SufA from *Erwinia chrysanthemi*

CHARACTERIZATION OF A SCAFFOLD PROTEIN REQUIRED FOR IRON-SULFUR CLUSTER ASSEMBLY*

Sandra Ollagnier-de Choudens‡, Laurence Nachini§, Yiannis Sanakis‡, Laurent Loiseau**, and Marc Fontecave‡ ‡‡

From the ¤Laboratoire de Chimie et Biochimie des Centres Rédox Biologiques, Département Réponse et Dynamique Cellulaire-Chimie Biochimie, CEACNRS/Université Joseph Fourier, UMR 5047, 17 Ave. des Martyrs, Grenoble 38054, cédex 09, France, the bICell and Molecular Biology, Göteborg University, Box 462, Göteborg 40530, Sweden, the cNational Center for Scientific Research, Demokritos, Institute of Materials Science, Ag. Paraskevi, Attiki 15310, Greece, the dDepartment of Biological Applications And Technologies, University of Ioannina, Ioannina 45110, Greece, and the e**Laboratoire de Chimie Bacterienne-CNRS, IBSM, 31 Chemin Joseph Aiguier, Marseille 13402, cédex 20, France

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SufA is a component of the recently discovered suf operon, which has been shown to play an important function in bacteria during iron-sulfur cluster biosynthesis and resistance to oxidative stress. The SufA protein from *Erwinia chrysanthemi*, a Gram-negative plant pathogen, has been purified to homogeneity and characterized. It is a homodimer with the ability to assemble rather labile [2Fe-2S] and [4Fe-4S] clusters as shown by Mössbauer spectroscopy. These clusters can be transferred to apoproteins such as ferredoxin or biotin synthase during a reaction that is not inhibited by bathophenanthroline, an iron chelator. Cluster assembly in these proteins is much more efficient when iron and sulfur are provided by holoSufA than by free iron sulfate and sodium sulfide. We propose the function of SufA is that of a scaffold protein for [Fe-S] cluster assembly and compare it to IscA, a member of the isc operon also involved in cluster biosynthesis in both prokaryotes and eukaryotes. Mechanistic and physiological implications of these results are also discussed.

Iron-sulfur [Fe-S] proteins play important roles in electron transfer, in redox and non-redox catalysis, in regulation, and as sensors within all living organisms, prokaryotes and eukaryotes (1, 2). The biosynthetic process by which defined proportions of iron and sulfur atoms are mobilized from their storage sources and combined in a controlled way to generate the various iron-sulfur cluster prosthetic groups is still far from understood. It requires a complex protein machinery that is only now becoming identified and characterized.

In the bacteria *Escherichia coli* and *Azotobacter vinelandii*, from which most of the available information is derived, this machinery has been found to be encoded by a highly conserved cluster of at least seven genes, *iscRSUA-hscBA-fdx*, also named the ISC (for iron-sulfur cluster) machinery (3, 4). Disruption of these genes generally results in both decreased cluster content and activity of many important [Fe-S] enzymes such as ascorbate or succinate dehydrogenase, whereas overexpression of the operon yields increased production of recombinant [Fe-S] proteins (5–9). Homologs to the proteins of the isc operon from *E. coli* have been found in the mitochondria of eukaryotes and, in yeast, shown by genetic experiments to play crucial roles in [Fe-S] cluster assembly (10–20). IscS proteins are pyridoxal-phosphate (PLP)-dependent cysteine desulfurases that catalyze the mobilization of sulfur atom from cysteine for incorporation into clusters (21, 22). IscU and IscA proteins are able to assemble transient and labile [2Fe-2S] and [4Fe-4S] clusters, as shown by Mössbauer and Raman resonance spectroscopy (23–31). These clusters can be rather efficiently transferred to apoferredoxins in vitro, and both IscU and IscA were proposed to function as scaffold proteins for mediating general [Fe-S] cluster assembly (23, 27, 30–32). HscA and HscB proteins are molecular chaperones (33). They both interact with IscU, and a complex has been detected in two-hybrid experiments between HscA and IscA (34–37). As a consequence they are supposed to be required for optimizing conformations that facilitate [Fe-S] cluster assembly and transfer from IscU/IscA to the target apoproteins. IscU but not IscA also makes a complex with IscS from which it directly gets the sulfur atoms required for [Fe-S] cluster synthesis (25, 38–40). Finally, both IscU and IscA can form complexes with Fdx, the product of another gene of the ISC machinery (23, 29, 30). Fdx is a [2Fe-2S] ferredoxin, thus suggesting that electron transfer steps are part of the cluster assembly process (41). Even though all of these proteins have been isolated in pure form and extensively characterized, the detailed mechanism by which they work together to incorporate an [Fe-S] cluster into an apoprotein is not known and is the subject of intense studies in several laboratories.

As part of our efforts to understand this important biological reaction, we studied the properties of the IscA protein from *E. coli* (23). There are many reasons for this. First, it is important to understand why most organisms contain both IscA and IscU proteins, with apparently similar functions. It is generally assumed that IscU proteins are the key players in the cluster assembly process, even though there is no clear evidence for it. In support to this notion is the observation that, in *Saccharomyces cerevisiae*, a knockout of both iscU homologs, *ISU1* and *ISU2*, is lethal, whereas a knockout of both iscA homologs, *ISA1* and *ISA2*, only results in retarded growth on non-fermentable carbon sources, accumulation of iron in mitochondria, and marked decrease in the activities of mitochondrial and cytosolic [Fe-S] enzymes (12, 15, 16, 42). On the other
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hand, it was recently shown, using an in vitro [Fe-S] cluster assembly assay, that extracts from *S. cerevisiae* mitochondria depleted in ISA1 displayed severely decreased activities during cluster assembly in biotin synthase, a [4Fe-4S] enzyme, when compared with wild-type extracts (43). A second indication for the importance of IscA-type proteins is the recent discovery of an additional operon in bacteria and intracellular parasites, named *suf*, also involved in iron and sulfur metabolism. The *suf* operon contains six genes, *sufABCDSE*, including an iscA homolog, *sufA*, and an iscS homolog, *sufS* (44–46).

