub>2</sub>D 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 FADH<sub>2</sub>, albeit to a lesser extent (0.1–0.3 mol of FADH<sub>2</sub> per mol of protein, respectively). SufC alone and SufD were shown not to bind the flavin. Thus, only the whole SufBC<sub>2</sub>D 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 FADH<sub>2</sub>.

To determine the dissociation constant ( $K_d$ ) for the binding of FADH<sub>2</sub> to SufBC<sub>2</sub>D, an ultrafiltration assay was used as described under “Experimental Procedures.” The flavin-free apo-SufBC<sub>2</sub>D complex (20  $\mu$ M) was co-incubated at 18 °C anaerobically with different concentrations of FADH<sub>2</sub> (0–200  $\mu$ M) obtained by photo-induced reduction of FAD. After co-incubation, the samples were transferred to a 100,000 molecular weight cutoff concentrator, and unbound FADH<sub>2</sub> was separated from SufBC<sub>2</sub>D by ultrafiltration. As a control, the same experiment was performed in the absence of apo-SufBC<sub>2</sub>D. The flavin content of the flow-through fraction of the samples incubated in the presence of apo-SufBC<sub>2</sub>D was determined as described under “Experimental Procedures.” The amount of complex (SufBC<sub>2</sub>D-FADH<sub>2</sub>) and the concentrations of free SufBC<sub>2</sub>D were determined according to the calculated concentrations of free and total FADH<sub>2</sub> and the known amount of total apo-SufBC<sub>2</sub>D. Under these conditions, a dissociation constant of 12  $\mu$ M was determined (Fig. 4).

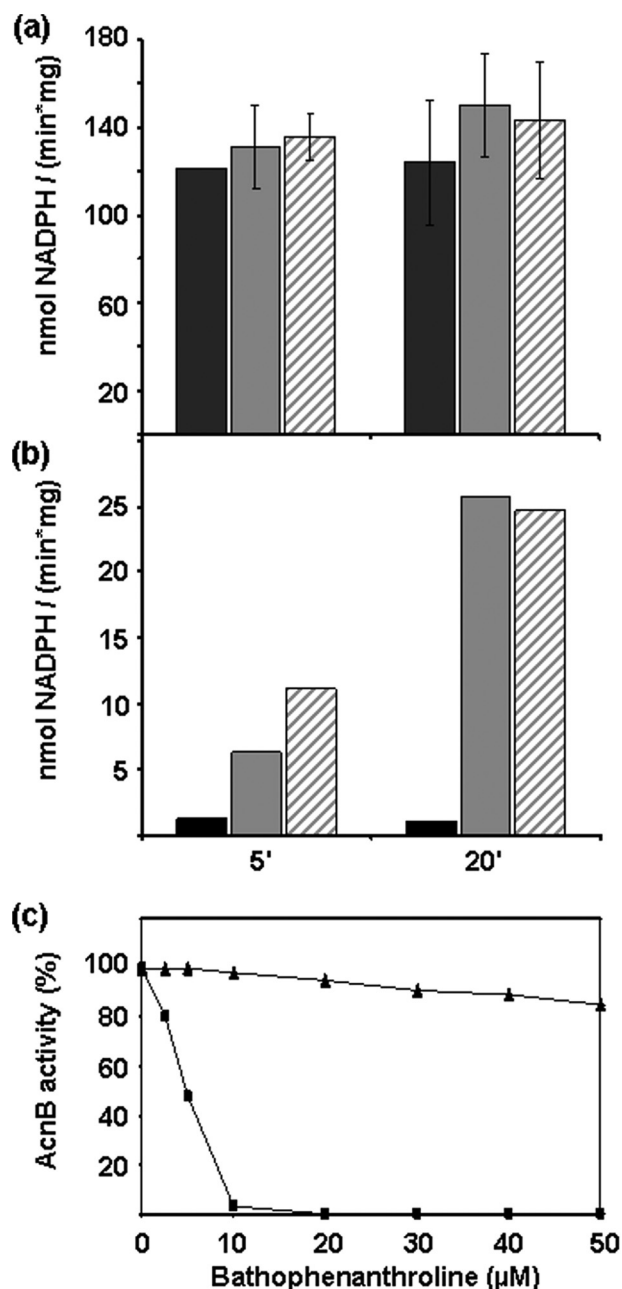
**Bound Flavin Is Not Required for SufBC<sub>2</sub>D [4Fe-4S] Cluster Transfer—Aconitase B (AcnB),** an enzyme containing a [4Fe-4S] cluster in its active form, was used as a target for Fe-S transfer experiments. Both forms of holo-SufBC<sub>2</sub>D ([Fe-S] and [Fe-S] + FADH<sub>2</sub>) were used as a potential source of clusters. In a typical experiment, an excess of holo-SufBC<sub>2</sub>D (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-SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D-[Fe-S] and SufBC<sub>2</sub>D-[Fe-S] + FADH<sub>2</sub> even in the absence of DTT (Fig. 5b). Thus, these data show for the first time a cluster transfer from SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D-dependent and the chemical aconitase activation. Indeed, addition of increasing concentrations of a strong iron chelator,



