FEBS Letters 581 (2007) 1362–1368

The SUF iron–sulfur cluster biosynthetic machinery: Sulfur transfer from the SUFS–SUFE complex to SUFA

Maïté Sendra[a], Sandrine Ollagnier de Choudens[a,*], David Lascoux[b], Yiannis Sanakis[c], Marc Fontecave[a,*]

[a] *Laboratoire de Chimie et Biologie des Métaux, iRTSV/LCBM, UMR 5249, CEA-Grenoble, 17 Avenue des Martyrs, 38054 Grenoble Cedex 09, France*
[b] *Laboratoire de spectrométrie de masse des protéines, Institut de Biologie Structurale CNRS/CEA/UJF, 41 rue J. Horowitz, 38027 Grenoble Cedex 1, France*
[c] *NCSR, Demokritos, Institute of Materials Science, 15310 Ag. Paraskevi, Attiki, Greece*

Received 9 January 2007; revised 23 February 2007; accepted 26 February 2007

Available online 5 March 2007

Edited by Hans Eklund

Abstract **Iron–sulfur cluster biosynthesis depends on protein machineries, such as the ISC and SUF systems. The reaction is proposed to imply binding of sulfur and iron atoms and assembly of the cluster within a scaffold protein followed by transfer of the cluster to recipient apoproteins. The SufA protein from *Escherichia coli*, used here as a model scaffold protein is competent for binding sulfur atoms provided by the SufS–SufE cysteine desulfurase system covalently as shown by mass spectrometry. Investigation of site-directed mutants and peptide mapping experiments performed on digested sulfurated SufA demonstrate that binding exclusively occurs at the three conserved cysteines (cys50, cys114, cys116). In contrast, it binds iron only weakly ($K_a = 5 \times 10^5\ M^{-1}$) and not specifically to the conserved cysteines as shown by Mössbauer spectroscopy. [Fe–S] clusters, characterized by Mössbauer spectroscopy, can be assembled during reaction of sulfurated SufA with ferrous iron in the presence of a source of electrons.**

Keywords: Biosynthesis; Iron–sulfur cluster; Mass spectrometry; Mössbauer spectroscopy; Site-directed mutagenesis

1. Introduction

Iron–sulfur clusters serve in a variety of biological functions including electron transfer, regulation, protein structure stabilization, redox and non-redox catalysis [1,2]. It is now clear that the formation of iron–sulfur clusters is not spontaneous in vivo. *Escherichia coli* contains machineries involved in this process. The first one, referred as ISC (*iscS–iscU–iscA–hscB–hscA–fdx*), is essential for general biosynthesis of [Fe–S] clusters in bacteria [3,4]. Homologues of these proteins have also been identified in eukaryotes suggesting a highly conserved mechanism [5,6]. The second machinery, SUF (*sufA–sufB–sufC–sufD–sufS–sufE*), works under iron limitation and oxidative stress [7,8]. They have in common the involvement of a cysteine desulfurase (IscS, SufS/E) for the utilization of cysteine as a source of sulfur [9,10]. Sulfur atoms from free cysteine are transferred to an essential cysteine of the cysteine desulfurase thus generating persulfide/polysulfide intermediate forms from which they can be mobilized either as sulfide by reduction or by reaction with another protein containing a nucleophilic cysteine (transpersulfuration) [11]. These systems contain also scaffold proteins (SufA, IscA/U) which provide an intermediate assembly site for [Fe–S] clusters [12–15]. These proteins may be viewed as "cluster factories" in which clusters are assembled and from which they are subsequently transferred to apo recipient proteins. This notion has been challenged in the case of IscA since IscA was also shown to be an Fe-binding protein and was proposed to function as an Fe donor [16]. On the other hand the presence of a specific [Fe–S] cluster binding site in IscA consisting of the three conserved cysteines was demonstrated by X-ray crystallography [17]. Finally, the SUF and ISC systems contain helper proteins (HscA/B, Ferredoxin, SufBCD) which are endowed with ATPase or electron transfer activity [4,7,18,19].

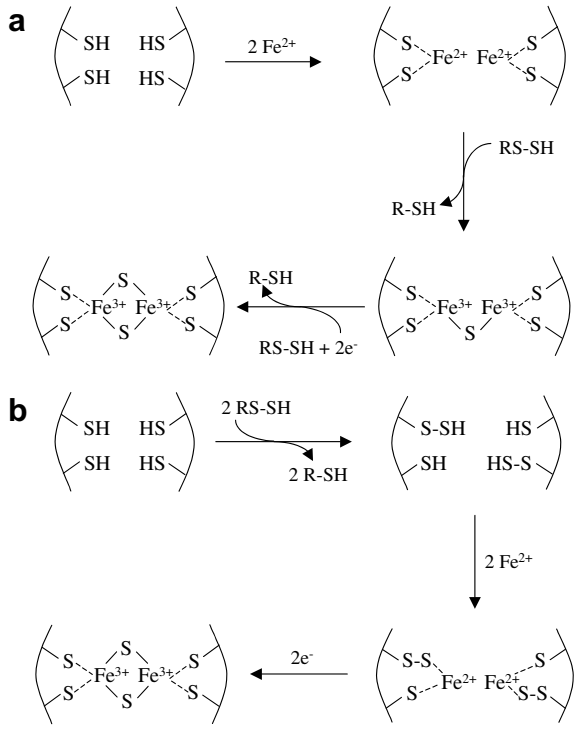
All scaffold proteins known so far are able to chelate labile iron–sulfur clusters during treatment of the apoprotein form with iron and sulfide under anaerobic conditions. Under these conditions they generally assemble at conserved cysteines a mixture of [4Fe–4S] and [2Fe–2S] clusters which are transferable to apoprotein targets through a concerted pathway not inhibited by iron chelators [12,13,20,21]. The origin and relevance of this cluster heterogeneity are still not understood. Furthermore, the molecular mechanism by which Fe and S are assembled into the scaffold protein at the cluster binding site is unknown.