In contrast to *S. cerevisiae*, the genes of the *isc* operon are necessary for optimal growth but are not essential for the viability of *E. coli* cells. In an *E. coli* mutant, in which the entire *isc* operon has been deleted, the activity of [Fe-S] proteins is only 2–10% of their activity in wild-type cells (46). This is presumably due to the presence of the *suf* genes as supported by the fact that overexpression of *suf operon* restores the growth phenotype and activity of [Fe-S] proteins in the mutant cells lacking all components of the ISC machinery. Furthermore, lethality was observed when both the *isc* and *suf* operons were inactivated (46).

Genetic experiments using Erwinia chrysanthemi, a Gram-negative pathogen, and *E. coli*, have recently provided more detailed information on the *suf* genes. In both species the *suf* operon was found to be under the Fe-dependent repressor Fur, and, in *E. coli* at least, it was also found to belong to the oxidative stress OxyR-dependent regulon (47–49). This suggests a specific function of the SUF machinery during repair of [Fe-S] clusters as a consequence of oxidative stress and a strong iron limitation. SufC appears to be the most critical protein in this system, because inactivation of *sufC* in *E. chrysanthemi* resulted in (i) decreased Fe uptake, (ii) increased accumulation of free intracellular iron levels, (iii) increased sensitivity to oxidative stress, (iv) delay of the induction of the transcriptional activator SoxS by SoxR, a homodimeric [2Fe-2S] redox-regulated transcription factor, (v) decrease activities of enzymes containing oxygen labile [Fe-S] clusters, under oxidative stress, and (vi) decreased virulence of *E. chrysanthemi* (44, 48, 50). Decreased iron uptake was shown to be a consequence of a decreased ability to obtain iron from ferri-siderophores, such as chrysobactin or ferroximin, possibly because [Fe-S] clusters in ferrisiderophore reductases are not correctly assembled in this mutant (44, 48, 50).

With the exception of SufS, the Suf proteins have been very little studied at the biochemical level so far (51–53). However, this system provides a unique tool to understand mechanisms of [Fe-S] cluster assembly, because it is simpler than the ISC system. Indeed, it does not contain equivalents of the ferredoxin protein (Fdx) and of the molecular chaperones (HscA and HscB). As discussed above we were intrigued by the absence of an IscU-type protein, whereas a protein (SufA) displaying significant sequence homology to IscA is part of the machinery. This gives an opportunity to investigate the specific function of an IscA-type protein. Fig. 1 shows the amino acid sequences of SufA proteins from a variety of bacterial and archaeal sources. Alignment with IscA proteins is possible demonstrating 52% identity between SufA and IscA from the same organism, *E. coli*. In particular, the three invariant cysteines of IscA, which have been shown by site-directed mutagenesis in the case of the yeast protein to be essential for function and were proposed to be involved in iron binding, are present in SufA proteins (15, 42). It is thus very tempting to suppose that SufA has the potential to assemble an [Fe-S] cluster and to serve in a way similar to IscA, as a scaffold protein, for mediating [Fe-S] cluster assembly in target proteins.

With this protein the only other protein that SUF has the potential to assemble an [Fe-S] cluster and to serve in a way similar to IscA, as a scaffold protein, for mediating [Fe-S] cluster assembly in target proteins.

with ISC is a cysteine desulfurase (SufS), which displays 23% identity to IscS. On the other hand, the *suf operon* contains proteins that are not present in cluster of ISC genes. The available data suggest that SufB, SufC, and SufD, which are encoded by highly conserved genes occurring in bacteria, archaea, plants, and parasites, work together to form a multimeric ABC transporter complex with ATPase activity (50). The role of the SufB, SufC, and SufD complex is still unclear. A possible function would be to provide energy to the SUF machinery for [Fe-S] cluster assembly. Additional characterization is needed to confirm the presence and the role of the complex. Finally, nothing is known about the function of SufE. In *E. coli*, a homolog of *sufE*, referred as ygdK, lies just after the third cysteine desulfurase of that microorganism, named *cysd*, indicating a conservation of genetic organization between *sufS*, *sufE* and *cysd-ygdK* (48). Thus it is tempting to suggest that SufE is also involved in [Fe-S] assembly. Similar examples of this association are found in *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Haemophilus influenzae* (45).

Here we report, for the first time, the isolation and characterization of SufA protein. We show that SufA from *E. chrysanthemi* can assemble iron-sulfur clusters, which can be efficiently transferred to target apoproteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were of reagent grade and obtained from Sigma–Aldrich Chemical Co. or Fluka, unless otherwise stated. 152FeOAc was converted into ferric chloride by dissolving it in hot concentrated (35%) hydrochloric acid of analytical grade (Carlo Erba) and repeatedly concentrated in water. 5-Deaza-7,8-dimethyl-10-methylisoalloxazine (DAF) was prepared according to Ashton et al. (54). S-Adenosylmethionine was from Roche Applied Science. Pyridoxal 5'-phosphate was from Interchim. IscA, flavodoxin, and flavodoxin reductase were available in our laboratory. Enzymes, oligonucleotides, and culture media were purchased from New England BioLabs, Oligo Express, and Difco, respectively. Pco DNA polymerase and the High Pure Plasmid Isolation kit, for plasmid DNA purification, were from Roche Diagnostics (Mannheim, Germany). Isolation of DNA fragments from agarose gels was performed using a QIAquick gel extraction kit (Qiagen). DNA sequencing was performed by Genome Express (France). Antibodies against VSV-G were obtained from Roche Applied Science. *E. coli* MG1655Δsuf was described previously (50).