**FIGURE 4. Binding affinity of FADH<sub>2</sub> to the apo-SufBC<sub>2</sub>D complex.** The apo-SufBC<sub>2</sub>D (20 μM) complex was incubated anaerobically with different concentrations of FADH<sub>2</sub> (0–200 μM) obtained by photo-induced reduction of FAD. A molecular weight cutoff concentrator was used to separate unbound FADH<sub>2</sub> from protein-bound FADH<sub>2</sub>, and the flavin content of each was determined outside the glove box after oxidation, heat denaturation, centrifugation, and UV-visible analysis of the supernatant. The concentration of SufBC<sub>2</sub>D-FADH<sub>2</sub> ([FAD<sub>bound</sub>]) and that of apo-SufBC<sub>2</sub>D was determined according to the calculated concentrations of unbound and total FADH<sub>2</sub> and the known amount of total apo-SufBC<sub>2</sub>D. The protein-bound FADH<sub>2</sub> as a function of unbound FADH<sub>2</sub> in solution was then plotted. The data have been fitted by a saturation hyperbola according to Equation 1.

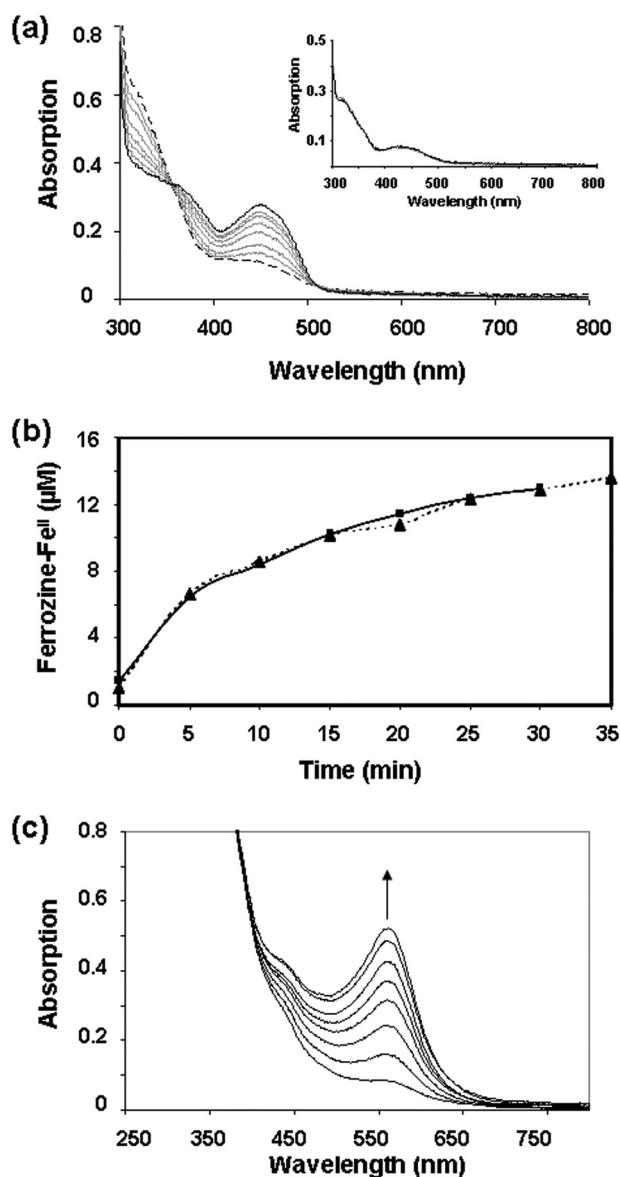
bathophenanthroline, to the standard reaction mixture had very little effect on the Fe-S cluster transfer from SufBC<sub>2</sub>D to AcnB, although it completely inhibited the chemical reconstitution of the aconitase. These data, the lack of requirement for DTT and inhibition by a chelator, thus show that cluster transfer from SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D-FADH<sub>2</sub> Complex**—Reduced flavins are excellent ferric iron-reducing agents (36, 37). We thus investigated the potential of the reduced flavin of the SufBC<sub>2</sub>D 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. SufBC<sub>2</sub>D-FADH<sub>2</sub> complex (30 μM, 1 FADH<sub>2</sub>/complex) was incubated anaerobically with ferric citrate (300 μM), and electron transfer from FADH<sub>2</sub> 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, SufBC<sub>2</sub>D-FADH<sub>2</sub> complex was incubated with buffer instead of ferric citrate. A time-dependent ( $t_{1/2}$  = 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 FADH<sub>2</sub> cofactor using ferrozine in excess as an effective Fe<sup>2+</sup> chelator and an Fe<sup>2+</sup> 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 SufBC<sub>2</sub>D-