We used the SUF system from *E. coli* as a model in order to address this last issue. Two tentative mechanisms have been previously postulated [22,23] and are illustrated in Scheme 1 in the case of [2Fe–2S] cluster assembly. In the first one ("Fe first, S second") (Scheme 1a) two iron atoms, in the ferrous redox state, are first chelated by the cysteine ligands. A sulfur atom is then, in a second step, transferred from the persulfide of a cysteine desulfurase to the scaffold Fe form generating a sulfide-bridged diferric species, during a reaction implying a 2-electron reduction of the sulfane sulfur to sulfide by the ferrous ions. A second S atom needs to be provided by the cysteine desulfurase and reduced by two additional electrons. In the second mechanism ("S first, Fe second") (Scheme 1b), the reaction starts with a transpersulfuration reaction during

*Corresponding authors. Fax: +33 4 38 78 91 24.
E-mail addresses: sollagnier@cea.fr (S. Ollagnier de Choudens), mfontecave@cea.fr (M. Fontecave).

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic; PCR, polymerase chain reaction

0014-5793/$32.00
doi:10.1016/j.febslet.2007.02.058



Scheme 1. Two mechanisms proposed for [2Fe–2S] cluster assembly within a scaffold protein. (a) the "Fe first, S second" model. (b) the "S first, Fe second" model (R–SH = the active cysteine of a cysteine desulfurase enzyme).

which the nucleophilic cysteines of the scaffold protein acquire the S atoms from the cysteine desulfurase by the attack of the persulfides, generating persulfides on the scaffold protein. In a second step, two ferrous ions get chelated by these persulfide moieties. To complete the reaction a 2-electron reduction of each of the sulfane S atoms are required.

Here, we show that SufA reacts with the sulfurated form of the SufE component of the cysteine desulfurase. The reaction consists in transfer of S atoms from persulfide/polysulfide species of SufE to the three conserved cysteines of SufA (cysteines 50, 114 and 116), as shown by mass spectrometry and site-directed mutagenesis. Our results favor the "S first, Fe second" mechanism.

2. Materials and methods

2.1. Materials and plasmids

All chemicals were of reagent grade and obtained from Sigma–Aldrich chemical Co. or Fluka unless otherwise stated. Cysteine was from Boehringer Mannheim. Plasmids pET-Shis, pET-Ehis and pET-Ahis, encoding the His-tagged SufS, SufE and SufA proteins, respectively, were obtained as previously described [11,20].

Plasmids pET-A_{C50S}his, pET-A_{C114S}his and pET-A_{C116S}his were obtained as follows using the QuickChange technique (Stratagene). Complementary mutagenic oligonucleotides (2 μM), designed such as cysteine is changed to a serine residue (see below), were used for polymerase chain reaction (PCR) amplification step, in the presence of the *Pfu* DNA polymerase (2.5 U), 1× Pfu Buffer, dNTP mix (0.4 mM) and pET-Ahis (20 ng) used as a matrix. The PCR was run as follows: the template pET-Ahis was denatured for 30 s at 94 °C, then the second step was performed using 18 cycles (30 s at 94 °C, 1 min at 45 °C, 13 min at 68 °C), followed by a final 10 min elongation step at 68 °C. The PCR product was digested with DpnI (10 U) for 1 h at 37 °C. Subsequently, competent DH5α were transformed with the mutant constructs. The cloned gene was then sequenced to ensure that no error was introduced during PCR reaction. The oligonucleotides are (the bold position indicates the mutations):

C50S-1: 5′-AAGCAAACGGGC**TCC**GCGGGCTTTGGC-3′
C50S-2: 5′-GCCAAAGCCCGC**GGA**GCCCGTTTGCTT-3′
C114S-1: 5′-GCCCAGAATGAA**TCT**GGCTGTGGCGAA-3′
C114S-2: 5′-TTCGCCACAGCC**AGA**TTCATTCTGGGC-3′
C116S-1: 5′-AATGAATGTGGC**TCT**GGCGAAAGCTTT-3′
C116S-2: 5′-AAAGCTTTCGCC**AGA**GCCACATTCATT-3′

Plasmid pET-A_{STOP} encoding the SufA protein lacking the His-tag ($SufA_{STOP}$) was also obtained using the QuickChange technique (Stratagene). The complementary oligonucleotides were designed such as a STOP codon is inserted before the bases encoding the histidine-tag. Then the conditions for the PCR amplification step are the same than above. The oligonucleotides are

STOP-1: GGCGAAAGCTTTGGGGTA**TAG**CTCGAGCACCAC-CACCAC
STOP-2: GTGGTGGTGGTGCTCGAG**CTA**TACCCCAAAGCTT-TCGCC

2.1.1. Purification of SufS, SufE, SufA, $SufA_{C50S}$, $SufA_{C114S}$ and $SufA_{C116S}$. *E. coli* SufS, SufE, SufA, $SufA_{C50S}$, $SufA_{C114S}$ and $SufA_{C116S}$ containing a his-tag at the C-terminus were isolated from *E. coli* (strain BL21(DE3)) as previously described [10,13].

2.1.2. Purification of $SufA_{STOP}$. *E.coli* BL21(DE3) cells were transformed with pET-A_{STOP} and $SufA_{STOP}$ expression was induced by adding 0.5 mM IPTG at OD_{600} = 0.5. After 3 h at 37 °C, the pellet from a 5-L culture was resuspended in buffer A (25 mM Tris–HCl, pH 7.5, 25 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM PMSF). After sonication (10 s × 12 times) and centrifugation (45 000 rpm for 1.5 h at 4 °C), the soluble proteins were treated with 2% streptomycine sulfate (30 min) and the solution centrifuged (10 000 rpm at 4 °C). The proteins (700 mg) were then precipitated with 40% ammonium sulfate. After centrifugation (10 000 rpm; 30 min) the pellet was resuspended in buffer B (25 mM Tris–HCl pH 7.5) and the resulting solution loaded onto a Superdex-75 column equilibrated with buffer C (100 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM DTT) at flow rate of 0.8 ml/min. The $SufA_{STOP}$ enriched fractions were pooled, concentrated and the pure protein (200 mg) stored at −80 °C.