Cloning of the *sufA Gene and Construction of the Overexpressing Plasmids—The *sufA* gene, encoding the SufA protein, was amplified by PCR using *E. chrysanthemi* RS972 DNA as a template. Primers used for *sufA* amplification were 5'-CCGCAATGGAAACCAAGCTTTGAGG-3' (forward primer, underlined bases indicate a *NdeI* site) and 5'-GGTTCGAGAAGCCCAAACATTTGCCGCCG-3' (reverse primer, underlined bases indicate an *XhoI* site). PCR was run as follows: genomic DNA was denatured for 10 min at 94 °C. The Pco DNA polymerase (0.5 unit), deoxynucleotide mix (0.2 mM each), and the primers (0.5 mM final concentration) were added, and 30 cycles (30 s at 94 °C, 30 s at 50 °C, then 30 s at 72 °C) were then performed, followed by a final 10-min elongation step at 72 °C. The PCR product was digested with *NdeI* and *XhoI* and then ligated into a pET22b(+) vector (Novagen), digested with *Ndel* and *XhoI* and then ligated into a pET22b(+) vector (Novagen), digested with *NdeI* and *XhoI*. The cloned gene was then sequenced to ensure that no error was introduced during PCR reaction. The plasmid was then named pET/SufA.

For the cross-linking experiment, a VSV-SufA encoding protein was constructed as follow. Primers used for *sufA* amplification were 5'-CCCTACCGCTCTAATGGATGAAAGGCGCCGTTGGAATGTCGAAAGCAGATGATGAG-3' (forward primer, underlined bases indicate a *NocI* site) and 5'-GCGGGAATTCCTACTTTGAGAAGCCCAAACATTTGCCGCGCGCCG-3' (reverse primer, underlined bases indicate a *XhoI* site). PCR was run as follows: genomic DNA was denatured for 2 min at 94 °C. The Pco DNA polymerase (0.5 unit), deoxynucleotide mix (0.2 mM each), and the primers (0.5 mM final concentration) were added, and 30 cycles (30 s at 94 °C, 30 s at 50 °C, then 30 s at 72 °C) were then performed, followed by a final 10-min elongation step at 72 °C. The PCR product was digested with *NcoI* and *XhoI* and then ligated into pBET22b(+) vector (Novagen), digested with the same restriction enzymes, for production of SufA with a histidine tag. The cloned gene was then sequenced to ensure that no error was introduced during PCR reaction. The plasmid was then named pET/SufA.
Overexpression and Purification of SufA—For overproduction of SufA, E. coli (competent BL21DE3) strains were transformed with pET/SufA vector, and 2 × 100 ml overnight cultures were used as an inoculum for 2 × 400 ml of LB medium (Difco) containing 100 μg/ml ampicillin. Cells were grown at 37 °C to an A600 of 0.5, and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Eugene) for 4 h at 37 °C. The bacterial pellet (7 g/800 ml culture) was resuspended in 50 ml of buffer A (100 mM Tris-HCl, pH 7.5, 50 mM NaCl) and treated twice with a French press for disruption. The cell lysate was centrifuged at high speed for 30 min, at 4 °C. The supernatant (180 mg of soluble proteins) was loaded onto a 5-ml Hi-trap column (Amersham Biosciences), charged with nickel, and equilibrated with buffer B (buffer A plus 1 M imidazole). The flow-through was washed twice with 10 ml of buffer B plus 1×106 M imidazole. SufA protein was then aliquoted and stored at −80 °C.

Aggregation State Analysis—Fast protein liquid chromatography gel filtration was used to load into a 5-ml Hi-trap column (Amersham Biosciences) at a flow rate of 0.5 ml/min equilibrated with buffer A (pH 7.5) and washed, resuspended in 1.5 ml of Tris buffer (pH 6.8), and centrifuged for 10,000 rpm, 10 min, 4 °C, supernatants were submitted to ultrafiltration with an analytical Superdex-75 (Amersham Biosciences) at a flow rate of 0.5 ml/min for the activity as described below. The conserved cysteines are in some other important regions. SufA from E. coli, V. cholerae, and Hs, Homo sapiens.

Iron and Sulfide Binding to SufA—The following procedure was made anaerobically inside a glove box (Jacomex B553 (NMT)). ApoSufA is obtained by irradiation with DAF in the presence of 10 mM EDTA. After 1 h of incubation, the colorless protein was purified on a Sephadex G-25 column equilibrated with buffer C (0.1 M Tris-HCl, pH 8.0). ApoSufA (30 μM monomer) was incubated in buffer C at 37 °C for 3–4 h with a 3- to 4-fold molar excess of both Na2S (Fluka) and either Fe(NH4)2(SO4)2 (Aldrich) or 57FeCl3 in the presence of 5 mM dithiothreitol (DTT). Then 2 mM EDTA was added, and the solution was further incubated for 30 min. The protein was desalted on Sephadex G-25 (80 ml, same buffer), and the colored fractions were concentrated on Nanocon 10 (Amicon).

Preparation of Reconstituted Biotin Synthase (recBioB)—Reconstitution of apoprotein was achieved with 35Fe as described previously (56) and desalted over Sephadex G-25 (equilibrated with buffer C) to remove adventitiously bound iron after reconstitution.

[Fe-S] Cluster Transfer from HoloSufA to RecA—Aporoferrodoxin was obtained from holoferredoxin as already described (32). Typically, apoferrodoxin (76 nmol) was incubated with holoSufA (76 nmol) for 2 h at 25 °C in buffer D (0.1 M Tris-HCl, pH 8.0, 30 mM KCl). Transfer of the [Fe-S] cluster from holoSufA to ferredoxin was monitored at time intervals by EPR spectroscopy at liquid helium temperature, from the ferredoxin-characteristic EPR signal obtained after reduction of the cluster-bound dithionite.