**FIGURE 5. Iron-sulfur cluster transfer from SufBC<sub>2</sub>D to AcnB.** Holo-SufBC<sub>2</sub>D complex (0.3 nmol), [Fe-S] (gray bars), or [Fe-S] + FADH<sub>2</sub> (hatched bars), was co-incubated in 10 μl of 50 mM Tris-HCl, pH 7.6, with (a) and without (b) 5 mM DTT with apo-AcnB (0.2 nmol). After 5 and 20 min of incubation, the activity of AcnB was measured by monitoring the absorption at 340 nm. For this, a mixture of 1.2 mM MnCl<sub>2</sub>, 25 mM citrate, 0.5 unit of isocitrate dehydrogenase, and 0.25 mM NADP<sup>+</sup> was added to the protein mixture in a final volume of 100 μl. As a control, apo-AcnB was incubated with a 5 molar excess of iron and sulfide, and the activity was assayed (black bars). c, intact cluster transfer from SufBC<sub>2</sub>D to AcnB. Apo-AcnB (0.2 nmol) was incubated anaerobically with either [4Fe-4S] SufBC<sub>2</sub>D complex (0.3 nmol) (▲) or 5-fold molar excess of Fe<sup>2+</sup> and S<sup>2-</sup> (■) in 100 μl of 50 mM Tris-HCl, pH 7.6, 5 mM DTT with increasing amounts of bathophenanthroline, and the AcnB activity was measured after 20 min of incubation.

FADH<sub>2</sub> complex containing 0.74 FADH<sub>2</sub>/complex, we observed a time-dependent formation of the ferrozine-Fe<sup>2+</sup> complex ( $t_{1/2}$  = ~5 min) indicating reduction of ferric citrate by FADH<sub>2</sub> and mobilization of Fe<sup>2+</sup> by ferrozine (Fig. 6b). At the end of the reaction, about 14 μM of ferrozine-Fe<sup>2+</sup> complex



**FIGURE 6. Ferric reductase activity of the SufBC<sub>2</sub>D-FADH<sub>2</sub> complex.** *a*, time-dependent oxidation ( $t = 0$ –65 min) of the flavin in the SufBC<sub>2</sub>D-FADH<sub>2</sub> complex (30  $\mu$ M) in the presence of ferric citrate (300  $\mu$ M) in 50 mM Tris-HCl, pH 7.5. The light absorption spectrum of the solution in the 300–800 nm range is recorded at 5, 10, 20, 30, 40, 50, and 65 min, and the amount of oxidized flavin was determined from the absorption at 450 nm. *Inset*, control experiment showing the time-dependent oxidation of the flavin in the SufBC<sub>2</sub>D-FADH<sub>2</sub> complex (30  $\mu$ M) in the absence of ferric citrate in 50 mM Tris-HCl, pH 7.5 buffer ( $t = 0$  and 65 min). *b*, reduction of ferric citrate (100  $\mu$ M) by the SufBC<sub>2</sub>D-FADH<sub>2</sub> complex (10  $\mu$ M, 0.74 FADH<sub>2</sub>/complex) in the presence of the Fe<sup>2+</sup>-chelator ferrozine (300  $\mu$ M) in the presence (■) and absence (▲) of ATP. The amount of ferrozine-Fe<sup>2+</sup> complex ( $\mu$ M), determined from the absorption at 562 nm, was plotted as a function of time (min). *c*, reduction of the CyaY-Fe<sup>3+</sup> protein (10  $\mu$ M, 20 iron/monomer) by the SufBC<sub>2</sub>D-FADH<sub>2</sub> complex (10  $\mu$ M with 9.3  $\mu$ M of FADH<sub>2</sub>) in the presence of ferrozine (1 mM). The light absorption spectrum of the solution was recorded every 10 min at 562 nm. The arrow indicates the increased formation of the ferrozine-Fe<sup>2+</sup> complex.