2.2. Sulfur transfer assay from SufSE to SufA

All the experiments were done under anaerobic conditions inside a glove box (<4 ppm O_2, 19 °C). Two methods were used to obtain sulfurated form of SufA: the "stoichiometric" and the "catalytic" ones. For the first method, the sulfur transfer reaction was carried out using preparations of SufSE preloaded with S atoms, as previously described [11]. Briefly, 300 μM SufS, 300 μM SufE and 4 mM cysteine were incubated in buffer D (50 mM Tris–HCl, pH 7.5) for 30 min. The reaction was initiated by addition of cysteine and was stopped by removing the cysteine by desalting over a Micro Bio-spin 6 column (Biorad). The resulting form (sulfurated SufSE) was incubated with a 2-fold excess of SufA (wild-type or mutated proteins) for 30 min. The reaction was stopped by freezing in liquid nitrogen and proteins analyzed by mass spectrometry. The second method used catalytic amounts of SufSE: 200 μM SufA was incubated with 6 μM SufS and 6 μM SufE in the presence of 10 mM cysteine. After 3 h incubation at 37 °C, the solution was desalted over a Micro Bio-spin 6 column (Biorad) and frozen in liquid nitrogen. In the first method, SufSE is the only S donor to SufA. In the second method SufSE mediates S transfer from cysteine to SufA.

2.3. Digestion experiment

Sulfurated SufA (SufA–SSH) was digested by endoproteinase Lys-C (Roche Diagnostics Gmbh) at room temperature for 18 h with a 1:10 enzyme: protein ratio.

2.4. Fe binding to SufA

SufA (500 μM) was incubated anaerobically with 3 mM DTT in a final volume of 100 μL buffer E (100 mM Tris–HCl, pH 8, 50 mM KCl) for 10 min. Then, different molar excess (2, 4 or 8-fold molar excess) of $Fe(NH_4)_2(SO_4)_2$ were added. The protein was desalted on a NAP10 column (Amersham) using buffer E. The apparent iron association constant of SufA was determined using different iron (II) chelators (histidine and citrate) as iron competitor. Iron-loaded SufA

(450 μM) was incubated anaerobically with (0–100 mM) chelators for 1 h before SufA was repurified using Nap10 column. The amount of iron was determined for each concentration of chelator according to Fish method [24].

2.5. Mössbauer experiments

The SufA-^{57}Fe form was prepared as follows: SufA (750 μM) was incubated with 4-fold molar excess (3 mM) of ^{57}Fe(II) in the presence of 4 mM DTT for 90 min. The protein was desalted on a NAP10 column (buffer E). Concentrated SufA-^{57}Fe (660 μM) was introduced into the 400-μL Mössbauer cup, and frozen anaerobically. For the [FeS] cluster assembly, sulfurated wtSufA (1 mM; 1.45 S/monomer) was incubated with ^{57}Fe(II) (1.8 mM) in the presence or in the absence of 6 mM DTT. After desalting the protein was concentrated to 1.75 mM and analyzed. Spectra were recorded on a spectrometer operating in a constant acceleration mode using an Oxford cryostat that allowed temperatures from 1.5 to 300 K and a ^{57}Co source in rhodium.

2.6. Alkylation of SufA

Alkylation experiments were performed at room temperature either aerobically or anaerobically. The proteins (wild-type and cysteine-to-serine proteins) were incubated in buffer D during 3 h in the dark, in the presence of 25 molar excess of a freshly prepared solution of iodoacetamide. The reaction was stopped by removing the excess of iodoacetamide by desalting over a Micro Bio-spin 6 column and the solutions frozen in liquid nitrogen before LC–MS analysis.

2.7. LC–MS analysis

A Q-Tof (Q-TOF Micro, Waters) coupled with a Capl LC (Waters) was used for the LC–MS analysis. All samples were desalted on a trap (Michrom BioResources protein cap trap) and eluted using an analytical column (Poroshell 300SB-C8 0.5 × 75 mm 5μ, Agilent technologies). The eluant from the analytical column was sprayed on-line. The ion spray voltage was set to 3000 V. Sample and extraction cone were set, respectively, at 40 V and 1 V. The mass spectra were acquired from *m/z* 500 to 2000 with a 1 s scan time and data were processed with MassLinx 4.0 (Waters). This method was used to analyze the alkylated protein. The data are presented as deconvoluted mass spectra.

2.8. Infusion-MS analysis

A Q-TOF Micro mass spectrometer equipped with a Z-spray ion source (Micromass, Manchester, UK), operating with a needle voltage of 3 kV was used to analyze few samples. Sample cone and extraction voltages were 70 and 3.5 V, respectively. Samples were infused continuously at a 5 μl/min flow rate with a concentration between 400 and 900 nM in water/acetonitrile (1/1, v/v) with 0.2% formic acid. The mass spectra were recorded in the 700–1600 range of mass-to-charge (*m/z*) with a 1 s scan time. A 1 μM solution of Glu-fibrinopeptide B was used to calibrate the instrument in the MS/MS mode and processed with MassLinx 4.0 (Waters). This method was used to monitor sulfur transfer from SufSE to SufA (wild-type and mutants). Complexity of spectra did not allow deconvolution. Then the results are presented as a diagram giving the different proportions of sulfurated forms vs apo, calculated on the basis of three consecutive charge states.

2.9. MALDI-TOF

MALDI-TOF analyses were carried out on an Applied Biosystems Voyager EliteXLmass spectrometer. Sample of SufA-SSH was deposited on the Maldi plate according to the dry droplet mode using a semi saturated solution of α-cyano-4-hydroxy-cinnamic acid.

2.10. Analysis

Protein concentration (by monomer) was determined by the method of Bradford, whereas iron and sulfide contents were determined according to Fish and Beinert methods [24–26].