[Fe-S] Cluster Transfer from HoloSufA to ApoBioB—ApoBioB was obtained by irradiation of the as-isolated enzyme with DAF in the presence of 10 mM EDTA and purified onto a Sephadex G-25 column equilibrated with buffer C. ApoBioB was then incubated in buffer C with either a 2-fold molar excess of holoSufA or a 4-fold molar excess of FeS cluster and SF2. At time intervals (5, 10, 20, 30, and 60 min) biotin synthase activity was measured by addition of all components required for the activity as described below. HoloBioB from E. coli, prepared as previously discussed (23), was also used in place of holoSufA.

Biotin Synthase Activity—The activity was assayed from the amount of biotin formed from dethiobiotin. The standard reaction mixture in a final volume of 50 μl of 0.1 M Tris·HCl, pH 8, and 30 mM KCl contained 1.7 nmol of biotin synthase monomer, 1 equivalent of pyridoxal 5-phosphate (PLP), 400 μM dethiobiotin, 1 mM DTT, 150 μM S-adenosylmethionine, 20 μM flavodoxin, 4 μM flavodoxin reductase, 1 mM NADPH, and 2 mM cysteine. The reaction was monitored at 37 °C. After 90 min of incubation, an aliquot was withdrawn and the reaction stopped by the addition of 10% (v/v) 1 M trichloroacetic acid. After centrifugation, the supernatant was analyzed for biotin formation. Biotin was measured by a microbiological method using Lactobacillus plantarum and a calibration curve in each experiment. All the data presented in this report represent the average of at least duplicate experiments.

UV-visible Spectroscopy—UV-visible spectra were recorded with a Cary 1 Bio (Varian) spectrophotometer. Mössbauer Spectroscopy—57Fe-Mössbauer spectra were recorded using 400-μl cuvettes containing 600–750 μg protein. Spectra were recorded on a spectrometer operating in constant acceleration mode using an Oxford cryostat that allowed temperatures from 1.5 to 300 K and a 57Co source in rhodium.

Analysis—Protein concentration (by monomer) was determined by a microbial method using Lactobacillus plantarum and a calibration curve in each experiment. All the data presented in this report represent the average of at least duplicate experiments.

Results

SufA Proteins: Sequence Analysis—Using the Blast search algorithm against the protein data base with the sequence of SufA from E. chrysanthemi we identified several proteins from different organisms that displayed sequence homology to E. chrysanthemi SufA (Fig. 1). These include SufA putative proteins as well as IsA proteins and ISAp eukaryotic homologs. Like IsA, SufA proteins are characterized by the presence of three conserved cysteines residues in the C-terminal region, two of which are in a CGCG motif. A high sequence conservation was present around these cysteines as well as in some other important regions. SufA from E. chrysanthemi
showed 59% identity with SufA from *E. coli* and 42% with IscA from *E. coli*. In eukaryotes, in which genes are not organized within operons, SufA and IscA homologs were also present (45). The proteins from *Homo sapiens* and *Arabidopsis thaliana* showed 59% identity with SufA from *E. chrysanthemi*, respectively.

**Cellular Location of SufA in *E. chrysanthemi***—Previous analyses revealed that SufB, SufC, and SufD proteins are located in the cytosol of *E. chrysanthemi*. Therefore, we were interested in analyzing the cellular location of SufA. A strain of *E. chrysanthemi* was transformed with a chimeric gene encoding a VSV-SufA-tagged protein, which can be detected by reaction with an antibody against the VSV region. Cell fractionation techniques revealed that SufA was indeed located exclusively in the cytosol (Fig. 2).

**Expression and Purification of SufA**—A SufA-(His)$_6$ protein from *E. chrysanthemi* was overproduced in BL21(DE3) *E. coli* cells. Analysis by SDS-electrophoresis gel of whole cell extracts revealed a Coomassie Blue-stained band at around 15,000 Da ([* Insets in Fig. 3, lane 2*]). This molecular mass is in agreement with the mass calculated from the DNA sequence (14,700 Da). After extraction, a high yield of soluble SufA was obtained, even though a significant amount of protein remained under unsoluble forms. Soluble SufA was purified with a Ni-NTA column that specifically retains proteins containing a cluster of histidines. A gel filtration step using Superdex 75 was used to determine the oligomerization state of SufA (Fig. 3). During that step, SufA eluted in a major peak corresponding to a dimer, but a minor amount of aggregated forms of larger size could also be observed. Combining the two Superdex-75 fractions containing SufA, 47 mg of SufA could be obtained from 180 mg of bacterial extracts, in a more than 95% pure form as judged by gel electrophoresis ([* Insets in Fig. 3, lanes 7 and 8*]). Edman degradation analysis revealed a MQTHD sequence that corresponded to the predicted N terminus sequence of SufA.

**Biochemical and Spectroscopic Characterization of SufA**—Concentrated purified solutions of SufA were slightly pink-colored with a UV-visible spectrum that indicated the presence of iron-sulfur clusters. Iron and sulfide analysis confirmed the presence of stoichiometric amounts of iron and sulfide but with only 0.06–0.08 iron and sulfide atom per polypeptide chain. This small amount of cluster could be removed by irradiation in the presence of deazaflavin (DAF) and EDTA, a method used to prepare apoSufA, the apoprotein form. Incubation of apoSufA inside an anaerobic glove box with a 5-fold molar excess of both ferrous sulfate and sodium sulfide, in the presence of dithiothreitol (DTT), followed by desalting on a Sephadex G-25 column, resulted in an EPR-silent brownish protein (holoSufA) containing a maximum amount of 1.7–2.1 atoms of iron and sulfide per polypeptide chain. ApoSufA was unable to chelate iron in the absence of sodium sulfide. Like the apo form, holoSufA eluted in two peaks during Superdex-75 chromatography, the major one corresponding to a dimer and the minor one to a trimer. The light absorption spectrum of holoSufA displayed bands at 330, 420, and 460 nm, indicating the presence of an iron-sulfur center (Fig. 4). This center was stable in the presence of 2 mM EDTA during 30 min under anaerobiosis.