were formed corresponding to 95% of reducing equivalents present on the SufBC<sub>2</sub>D complex under the FADH<sub>2</sub> form (14.8  $\mu$ M). Addition of ATP/MgCl<sub>2</sub> had neither an effect on the rate nor on the yield of the reaction (Fig. 6*b*). For comparison, free FADH<sub>2</sub> (30  $\mu$ M) reacted with ferric citrate (300  $\mu$ M) 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<sub>2</sub>D-FADH<sub>2</sub> complex (9.3  $\mu$ M FADH<sub>2</sub>) was incubated anaerobically with 10  $\mu$ M of the CyaY-Fe<sup>3+</sup>. The CyaY-Fe<sup>3+</sup> protein that we used contained about 20 iron/monomer protein (34). The reduction of Fe<sup>3+</sup> from CyaY to Fe<sup>2+</sup> and its release was monitored by UV-visible spectroscopy using 1 mM ferrozine. We also observed a time-dependent formation of the ferrozine-Fe<sup>2+</sup> complex, as shown by the increase of the absorption at 562 nm (Fig. 6*c*) and the concomitant oxidation of the flavin (increase of the absorption band at 450 nm) indicating reduction of CyaY-Fe<sup>3+</sup> by the reduced FADH<sub>2</sub> and mobilization of Fe<sup>2+</sup> 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  $\mu$ M Fe<sup>2+</sup>-ferrozine complex. This matches the amount of reducing equivalents available within the complex under the form of the reduced flavin FADH<sub>2</sub>. No reduction of CyaY-Fe<sup>3+</sup> could be observed in a reaction mixture containing apo-SufBC<sub>2</sub>D, CyaY-Fe<sup>3+</sup>, and ferrozine (supplemental Fig. 5). Cytochrome *c* was also shown to be an oxidant of the protein-bound reduced flavin (FADH<sub>2</sub>) (supplemental Fig. 6).

## DISCUSSION

The results presented here provide a thorough biochemical characterization of the SufBC<sub>2</sub>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<sub>2</sub>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<sub>2</sub> dimer, but not that of the SufB<sub>2</sub> dimer, have been determined, with SufD<sub>2</sub> showing a novel fold in which 20  $\beta$ -strands are assembled into a right-handed parallel  $\alpha$ -helix participating in dimerization (23, 28, 40). Clearly, formation of these dimers (SufB<sub>2</sub> and SufD<sub>2</sub>) under *in vitro* conditions makes it difficult for SufB, SufC, and SufD to assemble as a SufBC<sub>2</sub>D complex. Furthermore, it seems that SufB and SufD compete for SufC binding because some SufB<sub>2</sub>C and SufC<sub>2</sub>D<sub>2</sub> 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<sub>2</sub>C<sub>2</sub> 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<sub>2</sub>D complex (Fig. 7).

The SufC<sub>2</sub>D<sub>2</sub> 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