3. Results

3.1. Fe binding to SufA

Anaerobic incubation of the apoprotein his-tagged form of SufA (wtSufA) with an excess of ferrous or ferric iron (up to 8-fold) in the presence or in the absence of DTT followed by treatment with one equivalent of ethylenediaminetetraacetic (EDTA) with regard to Fe and desalting resulted in a protein essentially devoided of Fe. The same was observed with cysteine-to-serine mutant (mSufA) proteins. When treatment with EDTA was omitted wtSufA could retain after desalting approximately 1.5–2 Fe atoms/monomer. $SufA_{STOP}$ lacking the His-tag could bind similar amounts of iron/monomer under the same conditions. In addition, wtSufA and $SufA_{STOP}$ treated with ^{57}Fe displayed the same Mössbauer spectrum (see below). This ruled out the tag as being the Fe-binding site. The same amounts of protein-bound Fe were also obtained in the case of mSufA proteins and of alkylated SufA, obtained by reaction of wtSufA with iodoacetamide and complete alkylation of the three cysteines as shown by mass spectrometry (see Supplementary material 1). These results indicate that SufA cysteines are not or marginally involved in Fe binding.

To further characterize the Fe coordination the experiment using wtSufA and alkylated SufA was repeated with ferrous ^{57}Fe as an iron source and the samples analyzed by Mössbauer spectroscopy at 105 K and 4.2 K. The spectra at 105 K of both forms are shown in Fig. 1. In both cases characteristic doublets are observed with an average isomer shift which is consistent with high spin ferrous sites. In the alkylated form (lower pattern) the spectrum is analyzed assuming two doublets A and B with the parameters quoted in Table 1. These parameters are consistent with high spin ferrous sites in octahedral environment comprising N or O donors. For the non-alkylated sample (upper pattern) the spectrum is slightly more complicated and, in addition to doublets A and B, two other species are present. These species have been simulated assuming two additional doublets C and D with the parameters listed in Table 1. Doublet C has parameters which are consistent with tetrahedral, high spin Fe^{2+} with S donors accounting for less than 20% of total iron. The absence of doublet C in the alkylated sample suggests that this doublet is associated with the conserved cysteines. Finally doublet D, responsible for the weak absorption at ca. +0.7 mm s^{-1}, accounts for not more than 6% of iron, with parameters consistent with high spin ferric species. These results show that binding of Fe to SufA is unspecific and only marginally involves the conserved cysteines.

To examine iron binding by SufA further, we determined the iron association constant in competition experiments using citrate as an iron(II) chelator. Iron-loaded SufA (450 μM) was incubated anaerobically with (0–100 mM) of citrate for 1 h and repurified using a Nap10 column. The amount of iron was determined for each concentration of chelator. Using for the association constant for the citrate–Fe(II) complex the value of $10^{4.8}$ M^{-1} [27] we estimated that the apparent iron association constant of SufA is approximately 5×10^5 M^{-1}. A comparable value (2×10^5 M^{-1}) was determined with histidine used as Fe(II) chelator. This indicates a weak binding of Fe(II) to SufA.

3.2. Transfer of sulfur from SufSE complex to SufA

We then analyzed the ability of SufA to bind sulfur atoms, provided by the cysteine desulfurase SufSE complex. In the so-called "catalytic" experiment, wtSufA (200 μM) was incubated for 3 h at 37 °C with catalytic amounts of the SufS–SufE (6 μM each) system in the presence of an excess of cysteine (10 mM). After desalting, SufA was analyzed by infusion-MS as described in the experimental section. Direct sulfur transfer

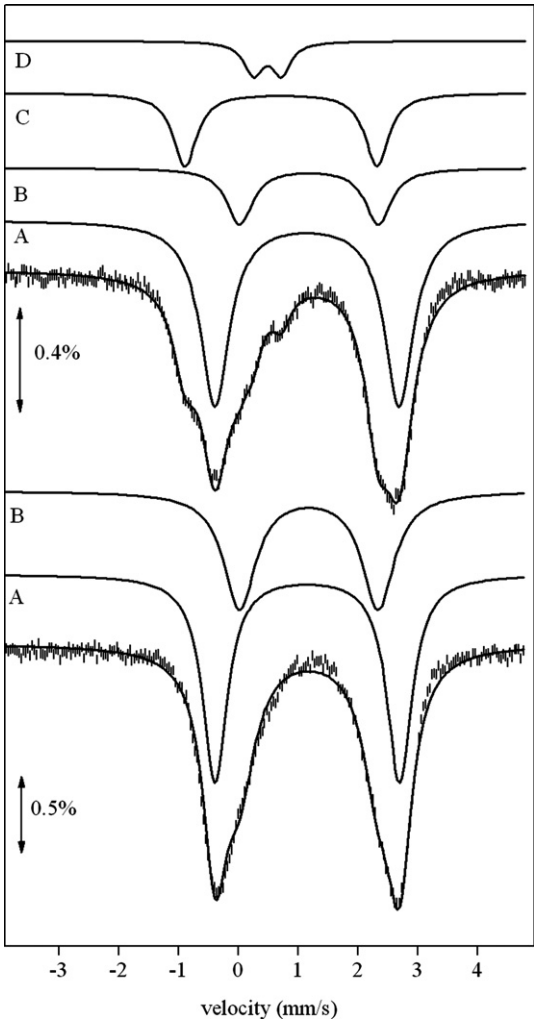


Fig. 1. Mössbauer spectra of the SufA-^{57}Fe form (660 μM, 1.5 iron/monomer) recorded at 105 K. The upper and lower patterns correspond to the non-alkylated and alkylated forms, respectively. Solid lines represent theoretical simulations assuming the doublets defined in Table 1.