The clusters were unstable both in aerated solutions or during anaerobic reduction. In the presence of oxygen we observed a bleaching of the protein solution, and the cluster had a half-life time of about 20 min at 25 °C. Addition of DTT did not stabilize the cluster under aerobic conditions. In contrast, the light absorption spectrum did not change when the protein was left overnight in the anaerobic glove box (data not shown). Treatment of SufA with strong reducing agents (2 mM dithiothreitol) led to a metal-free protein. We were unable to detect an EPR-active form under either oxidative or reductive conditions.

We used Mössbauer spectroscopy to characterize the iron sites in holoSufA preparations after reconstitution with $^{57}$Fe and sulfide and treatment with 2 mM EDTA and desalting on Sephadex G-25. Different conditions gave preparations with lower proportions of defined clusters. One of these, treated with larger concentrations of EDTA (10 mM), proved useful for analysis of the Mössbauer data, because it contained a larger proportion of [2Fe-2S] clusters and no more [4Fe-4S] clusters. Similar results were obtained whether sulfur was provided by sodium sulfide or by the SufS-dependent cysteine desulfurization reaction (data not shown). Fig. 5 ([* Insets in Fig. 3, lanes 7 and 8*]) shows the 77 K Mössbauer spectra, respectively, from a sample treated anaerobically with 2 mM EDTA (sample 1: 0.74 mm final concentration; 1.7 iron/monomer), and Fig. 5 ([* Insets in Fig. 3, lanes 7 and 8*]) shows the 77 K Mössbauer spectra corresponding to the predicted N terminus sequence of SufA.
and the broad peak at $-2.5$ mm/s can be assigned to mononuclear Fe$^{2+}$(S = 2) sites. We have simulated these peaks assuming two sites; site 1 with parameters $\delta = 0.71$ mm/s and $\Delta E_Q = 3.20$ mm/s and site 2 with $\delta = 1.20$ mm/s and $\Delta E_Q = 2.80$ mm/s. Site 1 contributes 13% to the spectrum and is attributed to Fe$^{2+}$(S = 2) in tetrahedral coordination comprising sulfur atoms as in sample 2. Site 2 represents 17% of total iron, and its parameters are consistent with Fe$^{2+}$(S = 2) in octahedral environment with a coordination of five or six N/O donors. The rest of the iron gives rise to a broad asymmetric doublet, confined in the region of 0–1 mm/s. We have simulated this part of the spectrum assuming two doublets. One doublet has parameters similar to the parameters of the [2Fe-2S]$^{2+}$ cluster observed in Fig. 5D. Its presence is justified from the 4.2 K spectrum (see below). The other doublet (dotted line in Fig. 5A) has apparent parameters $\delta = 0.49$ mm/s and $\Delta E_Q = 0.92$ mm/s.

Unlike sample 2, at 4.2 K, in Fig. 5B we still observe diamagnetic doublets in the central part of the spectrum. We simulated this part assuming three doublets. One doublet has parameters similar to the parameters of the [2Fe-2S]$^{2+}$ cluster observed in Fig. 5D of sample 2. The other two doublets (dotted lines in Fig. 5B) have apparent parameters $\delta_1 = 0.46$ mm/s, $\Delta E_Q = 1.26$ mm/s; $\delta_2 = 0.48$ mm/s, $\Delta E_Q = 0.82$ mm/s. On average, the values of the isomer shift ($-0.47$ mm/s) and quadruple splitting (1.04 mm/s) are consistent with a [4Fe-4S]$^{2+}$ cluster.

From an analysis of spectra in Figs. 5A and 5B we estimate that 11% of the iron belongs to [2Fe-2S]$^{2+}$ clusters and 40% to [4Fe-4S]$^{2+}$ clusters. 30% of the iron belongs to high spin mononuclear ferrous iron. A closer examination of the spectrum in Fig. 5B suggests that the remaining ~20% of the iron gives rise to a broad background extending from $-3$ to +3 mm/s. This species is attributed to paramagnetic iron-sulfide impurities.

Iron-Sulfur Cluster Transfer—The presence of iron sulfur clusters in holoSufA led us to analyze the ability of these clusters to be transferred to an acceptor apoprotein. To investigate this, two [Fe-S] proteins were chosen as model substrate proteins: ferredoxin (Fdx) and biotin synthase (BioB). In this study we present the results obtained with proteins from E. coli, which display very strong homologies to those from E. chrysanthemi (Fdx: 90% identity; BioB: 84% identity). Fdx is a 2[Fe-S] protein. The corresponding apo form of Fdx, apoFdx, was incubated with a stoichiometric amount of holoSufA under anaerobic conditions, and the reaction was monitored by UV-visible spectroscopy because holoferredoxin displays characteristic light absorption bands at 415 and 460 nm. Furthermore, the presence of the cluster in ferredoxin can be confirmed, after reduction with an excess of dithionite, both from its EPR signals, characteristic for a reduced [2Fe-2S]$^{2+}$ cluster, and its UV-visible spectrum with a new absorption band at 550 nm. From these assays we determined that 80% of iron and sulfide initially present in holoSufA was recovered as a [2Fe-2S] cluster in Fdx, after a 40-min reaction (data not shown). In comparison, formation of the cluster by incubation of apoFdx with a 2-fold molar excess of iron and sulfide was highly inefficient with a yield of 5% after a 3-h reaction.