## Biosynthesis of Iron-Sulfur Clusters by the Suf Machinery

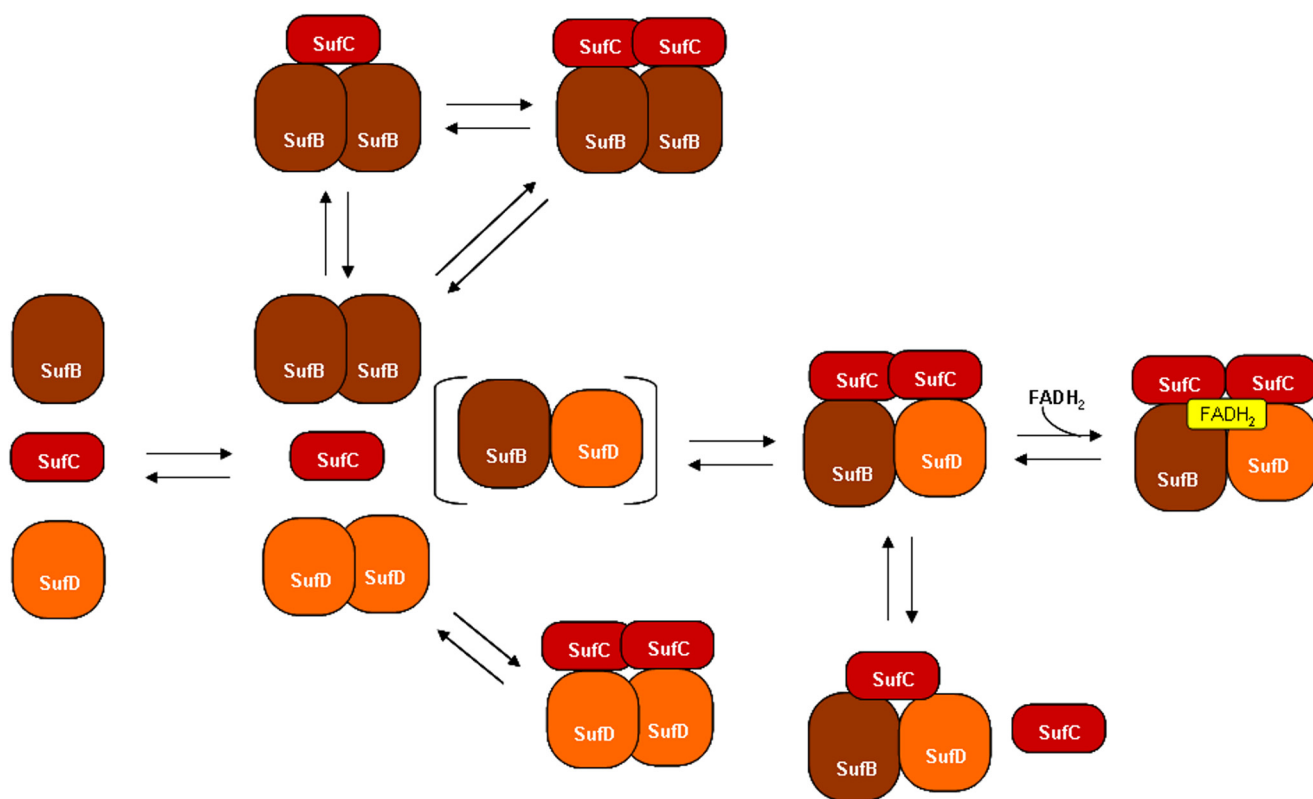


FIGURE 7. **Complexes formed upon incubation of SufB, SufC, and SufD proteins (from this study and from data obtained in Refs. 24, 29).** Only *in vivo* a SufBC<sub>2</sub>D stoichiometry may be obtained presumably through an intermediate SufBD, not observed *in vitro*.

the SufD homodimer. Because the structures of SufB and SufD were predicted to share a striking similarity, especially in the regions corresponding to the  $\beta$ -helix core domain and the C-terminal helical domain, which are involved in the inter-subunit interactions, the SufBC<sub>2</sub>D complex is likely to share a common architecture with the SufC<sub>2</sub>D<sub>2</sub> complex with SufB interacting with both SufD and SufC (Fig. 7).

The second property of the SufBC<sub>2</sub>D 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]<sup>2+</sup> cluster identical to the one found in the reconstituted SufB protein, so it is also tempting to suggest that in the SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D complex.

Even though the cluster in SufB or in SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D complex to SufA but not from SufA to SufBC<sub>2</sub>D. SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D complex being the scaffold within the SUF system. This function would explain why under normal growth conditions SufBC<sub>2</sub>D contains no or little cluster because it is designed to bind it only transiently.

Finally, a major discovery is the finding that SufBC<sub>2</sub>D is a flavin-binding protein. Characterization of the flavin-protein interaction clearly established that the SufBC<sub>2</sub>D complex could bind 1 eq of reduced flavin adenine dinucleotide, FADH<sub>2</sub>. 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 SufBC<sub>2</sub>D-FADH<sub>2</sub> complex within cells is very likely because the anaerobically as-isolated protein contains FADH<sub>2</sub> exclusively even though SufBC<sub>2</sub>D has the potential to bind reduced FMN and riboflavin



MSRNTAETDDVKTWTGGPLNYKEGFFTLQATDELAKGINEEVVRAISAKRNEPEWMLFRLNAYRAWLEMEEPHW  
 LKAHYDKLNYQDYSYYSAPSCGNCDDTCASEP**PGAVQQTGAN**AFLSKEVEAAFEQLGVPVREGKEVAVDALFDSVS  
 VATTYREKLAEGQIIIFCSFGEAIHDHPELVVRKYLGTVVPGNDNF FAALNAAVASDGTFIYVPGVRCPEMLSTYF  
 RINAETKQGF**ERTILVA**EDSVSYIEGCSAPVRDSYQLHAAVVEVIHKNAEVKYSTVQNWFGPDNNT**GGIL**NF  
 VTKRALCEGENSKMSWTQSETGSAITWKYPSCI LRGDNSIGEFYSVALTSGHQQADTGTKMIHIGKNTKSTIISK  
 GISAGHSQNSYRGLVKIMPTATNARNF TQCDMSLIGANC GAHTFPYVECRNNSAQLHEATTSRIGEDQLFYCLQ  
**RGI**SEEDAISMI VNGFCKDVFSELPEFAVEAQKLLAISLEHSVG