from SufSE to wtSufA, "stoichiometric" experiment, was also demonstrated. In that case, wtSufA was treated with one equivalent of the sulfurated form of SufSE, prepared as previously described [11], in the absence of cysteine for 30 min at 37 °C and then analyzed by mass spectrometry. Considering that for all mass spectra (spectra of apo and sulfurated forms), the charge states distribution and their relative abundances were the same, we compared the area of the peaks corresponding to apoSufA or sulfurated SufA to have an approximation of their relative abundances. Due to the complexity of the infusion-MS spectra (see Supplementary material 2) deconvoluted spectra could not be delivered. As a consequence, for each peak we checked that the width at half of the peaks were similar and then, for each sulfurated form, an average of the peak intensity was made for three consecutive charge states. Considering that the sum of all peaks is equal to 100 we obtained Figs. 2 and 3 which summarize the obtained results in terms of the proportions of the different sulfurated forms obtained in SufA (wild-type and mutants). In the "catalytic" experiment (Fig. 2 "cat") three sulfurated states for SufA appeared, corresponding to the addition of 1, 2 and 3 sulfur atoms to the protein whereas the peak corresponding to apoSufA greatly decreased. Quantitation of mass spectrometry data based on areas of individual peaks indicated a net addition corresponding to 1.5 S atoms/ SufA monomer. Addition of DTT (Fig. 2 "DTT") or treatment with NADPH–thioredoxin reductase–thioredoxin (data not shown) converted the protein back to the initial apoform. In the "stoichiometric" experiment as well (Fig. 2 "stoichio"), in addition to the apo form, the 3 forms corresponding to the addition of 1, 2 and 3 sulfur atoms to SufA were observed. Quantitation of mass spectrometry data indicated a net addition corresponding to 1.5 S atoms/SufA monomer. Sulfur transfer was fast since the same spectrum was already obtained after 5 min incubation. No addition of sulfur could be observed when SufE was omitted from the reaction mixtures described above or when wtSufA was simply treated with sodium sulfide as a

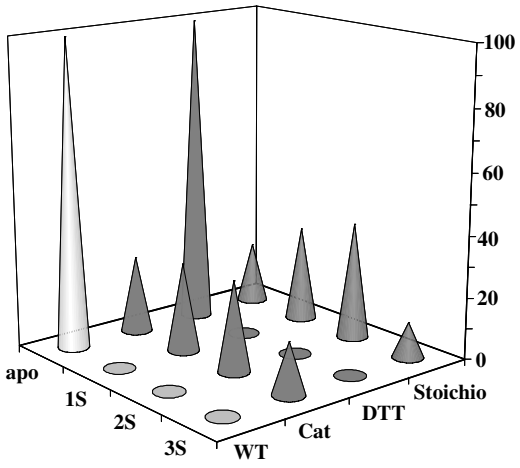


Fig. 2. SufA is able to bind sulfur atoms provided by SufSE. Pattern representing the relative abundance for the apo and sulfurated forms of wtSufA (200 μM) before ("WT") and after incubation with either a catalytic amount of SufSE (6 μM) and 10 mM cysteine ("Cat") or a stoichiometric amount of sulfurated SufSE (100 μM) in the absence of cysteine ("stoichio"). ("DTT"): experiment ("cat") incubated with 5 mM DTT for 10 min and desalted.

Table 1
Mössbauer parameters for the SufA-Fe samples

Sample	Site	Assignment	δ (mm s^{-1})	ΔE_Q (mm s^{-1})	Area (%)
Alkylated SufA-Fe	A	Fe^{2+}(S = 2)-octahedral N/O	1.15(3)	3.07(6)	58(3)
	B	Fe^{2+}(S = 2)-octahedral N/O	1.18(3)	2.30(6)	42(3)
Non-alkylated SufA-Fe	A	Fe^{2+}(S = 2)-octahedral N/O	1.15	3.07	59(5)
	B	Fe^{2+}(S = 2)-octahedral N/O	1.18	2.30	16(5)
	C	Fe^{2+}(S = 2)-tetrahedral S	0.71(4)	3.20(6)	19(5)
	D	Unknown ferric species	0.49(5)	0.46(10)	6(3)

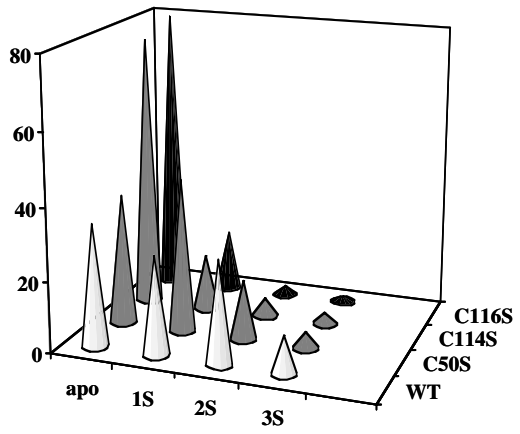


Fig. 3. Relative abundance for the sulfurated forms of wild-type and cysteine-to-serine mutant SufA proteins. Proteins (100 μM) were analyzed by infusion-MS after incubation with stoichiometric amounts of sulfurated SufSE for 30 min.

chemical sulfur donor. This clearly established the ability of wtSufA to extract sulfur from SufSE, probably through a transpersulfuration reaction.

The same experiment ("stoichiometric" experiment) was repeated with mSufA proteins and analyzed by infusion-MS as well. The results are summarized in Fig. 3. $SufA_{C50S}$ behaved as the wtSufA and could incorporate up to 3 S atoms on a monomer. This result thus indicated that more than one S atom could bind to a single cysteine and that, in all probability, polysulfide species rather than persulfide were generated under the reaction conditions. In contrast, in the case of $SufA_{C114S}$ and $SufA_{C116S}$ proteins the major peak was that corresponding to the apo form and very little sulfur could be incorporated. Quantitation of mass spectrometry data indicated an addition corresponding to 0.2 S atoms/SufA monomer. These results suggested that Cys114 and Cys116 were the sites of multiple S binding and that almost no S could be transferred directly to Cys50.

To further confirm the localization of the sulfur binding sites, the sulfurated form of wtSufA was digested with endoproteinase-Lys C and the resulting peptides analyzed by MALDI-MS (data not shown), following a protocol used to map sulfur binding sites in ThiI [28]. Spectra of the peptides containing either Cys50 or the C-terminal cysteines, Cys114 and Cys116, showed the presence of peaks corresponding to sulfurated forms of the peptides. All these experiments can be interpreted as follows: (i) the three conserved cysteines of SufA are the exclusive sulfur acceptors during sulfur transfer from SufSE, (ii) when Cys50 is substituted for serine, polysulfide species can be generated on Cys114 or Cys116, and (iii) sulfuration of Cys50 is very likely generated via an internal sulfur transfer from Cys114 or Cys116.