Biotin synthase (BioB), the product of the bioB gene, can be prepared in two different active forms. Form I, named recBioB, contains one catalytically essential [4Fe-4S] cluster per polypeptide chain and requires pyridoxal phosphate (PLP), cysteine, a source of electrons, and S-adenosylmethionine for activity (60, 61). Form II contains an additional [2Fe-2S] cluster and does not require PLP and cysteine (62, 63). However, it is important to note that in vitro and for unknown reasons both forms do not allow the production of more than 1 mol of biotin per mol of enzyme (61, 62, 64). In the experiment discussed.

**Fig. 4.** UV-visible absorption spectrum of reconstituted SufA protein (100 μM, 1.7 iron/polypeptide chain) in 0.1 M Tris-HCl, 50 mM KCl, pH 8.

**Fig. 5.** Zero field Mössbauer spectra of holoSufA from sample 1 (730 μM, 1.7 Fe/monomer) at 77 K (A) and 4.2 K (B) and from sample 2 (680 μM, 1.1 Fe/monomer) at 77 K (C) and 4.2 K (D). Solid and dashed lines represent theoretical simulations using the parameters quoted in the text. In B we show the theoretical contribution of the doublets belonging to the [2Fe-2S]$^{2+}$ (solid line) and [4Fe-4S]$^{2+}$ (dotted lines) clusters, respectively.
below we studied the formation of the [4Fe-4S] cluster of BioB. ApoBioB, the enzyme form lacking the cluster(s), was incubated with 2 molar excess of holoSufA, providing only a 4 molar excess of iron and sulfide, in the presence of DTT. Cluster transfer from SufA to BioB was then monitored by assaying the solution for biotin synthase activity. Because PLP was required in this assay, it is likely that under these reaction conditions the mechanism of the cluster transfer reaction only in the case of an indirect transfer mechanism, which implies that iron becomes transiently accessible.

ApoBioB and holoSufA (or holoIscA) were incubated anaerobically at 18 °C with increasing concentrations of BPS, and biotin synthase activity was measured after a 30-min reaction. As shown in Fig. 7, BPS did not inhibit the iron-sulfur cluster transfer from holoSufA (or holoIscA) to apoBioB, because the reaction proceeded equally well in the absence or in the presence of 150 μM of the iron chelator. On the contrary, reconstitution of the cluster of biotin synthase during incubation of apoBioB with 4 equivalents of iron and sulfide was inhibited by increasing concentrations of BPS.

Cluster transfer was also observed with comparable rates in the absence of DTT if apoBioB was first treated with 50 mM DTT anaerobically and then desalted. This shows that DTT was not required during the [Fe-S] transfer process but rather for reduction of disulfide bridges present in the aerobic preparation of apoBioB.

**DISCUSSION**

On the basis of its strong amino acid sequence similarity with IsaA (Fig. 1), a protein of the isc operon shown to be able to assemble an iron-sulfur center, we made the hypothesis that SufA, one of the components of the SUF machinery, would also have the potential to bind clusters. This is now unambiguously shown here for the first time with a purified preparation of the homodimeric recombinant SufA protein from *E. chrysanthemi*. Iron and sulfide analysis of both as-isolated and reconstituted proteins as well as UV-visible and Mössbauer spectroscopy are consistent with the presence of iron-sulfur clusters in this protein.

There are many similarities between IsaA and SufA proteins. These similarities are discussed in the following paragraphs. First, both purified proteins are found essentially in the homodimeric form with some tendency to aggregate into larger polymers (Fig. 3).

Second, IsaA and SufA proteins have in common three fully conserved cysteines (Cys-51, Cys-115, and Cys-117 for SufA and Cys-51, Cys-115, and Cys-117 for IsaA) from the yeast *S. cerevisiae*. These cysteines have been proposed to function as binding sites for an aldehyde oxidoreductase from *Desulfovibrio gigas*.

In this case X-ray crystallography has shown that these cysteines are bound to different iron clusters in the same [2Fe-2S] cluster (68). In the case of ISA1p and ISA2p from the yeast *S. cerevisiae*, these cysteines have been proposed to function as binding sites for an aldehyde oxidoreductase from *Desulfovibrio gigas*. In this case X-ray crystallography has shown that these cysteines are bound to different iron clusters in the same [2Fe-2S] cluster (68). In the case of ISA1p and ISA2p from the yeast *S. cerevisiae*, these cysteines have been proposed to function as binding sites for an aldehyde oxidoreductase from *Desulfovibrio gigas*.
residues for iron, because individual amino acid substitutions for each of the three cysteine residues yielded the same phenotypes as gene knockouts (15, 42). However, as yet the corresponding mutated proteins have not been purified and characterized. On the contrary, in the case of ISA1p from Schizosaccharomyces pombe, the corresponding mutated proteins were isolated in pure form and found to be able to coordinate a cluster under the in vitro reconstitution conditions employed for the wild-type protein (29). These clusters displayed UV-visible and Mössbauer spectroscopic properties quite comparable to those of the wild-type protein. Even though the stability of the clusters of the mutant proteins was found to be greatly reduced with regard to that of the wild-type protein, these results did not provide an unambiguous confirmation that these three conserved cysteines indeed are iron-binding residues. Nevertheless, at the present stage, we favor such an hypothesis and speculate that they play an iron-binding function in SufA, but this must be further investigated by site-directed mutagenesis. It is important to note that not only is there no other cysteine in SufA, but also two additional fully conserved residues, Asp-71 and Asp-97, are possible candidates as iron-binding residues and thus should be considered in future site-directed mutagenesis studies. At least one of these residues might provide the fourth ligand to a cluster bound by three cysteines.