FIGURE 8. **FAD-binding motifs in SufB from *E. coli***. These motifs that were originally found in the *p*-cresol-methylhydroxylase family (41) are depicted in boxes (conserved residues are in boldface).

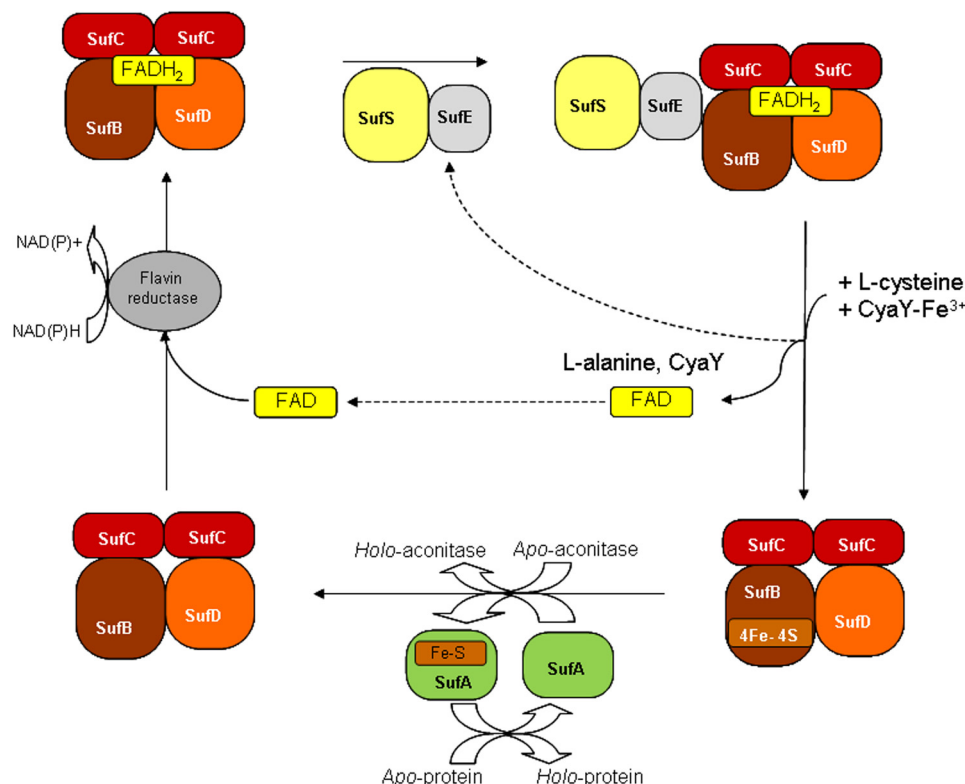


FIGURE 9. **Current view of the mechanism of Fe-S cluster assembly mediated by the Suf system.** In a first step, the SufBC<sub>2</sub>D complex binds 1 eq of FADH<sub>2</sub>, obtained via the action of a NAD(P)H:flavin oxidoreductase or flavin reductase. SufS and SufE transiently bind to the SufBC<sub>2</sub>D-FADH<sub>2</sub> complex. The whole complex reacts with cysteine to generate persulfides on SufB, and at the same time, the FADH<sub>2</sub> cofactor reduces the iron of a ferric iron source CyaY-Fe<sup>3+</sup>. The resulting ferrous ions react with persulfides to generate the [4Fe-4S] holo-form of SufB. This holo-form of SufBC<sub>2</sub>D then transfers its cluster to an apoprotein either directly or via SufA. During this process, the flavin is oxidized and released in solution where it can be reduced again by a flavin reductase.

to some extent *in vitro*. The site of flavin binding within the SufBC<sub>2</sub>D complex is suggested to reside on SufB because the SufB protein in the absence of SufC and SufD binds a small amount of FADH<sub>2</sub> *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)<sub>6</sub>G(A)XN motif, which forms a loop that binds to the adenine ring and compensates for the negative charge of the FAD molecule; the R(X)<sub>6</sub>EXXYXXXXXG(X)<sub>8</sub>Y 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 O<sub>2</sub> atom of the isoalloxazine ring and O<sub>3</sub> 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 FADH<sub>2</sub>.