We have also studied the sulfur transfer reaction from the sulfurated form of SufSE ("stoichiometric" conditions) to the iron-loaded form of SufA. After reaction the mass spectrum of SufA was identical to that of apoSufA treated similarly (Fig. 2), showing that iron does not prevent the cysteine residues from binding sulfur atoms derived from SufSE and thus supporting the conclusion that cysteines preferentially bind sulfur.

3.3. Reactivity of sulfurated SufA: [Fe–S] cluster assembly

When sulfurated wtSufA (1.45 S/monomer), obtained after treatment of wtSufA with catalytic amount of SufSE and cysteine in excess and a desalting step, was anaerobically treated with ferrous ^{57}Fe in slight excess (1.8 Fe/monomer) and analyzed by Mössbauer spectroscopy after desalting, no evidence for the formation of [Fe–S] clusters was observed (not shown). In contrast, when DTT or the NADPH–thioredoxin reductase–thioredoxin system was introduced in the reaction mix-

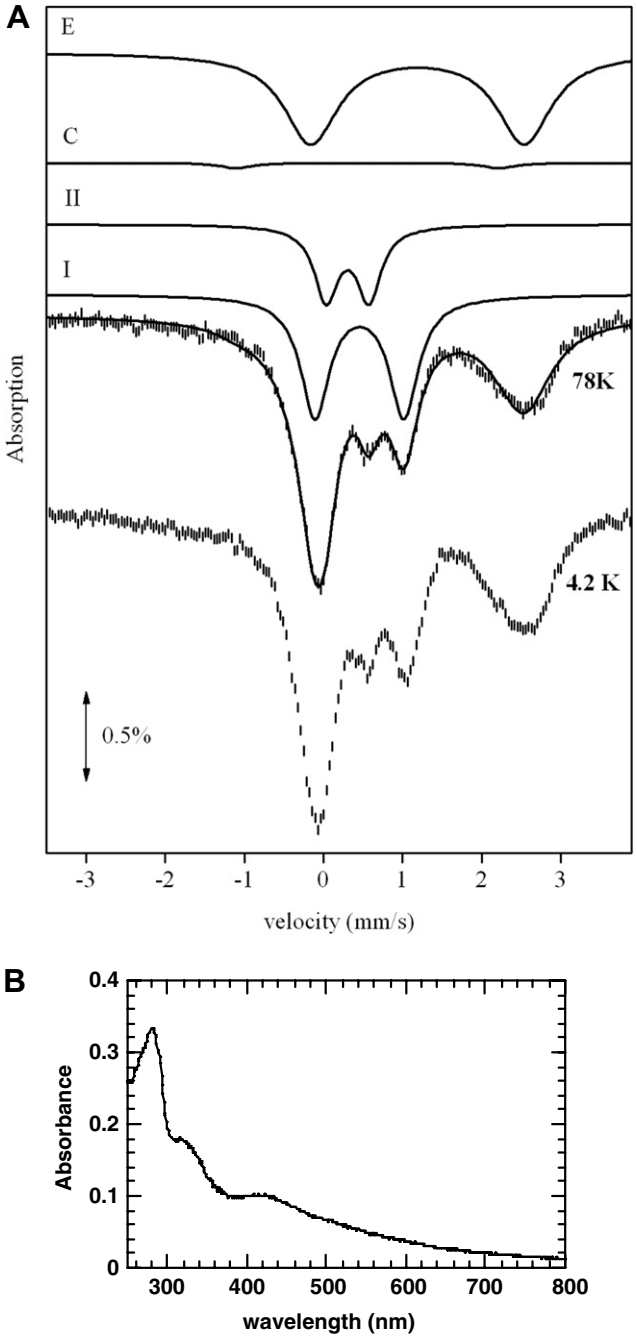


Fig. 4. (A) Mössbauer spectra recorded at 4.2 and 78 K of sulfurated SufA after a 5 min reaction with 1.8-fold excess of ferrous ^{57}Fe in the presence of 6 mM DTT and desalting. The final protein (1.75 mM) contains 1.5 iron/monomer. Solid lines represent theoretical simulations assuming the doublets as described in the text. (B) UV–visible spectrum of [Fe–S]-containing SufA Mössbauer sample (25 μM).

Table 2
Quantitation of [Fe–S] clusters (percentage of total iron) generated from sulfurated SufA or from apoSufA as starting material

	[4Fe–4S] Area (%)	[2Fe–2S] Area (%)	Fe^{2+} (octa and tetra) Area (%)
Sulfurated SufA + Fe^{2+} + e^-	35	15	50
SufA + Fe + S^{2-} + e^-	58	<2	40

ture efficient utilization of the SufA-bound sulfur atoms for fast formation of clusters was observed as shown by the appearance of an absorption band at 420 nm in UV–vis spectrum (Fig. 4B). In Fig. 4A we show Mössbauer spectra of the protein after 5 min reaction recorded at 4.2 K and 78 K. These spectra did not change upon further incubation. The fact that the two spectra were similar strongly suggested that the protein only contained diamagnetic (S = 0) species. The spectrum contains two doublets labelled I and II whose isomer shift and quadrupole splitting parameters are consistent with a $[4Fe–4S]^{2+}$ cluster (δ = 0.45 mm/s and ΔE_Q = 1.2 mm/s, accounting for 35(3) % of total Fe) for doublet I and a $[2Fe–2S]^{2+}$ (δ = 0.3 mm/s and ΔE_Q = 0.6 mm/s, accounting for 15(3) % of total Fe) for doublet II (Table 2). These parameters are comparable to those obtained previously for SufA [13]. The rest of the Fe (doublets C and E) corresponds to unreacted ferrous iron in excess, unspecifically bound to SufA in the form of tetrahedral Fe^{2+} (δ = 0.71 mm/s, ΔE_Q = 3.2 mm/s, 3–5% of total iron) and octahedral Fe^{2+} (doublet E) with N/O coordination (δ = 1.19 mm/s, ΔE_Q = 2.69 mm/s, 45–47% of total iron). This result shows that the sulfur atoms present in sulfurated SufA can be mobilized and react with Fe to assemble [4Fe–4S] and [2Fe–2S] clusters. In comparison, the standard reconstitution reaction of wtSufA (1.2 mM) with $^{57}Fe^{2+}$ (2.1 mM), sulfide (2.1 mM) and DTT (5 mM) (after 5 min incubation and desalting) resulted in significantly different proportions of clusters (Table 2).