Third, the clusters in SufA from E. chrysanthemei are air-sensitive and are rapidly decomposed during exposure to air. This probably explains why the protein was isolated mainly in the apoprotein form after chromatographic purification. Furthermore, the protein also loses its cluster during anaerobic reduction with dithionite. This oxidative and reductive lability of SufA clusters was also observed in the case of IscA from E. coli or Azotobacter vinelandii and ISA1p from S. pombe (23, 26, 29). It should be noted that, in both SufA and IscA, iron is tightly bound to the protein only in the presence of sulfide. These proteins are not able to, although in some cases only very weakly, chelate ferrous iron alone.

Fourth, the SufA protein can bind close to 2 iron and sulfur atoms per polypeptide chain mainly assembled within both [2Fe-2S]+ and [4Fe-4S]+ clusters, with typical Mössbauer parameters: $\delta = 0.27 \text{ mm/s}$, $\Delta E_Q = 0.59 \text{ mm/s}$ and $\delta = 0.47 \text{ mm/s}$, $\Delta E_Q = 1.04 \text{ mm/s}$, respectively. Whether the [2Fe-2S] clusters are intermediate species on the way to the [4Fe-4S] ones or they are degraded products of labile [4Fe-4S] clusters remains to be shown. Furthermore, all preparations of holo-SufA, even those extensively treated with EDTA, contained significant amounts of adventitiously bound iron, both high spin Fe$^{2+}$ mononuclear species and magnetic polymeric Fe-sulfides, as shown by Mössbauer spectroscopy (Fig. 5). Thus, despite great efforts to optimize the in vitro reconstitution process, we failed to set up the conditions for an efficient generation of clusters in SufA. IscA proteins from various origins have been shown to chelate the same amount of iron and sulfur atoms per polypeptide chain during reconstitution of the apo forms (23, 26, 29). On the other hand, contradictory results have been reported in the literature concerning the type of clusters present in IscA proteins (23, 26, 29). Our analysis of the protein from E. coli by Raman resonance spectroscopy led to the conclusion of the presence of [2Fe-2S] clusters (23). However, this spectroscopy is not as efficient for detection of [4Fe-4S] clusters in proteins, and we do not completely exclude the possibility that [4Fe-4S] clusters are also present in E. coli IscA. Mössbauer spectroscopy, which is the ideal method to discriminate between [2Fe-2S] and [4Fe-4S] clusters, has not been applied to this particular protein. Mössbauer spectroscopy has been used to investigate the clusters in two cases: ISA1p from S. pombe and IscA from A. vinelandii (26, 29). In the first case, only [2Fe-2S] clusters (one per monomer) could be detected with typical Mössbauer parameters ($\delta = 0.27 \text{ mm/s}$ and $\Delta E_Q = 0.56 \text{ mm/s}$) (29). On the contrary, in the case of IscA from A. vinelandii, a detailed study of the metal centers generated during reconstitution with free iron and sulfide provided by enzymatic cysteine desulfurization revealed that the protein can assemble both [2Fe-2S] and [4Fe-4S] clusters, with Mössbauer parameters comparable to those of the clusters of SufA ($\delta = 0.26 \text{ mm/s}$, $\Delta E_Q = 0.55 \text{ mm/s}$) for [2Fe-2S] and $\delta = 0.44 - 0.46 \text{ mm/s}$, $\Delta E_Q = 1.04 - 1.25 \text{ mm/s}$ for [4Fe-4S]) (26). The [2Fe-2S] clusters have been unambiguously shown to be transient species during the cluster assembly process, but the final [4Fe-4S] clusters were shown to be degraded under prolonged incubation into polymeric Fe-sulfides, confirming their significant instability. This instability of the metal centers is probably at the origin of the discrepancies described above. On the other hand, this instability might also be an intrinsic functional property of these proteins in relation to their role as sources of clusters during [Fe-S] assembly in apoproteins (see below). Nevertheless, a more definitive characterization of the iron centers in both IscA and SufA require a better control of the reconstitution conditions that need to be redefined.

Fifth, both SufA and IscA are able, at least in vitro, to efficiently transfer its iron and sulfide atoms to apoferrredoxin (23). The high transfer yield suggests that ferredoxin can take iron and sulfur not only from [2Fe-2S] and [4Fe-4S] clusters preassembled in SufA but also from unspecifically bound iron-sulfide species. These indeed represent a significant proportion of total iron and sulfur in holoSufA, as shown by Mössbauer spectroscopy. The formation of the [2Fe-2S] cluster of ferredoxin is greatly accelerated when iron and sulfur are preassembled in SufA (or IscA) when they are provided in the form of free iron and sulfide salt. This is consistent with the working hypothesis that SufA indeed plays the role of a scaffold protein for assembly of [Fe-S] clusters, providing a mechanism for bringing together elemental sulfur and iron into transient assembled clusters. This function would not be a specific property of the IscA-SufA proteins because IscU (bacteria), ISU (yeast), and NifU (nitrogen-fixing bacteria) proteins were also suggested to play this role (23, 27, 30–32, 67, 68). Because there is no homolog of these proteins in the suf operon and because the other components of that operon are unlikely to bind an [Fe-S] cluster, it is likely that SufA plays the specific and unique function of a scaffold protein during assembly of [Fe-S] clusters that is dependent on the SUF machinery.