It is worth noting that although SufB alone is able to bind small amounts of flavin, only the whole SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D-FADH<sub>2</sub> 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D plays a role in oxygen activation and oxidation of a substrate. However, it may suggest that SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D? Our results exclude a role of the flavin in the transfer of the clusters from SufBC<sub>2</sub>D 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 FADH<sub>2</sub> cofactor of SufBC<sub>2</sub>D 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 ( $E = -250$  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<sup>2+</sup> only very weakly (34). Reduced flavins display comparably low redox potentials, and thus, even though we have not measured that of the SufBC<sub>2</sub>D-FADH<sub>2</sub> 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D 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 SufBC<sub>2</sub>D complex binds 1 eq of FADH<sub>2</sub>. 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 SufBC<sub>2</sub>D-FADH<sub>2</sub> 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 FADH<sub>2</sub> 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 SufBC<sub>2</sub>D 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

1. Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005) *Annu. Rev. Biochem.* **74**, 247–281
2. Fontecave, M., and Ollagnier-de-Choudens, S. (2008) *Arch. Biochem. Biophys.* **474**, 226–237
3. Ayala-Castro, C., Saini, A., and Outten, F. W. (2008) *Microbiol. Mol. Biol. Rev.* **72**, 110–125
4. Tokumoto, U., Kitamura, S., Fukuyama, K., and Takahashi, Y. (2004) *J. Biochem.* **136**, 199–209
5. Lill, R., and Mühlenhoff, U. (2008) *Annu. Rev. Biochem.* **77**, 669–700
6. Balk, J., and Lobréaux, S. (2005) *Trends Plant Sci.* **10**, 324–331
7. Huet, G., Daffé, M., and Saves, I. (2005) *J. Bacteriol.* **187**, 6137–6146
8. Outten, F. W., Djaman, O., and Storz, G. (2004) *Mol. Microbiol.* **52**, 861–872
9. Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) *EMBO J.* **22**, 427–437
10. Ranquet, C., Ollagnier-de-Choudens, S., Loiseau, L., Barras, F., and Fontecave, M. (2007) *J. Biol. Chem.* **282**, 30442–30451
11. Mihara, H., Fujii, T., Kato, S., Kurihara, T., Hata, Y., and Esaki, N. (2002) *J. Biochem.* **131**, 679–685
12. Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) *J. Biochem.* **127**, 559–567
13. Ollagnier-de-Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E., and Fontecave, M. (2003) *FEBS Lett.* **555**, 263–267
14. Outten, F. W., Wood, M. J., Munoz, F. M., and Storz, G. (2003) *J. Biol. Chem.* **278**, 45713–45719
15. Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) *J. Biol. Chem.* **278**, 38352–38359
16. Lu, J., Yang, J., Tan, G., and Ding, H. (2008) *Biochem. J.* **409**, 535–543
17. Ollagnier-de-Choudens, S., Nachin, L., Sanakis, Y., Loiseau, L., Barras, F., and Fontecave, M. (2003) *J. Biol. Chem.* **278**, 17993–18001
18. Ollagnier-de-Choudens, S., Sanakis, Y., and Fontecave, M. (2004) *J. Biol. Inorg. Chem.* **9**, 828–838
19. Sendra, M., Ollagnier de Choudens, S., Lascoux, D., Sanakis, Y., and Fontecave, M. (2007) *FEBS Lett.* **581**, 1362–1368
20. Gupta, V., Sendra, M., Naik, S. G., Chahal, H. K., Huynh, B. H., Outten, F. W., Fontecave, M., and Ollagnier de Choudens, S. (2009) *J. Am. Chem. Soc.* **131**, 6149–6153
21. Vinella, D., Brochier-Armanet, C., Loiseau, L., Talla, E., and Barras, F. (2009) *PLoS Genet.* **5**, e1000497
22. Takahashi, Y., and Tokumoto, U. (2002) *J. Biol. Chem.* **277**, 28380–28383
23. Kitaoka, S., Wada, K., Hasegawa, Y., Minami, Y., Fukuyama, K., and Takahashi, Y. (2006) *FEBS Lett.* **580**, 137–143
24. Petrovic, A., Davis, C. T., Rangachari, K., Clough, B., Wilson, R. J., and Eccleston, J. F. (2008) *Protein Sci.* **17**, 1264–1274
25. Eccleston, J. F., Petrovic, A., Davis, C. T., Rangachari, K., and Wilson, R. J.