4. Discussion

Here, we address the question of the mechanism of iron–sulfur cluster assembly within scaffold proteins. This class of proteins belongs to the complex cellular assembly machineries involved in the maturation of [Fe–S] enzymes. Their specific function resides in the mobilization of Fe and sulfur atoms from corresponding sources, the assembly of defined clusters from these precursors and finally transfer of the newly formed clusters to apotargets [23]. Using SufA, the scaffold protein from the SUF system, as a model we have clearly established the following fact.

Binding of S atoms provided by the cysteine desulfurase SufS–SufE system specifically occurs at the three conserved cysteines (cys50, cys114, cys116) involved in cluster chelation. The finding that a scaffold protein, here SufA, mobilizes S atoms from persulfide-polysulfide species bound to cysteine desulfurases, here the SufS–SufE system, to generate persulfide–polysulfides on the three conserved cysteines, as shown by mass spectrometry, has a precedent. Indeed it has been recently shown, also by mass spectrometry, that S atom transfer occurs from the cysteine desulfurase IscS to the scaffold protein IscU [29]. However, we found at least one difference between the two systems. Indeed, whereas all cysteines of IscU can be sulfurated independently, it seems that only the C-terminal cysteines of the conserved CGC sequence of SufA, Cys114 and Cys116, can directly take S atoms from SufE, as shown by site-directed mutagenesis and that Cys50 takes sulphur atoms indirectly via Cys114 or Cys116. Interestingly, this differentiation with regard to cysteines in SufA (Cys114/116 and Cys50) is structurally relevant. Indeed, the recently reported crystal structure of apoSufA clearly shows that Cys114 and Cys116, close to each other, set apart from Cys50 [30]. These observations likely suggest that Cys114 and Cys116 are the sulfur atom recipients while Cys50 is too far away and more likely gets sulfurated by subsequent intramolecular sulfur transfer. The significance of this is not understood yet. Furthermore, our results are consistent with a single cysteine of SufA being able to bind several S atoms. Thus, polysulfide species have to be considered. This is not new either. Polysulfides have been observed in the sulfurated form of IscU [29]. This is also true in the case of SufE and CsdE which contain a single conserved cysteine and can bind up to 5 and 2 S atoms during reaction with SufS and CsdA, respectively [11,31]. The question is whether these polysulfide species are physiologically relevant or instead are due to the in vitro conditions which do not allow a tight control of S transfer reactions.

In contrast, SufA binds Fe only weakly and unspecifically. Indeed, we determined a binding constant of $5 \times 10^5\ M^{-1}$, which is much less than that for IscA whose K_a was determined to be $3 \times 10^{19}\ M^{-1}$ [16]. In addition, Mössbauer experiments performed on SufA indicate that Fe is coordinated mainly by N/O atoms in agreement with the weak K_a value whereas in IscA iron is coordinated by thiolate from conserved cysteines as suggested by site-directed mutagenesis experiments [32]. Clearly, SufA and IscA proteins have different iron binding properties (affinity and ligands). Considering the homology between the two proteins, this difference is intriguing. However, it should be mentioned that IscA displays specific Fe binding properties only in the presence of reducing agents [16,32,33], and that no Mössbauer spectroscopic characterization of the IscA-Fe form has been carried out so far.

The properties of Fe-SufA mentioned above lead us to propose that the cysteines of SufA are S atom acceptors rather than Fe ligands. As a consequence we favour the "S first, Fe second" mechanism (Scheme 1b) for the assembly of [Fe–S] clusters in SufA active site. We are nevertheless cautious that the Fe binding properties of SufA might be significantly changed under more complex conditions, reflecting the physiological conditions more precisely. Preliminary experiments using a physiological iron donor such as CyaY, the frataxin homolog [34], or a mixture of proteins likely to be associated with SufA, such as the SufBCD complex, do not show changes in the Fe binding properties of SufA (not shown).

In this context, it is interesting to observe that the reaction of the SufA-bound S atoms with ferrous iron, in the presence of a reducing agent, results in [4Fe–4S] and [2Fe–2S] clusters in SufA. This is the first example of the assembly of defined clusters within a scaffold protein during reaction of a sulfurated form of the protein with ferrous iron. This reaction requires an additional source of electrons since ferrous Fe seems to be not competent for reduction of the intermediate persulfide-polysulfide species. No cluster was formed either when sulfurated IscU was reacted with ferrous iron [29]. The reaction reported here results in a mixture of [4Fe–4S] and

[2Fe–2S] clusters in SufA. The fact that a different proportion of clusters is produced when apoSufA was treated with ferrous iron, sulfide and electrons suggests that the reaction does not proceed just through redox-dependent liberation of sulfide in solution (Table 2). The detailed mechanisms of cluster formation should be further investigated.

Acknowledgement: We thank Guy Serratrice for help and advices for the determination of iron binding constant of SufA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.02.058.