It can be speculated that, depending on the scaffold protein, IscA, IscU, or SufA, different clusters, [2Fe-2S] or [4Fe-4S], might be assembled in target proteins. So far this seems rather unlikely considering the ability of these proteins to transiently assemble both types of clusters (see above). Furthermore, this hypothesis is not supported by the phenotypes of the knockout bacterial and yeast strains where reduced activities of both [4Fe-4S] and [2Fe-2S] proteins were observed (5, 7, 8, 69). In this study we show that SufA not only serves as a [2Fe-2S] cluster donor to ferredoxin, as discussed above, but also as a [4Fe-4S] donor for biotin synthase, BioB (Fig. 6). In this case also cluster formation was achieved at a greater rate when iron and sulfur atoms were provided by holoSufA as compared with free iron and sulfide. Furthermore, we show here that both holoSufA and holoIscA can provide their clusters to BioB. Thus a single protein can deliver both types of clusters to different proteins and, furthermore, a given target can get its clusters from different scaffold proteins. These in vitro results thus
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seem to rule out the notion that the [Fe-S] cluster assembly process depends on specific combinations of cluster donors and acceptors.

Besides the lack of U-type proteins, there are two major differences between the ISC and the SUF systems. First, the SUF system lacks molecular chaperones, corresponding to those of the ISC system: DnaK/HscA and DnaJ/HscB in bacteria or Hsp70/SSQ1 and Hsc20/JAC1 in yeast. On the other hand, the SUF system contains three cytoplasmic proteins, SufB, SufC, and SufD, forming a complex and working together as an ABC/ATPase machinery, suggesting a requirement for ATP during SUF-dependent cluster synthesis and that SufBCD provides energy to this pathway (50). Whether SufBCD also has a molecular chaperone function is a possibility that needs to be studied. The second difference is the absence in the SUF system of an electron transfer protein such as ferredoxin. In yeast, mitochondrial ferredoxin and ferredoxin reductase are essential components of the [Fe-S] cluster assembling machinery, their deletion being lethal (14, 70, 71). Furthermore, requirement for a potent reduct system in this biosynthetic pathway is not surprising because electrons might be required for reduction and release of iron from storage or transport sources, generation of sulfide, assembly of the cluster itself, and release of the [Fe-S] cluster from its transient association with a scaffold protein. Thus either the SUF system does not require electrons and operates by a different mechanism or it uses another reductase system that needs to be identified.

We believe that SUF is an interesting system to investigate because it uses a limited number of components for assembly of [Fe-S] clusters and might represent the minimal combination for that process. In Scheme 1 we propose a hypothetical SUF-dependent mechanism for [Fe-S] cluster biosynthesis. In this mechanism SufA would serve as a unique scaffold protein assembling iron and sulfur atoms into transient clusters. SufS, possibly in association with SufE, would serve to generate sulfur from cysteine. The source of iron is still unknown. Even though, in the experiments reported here, SufBCD was not required for cluster transfer reactions from SufA, it is tentatively included in Scheme 1. Genetic studies have indeed shown the importance of this protein complex during [Fe-S] cluster assembly (46, 48, 50). Whether SufBCD has an effect on cluster assembly in SufA or on cluster transfer in vitro needs to be further studied. It might be relevant to note that the cellular location of the SufBCD proteins is still a matter of controversy, because SufC was found in the membrane in E. coli using microscopy-based technology and in the cytosol in both E. coli and E. chrysanthemi using conventional cell fractionation techniques (45, 48). Assuming that all Suf proteins work together, the fact that SufA was found to be located in the cytosol in the present study supports the later view. In Scheme 1 no electron source is involved, but one cannot exclude, as discussed above, that a reductase not encoded by the suf operon participates in the process. Mycoplasma sp., which are considered to be the most primitive parasitic cells, might tell us that it is even possible to do with less. These organisms contain in their genomes only three proteins of the ISC machinery, IscU, IscS, and HscB, and no bacterial-like ferredoxin reductase/ferredoxin system (45).

Of note, a final aspect of our work involves the mechanism of cluster transfer from holoSufA to BioB. Our results suggest that this transfer is not a two-step process, with first a release of iron and sulfur atoms from holoSufA in solution followed by their uptake by the apoprotein target. A concerted mechanism is more consistent with the inefficiency of BioB reconstitution with free iron and sulfide as compared with holoSufA and the absence of inhibitory effects of bathophenanthroline, a strong ferrous chelator, on the transfer process. In contrast, the inefficient cluster formation by free iron and sulfur is greatly inhibited by this chelator. The hypothesis of a concerted transfer process is reasonable, considering the need for scaffold proteins for assembly of [Fe-S] clusters in cells. In other words, why should a cluster be transiently pre-assembled into SufA (or IscA) on the way to its final destination if this cluster would be disassembled before transfer to the target protein? We are interested in understanding the molecular details of the cluster transfer reaction, but this requires a better control of cluster assembly in SufA, which is currently under study.

Cluster assembly in biotin synthase Bio2p, the yeast equivalent of BioB, has also been investigated in yeast. Using detergent extracts from S. cerevisiae mitochondria, Mülhenhoff et al. (43) have shown that cysteine, NADH, ATP, and DTT greatly stimulated formation of clusters. Cysteine is the substrate of cysteine desulfurase and is the precursor of sulfide, whereas NADH is the electron source, in agreement with the requirement for ferredoxin reductase/ferredoxin electron transfer chain. The requirement for ATP is consistent with the fact that chaperones are involved in cluster formation. The role of DTT has been discussed, but the possibilities that Bio2p could be oxidized in the mitochondrial extracts and that DTT could be required for reducing disulfide bridges, as a prerequisite for enzyme activity, were not considered. Our results show that DTT is not required anymore during cluster transfer from SufA to BioB if BioB has been pretreated with DTT first and then desalted. The most interesting result in this study of Bio2p activation, in the context of our work on IscA and SufA proteins, is that a deficiency in ISA1 caused a strong decline in [Fe-S] assembly. In contrast, deletion of NFU1 resulted in only moderate defects. This, together again with the fact that there are no U proteins (NifU or IscU) in the suf operon, supports our hypothesis of A-type proteins (IscA and SufA) playing a major role during [Fe-S] cluster biosynthesis, probably as a scaffold protein for transient [Fe-S] cluster assembly and delivery to target proteins.

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