- (2006) *J. Biol. Chem.* **281**, 8371–8378
26. Layer, G., Gaddam, S. A., Ayala-Castro, C. N., Ollagnier-de Choudens, S., Lascoux, D., Fontecave, M., and Outten, F. W. (2007) *J. Biol. Chem.* **282**, 13342–13350
  27. Chahal, H. K., Dai, Y., Saini, A., Ayala-Castro, C., and Outten, F. W. (2009) *Biochemistry* **48**, 10644–10653
  28. Badger, J., Sauder, J. M., Adams, J. M., Antonysamy, S., Bain, K., Bergseid, M. G., Buchanan, S. G., Buchanan, M. D., Batiyenko, Y., Christopher, J. A., Emtage, S., Eroshkina, A., Feil, I., Furlong, E. B., Gajiwala, K. S., Gao, X., He, D., Hendle, J., Huber, A., Hoda, K., Kearins, P., Kissinger, C., Laubert, B., Lewis, H. A., Lin, J., Loomis, K., Lorimer, D., Louie, G., Maletic, M., Marsh, C. D., Miller, I., Molinari, J., Muller-Dieckmann, H. J., Newman, J. M., Noland, B. W., Pagarigan, B., Park, F., Peat, T. S., Post, K. W., Radojicic, S., Ramos, A., Romero, R., Rutter, M. E., Sanderson, W. E., Schwinn, K. D., Tresser, J., Winhoven, J., Wright, T. A., Wu, L., Xu, J., and Harris, T. J. (2005) *Proteins* **60**, 787–796
  29. Wada, K., Sumi, N., Nagai, R., Iwasaki, K., Sato, T., Suzuki, K., Hasegawa, Y., Kitaoka, S., Minami, Y., Outten, F. W., Takahashi, Y., and Fukuyama, K. (2009) *J. Mol. Biol.* **387**, 245–258
  30. Jordan, P. A., Tang, Y., Bradbury, A. J., Thomson, A. J., and Guest, J. R. (1999) *Biochem. J.* **344**, 739–746
  31. Song, S. H., Dick, B., Penzkofer, A., and Hegemann, P. (2007) *J. Photochem. Photobiol. B* **87**, 37–48
  32. Mickowska, B., Dulinski, R., and Kozik, A. (2000) *J. Biochem. Biophys. Methods* **44**, 95–107
  33. Gardner, P. R., and Fridovich, I. (1992) *J. Biol. Chem.* **267**, 8757–8763
  34. Layer, G., Ollagnier-de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) *J. Biol. Chem.* **281**, 16256–16263
  35. Massey, V., and Palmer, G. (1966) *Biochemistry* **5**, 3181–3189
  36. Hallé, F., and Meyer, J. M. (1992) *Eur. J. Biochem.* **209**, 621–627
  37. Fontecave, M., Covès, J., and Pierre, J. L. (1994) *Biometals* **7**, 3–8
  38. Coves, J., and Fontecave, M. (1993) *Eur. J. Biochem.* **211**, 635–641
  39. Bedekovics, T., Gajdos, G. B., Kispal, G., and Isaya, G. (2007) *FEMS Yeast Res.* **7**, 1276–1284
  40. Watanabe, S., Kita, A., and Miki, K. (2005) *J. Mol. Biol.* **353**, 1043–1054
  41. Dym, O., and Eisenberg, D. (2001) *Protein Sci.* **10**, 1712–1728
  42. Valtou, J., Filisetti, L., Fontecave, M., and Nivière, V. (2004) *J. Biol. Chem.* **279**, 44362–44369
  43. Galán, B., Díaz, E., Prieto, M. A., and García, J. L. (2000) *J. Bacteriol.* **182**, 627–636
  44. Chandramouli, K., Unciuleac, M. C., Naik, S., Dean, D. R., Huynh, B. H., and Johnson, M. K. (2007) *Biochemistry* **46**, 6804–6811
  45. Covès, J., Nivière, V., Eschenbrenner, M., and Fontecave, M. (1993) *J. Biol. Chem.* **268**, 18604–18609
  46. Imlay, J. A., and Linn, S. (1988) *Science* **240**, 1302–1309
  47. Andrews, S. C., Robinson, A. K., and Rodríguez-Quinones, F. (2003) *FEMS Microbiol. Rev.* **27**, 215–237
  48. Sancho, J. (2006) *Cell. Mol. Life Sci.* **63**, 855–864