References

[1] Johnson, M.K. (1998) Iron-sulfur proteins: new roles for old clusters. Curr. Opin. Chem. Biol. 2, 173–181.
[2] Fontecave, M., Choudens, S.O., Py, B. and Barras, F. (2005) Mechanisms of iron-sulfur cluster assembly: the SUF machinery. J. Biol. Inorg. Chem., 1–9.
[3] Zheng, L., Cash, V.L., Flint, D.H. and Dean, D.R. (1998) Assembly of iron–sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from *Azotobacter vinelandii*. J. Biol. Chem. 273, 13264–13272.
[4] Takahashi, Y. and Nakamura, M. (1999) Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe–S clusters in Escherichia coli. J. Biochem. (Tokyo) 126, 917–926.
[5] Lill, R. and Kispal, G. (2000) Maturation of cellular Fe–S proteins: an essential function of mitochondria. Trends Biochem. Sci. 25, 352–356.
[6] Tong, W.H. and Rouault, T. (2000) Distinct iron–sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. 19, 5692–5700.
[7] Nachin, L., Loiseau, L., Expert, D. and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe–S] biogenesis under oxidative stress. EMBO J. 22, 427–437.
[8] Outten, F.W., Djaman, O. and Storz, G. (2004) A suf operon requirement for Fe–S cluster assembly during iron starvation in *Escherichia coli*. Mol. Microbiol. 52, 861–872.
[9] Mihara, H. and Esaki, N. (2002) Bacterial cysteine desulfurases: their function and mechanisms. Appl. Microbiol. Biotechnol. 60, 12–23.
[10] Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M. and Barras, F. (2003) Biogenesis of Fe–S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. J. Biol. Chem. 278, 38352–38359.
[11] Ollagnier-de-Choudens, S., Lascoux, D., Loiseau, L., Barras, F., Forest, E. and Fontecave, M. (2003) Mechanistic studies of the SufS–SufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE. FEBS Lett. 555, 263–267.
[12] Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y. and Fontecave, M. (2001) Iron-sulfur cluster assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. J. Biol. Chem. 276, 22604–22607.
[13] Ollagnier-de Choudens, S., Nachin, L., Sanakis, Y., Loiseau, L., Barras, F. and Fontecave, M. (2003) SufA from *Erwinia chrysanthemi*. Characterization of a scaffold protein required for iron–sulfur cluster assembly. J. Biol. Chem. 278, 17993–18001.
[14] Krebs, C., Agar, J.N., Smith, A.D., Frazzon, J., Dean, D.R., Huynh, B.H. and Johnson, M.K. (2001) IscA, an alternate scaffold for Fe–S cluster biosynthesis. Biochemistry 40, 14069–14080.
[15] Agar, J.N., Krebs, C., Frazzon, J., Huynh, B.H., Dean, D.R. and Johnson, M.K. (2000) IscU as a scaffold for iron–sulfur cluster biosynthesis: sequential assembly of [2Fe–2S] and [4Fe–4S] clusters in IscU. Biochemistry 39, 7856–7862.
[16] Ding, H. and Clark, R.J. (2004) Characterization of iron binding in IscA, an ancient iron–sulphur cluster assembly protein. Biochem. J. 379, 433–440.
[17] Morimoto, K., Yamashita, E., Kondou, Y., Lee, S.J., Arisaka, F., Tsukihara, T. and Nakai, M. (2006) The asymmetric IscA homodimer with an exposed [2Fe–2S] cluster suggests the structural basis of the Fe–S cluster biosynthetic scaffold. J. Mol. Biol. 360, 117–132.
[18] Outten, F.W., Wood, M.J., Munoz, F.M. and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe–S cluster assembly in *Escherichia coli*. J. Biol. Chem. 278, 45713–45719.
[19] Silberg, J.J., Tapley, T.L., Hoff, K.G. and Vickery, L.E. (2004) Regulation of the HscA ATPase reaction cycle by the co-chaperone HscB and the iron-sulfur cluster assembly protein IscU. J. Biol. Chem. 279, 53924–53931.
[20] Ollagnier-de-Choudens, S., Sanakis, Y. and Fontecave, M. (2004) SufA/IscA: reactivity studies of a class of scaffold proteins involved in [Fe–S] cluster assembly. J. Biol. Inorg. Chem. 9, 828–838.
[21] Wollenberg, M., Berndt, C., Bill, E., Schwenn, J.D. and Seidler, A. (2003) A dimer of the FeS cluster biosynthesis protein IscA from cyanobacteria binds a [2Fe2S] cluster between two protomers and transfers it to [2Fe2S] and [4Fe4S] apo proteins. Eur. J. Biochem. 270, 1662–1671.
[22] Fontecave, M., Choudens, S.O., Py, B. and Barras, F. (2005) Mechanisms of iron–sulfur cluster assembly: the SUF machinery. J. Biol. Inorg. Chem. 10, 713–721.
[23] Johnson, D.C., Dean, D.R., Smith, A.D. and Johnson, M.K. (2005) Structure, function, and formation of biological iron–sulfur clusters. Annu. Rev. Biochem. 74, 247–281.
[24] Fish, W.W. (1988) Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. Meth. Enzymol. 158, 357–364.
[25] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
[26] Beinert, H. (1983) Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron–sulfur proteins. Anal. Biochem. 131, 373–378.
[27] Field, T.M., McCourt, J.L. and McBryde, W.A.E. (1974) Composition and stability of iron and copper citrate complexes in aqueous solution. Can. J. Chem. 52, 3119–3124.
[28] Wright, C.M., Christman, G.D., Snellinger, A.M., Johnston, M.V. and Mueller, E.G. (2006) Direct evidence for enzyme persulfide and disulfide intermediates during 4-thiouridine biosynthesis. Chem. Commun. (Camb.), 3104–3106.
[29] Smith, A.D., Frazzon, J., Dean, D.R. and Johnson, M.K. (2005) Role of conserved cysteines in mediating sulfur transfer from IscS to IscU. FEBS Lett. 579, 5236–5240.
[30] Wada, K., Hasegawa, Y., Gong, Z., Minami, Y., Fukuyama, K. and Takahashi, Y. (2005) Crystal structure of *Escherichia coli* SufA involved in biosynthesis of iron–sulfur clusters: implications for a functional dimer. FEBS Lett. 579, 6543–6548.
[31] Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M. and Barras, F. (2005) Analysis of the heteromeric CsdA–CsdE cysteine desulfurase, assisting Fe–S cluster biogenesis in *Escherichia coli*. J. Biol. Chem. 280, 26760–26769.
[32] Ding, H., Clark, R.J. and Ding, B. (2004) IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible free iron conditions. J. Biol. Chem. 279, 37499–37504.
[33] Ding, H., Harrison, K. and Lu, J. (2005) Thioredoxin reductase system mediates iron binding in IscA and iron delivery for the iron-sulfur cluster assembly in IscU. J. Biol. Chem. 280, 30432–30437.
[34] Layer, G., Ollagnier-de Choudens, S., Sanakis, Y. and Fontecave, M. (2006) Iron–sulfur cluster biosynthesis: characterization of *Escherichia coli* CYaY as an iron donor for the assembly of [2Fe–2S] clusters in the scaffold IscU. J. Biol. Chem. 281, 16256–16263.