Crystal Structure of *Bacillus subtilis* Cysteine Desulfurase SufS and Its Dynamic Interaction with Frataxin and Scaffold Protein SufU

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**Abstract**

The biosynthesis of iron sulfur (Fe-S) clusters in *Bacillus subtilis* is mediated by a SUF-type gene cluster, consisting of the cysteine desulfurase SufS, the scaffold protein SufU, and the putative chaperone complex SufB/SufC/SufD. Here, we present the high-resolution crystal structure of the SufS homodimer in its product-bound state (i.e., in complex with pyrodoxal-5'-phosphate, alanine, Cys361-persulfide). By performing hydrogen/deuterium exchange (H/DX) experiments, we characterized the interaction of SufS with SufU and demonstrate that SufU induces an opening of the active site pocket of SufS. Recent data indicate that frataxin could be involved in Fe-S cluster biosynthesis by facilitating iron incorporation. H/DX experiments show that frataxin indeed interacts with the SufS/SufU complex at the active site. Our findings deepen the current understanding of Fe-S cluster biosynthesis, a complex yet essential process, in the model organism *B. subtilis*.

**Introduction**

Iron sulfur (Fe-S) clusters are amongst the most versatile enzyme cofactors in Nature, as they are involved in cellular respiration, carbohydrate metabolism, DNA repair and various other vital functions throughout all kingdoms of life [1–4]. The biosynthesis of Fe-S clusters must be tightly regulated because of the toxicity of free sulfur and iron. Therefore, the systems for Fe-S cluster biogenesis are mainly conserved from bacteria to human, although elaborate transport systems have diverged through evolution [5–7]. Three distinct systems have been described for prokaryotic cells: i.) the NIF system (nitrogen fixation, [8]), ii.) the ISC system (iron-sulfur cluster, [9]) and iii.) the SUF system (sulfur mobilization, [10]). While the NIF system is specific for nitrogenase maturation in azototrophic bacteria, the ISC and/or SUF systems function in housekeeping in most—if not all—bacteria. In some bacterial species (*e.g.*, *Escherichia coli*), both systems are found, while others (*e.g.*, *B. subtilis*) rely on a single type of Fe-S machinery.
In eukaryotic cells, Fe-S biosynthesis takes place in the mitochondria in an ISC-like system [6] and in plastids in a SUF-like system [11]. In all of these systems, the Fe-S cluster is formed on a scaffold protein before it is transferred to a target apoprotein [12]. A cysteine desulfurase acquires sulfur from cysteine in a pyrodoxal-5'-phosphate (PLP)-dependent reaction and then transfers it as persulfide to the scaffold protein [13,14]. In the E. coli SUF and eukaryotic systems, auxiliary proteins (SufS/SufE and Nfs1/Isd11, respectively) were found to enhance the activity of the cysteine desulfurase and aid in persulfide transfer [15,16]. The assembly also relies on electron transport, most likely for the reduction of sulfane (S0) to sulfide (S2). For this purpose, it was shown that the E. coli ISC system utilizes ferredoxin [17], while the E. coli SUF system relies on a scaffold protein (SufB) associated with FAD [15] (in the latter case, however, in vitro experiments suggested a role in reduction of ferric iron rather sulfur) [18]. The mechanism of iron insertion remains elusive. It has been suggested that the highly conserved protein frataxin might act as the iron donor, although this role is still under debate. Structural analysis showed that frataxin assumes an α/β fold in which the N-terminal α-helix consists of several acidic residues, commonly referred to as the ‘acidic ridge’ [19–23]. Deletions of frataxin in Saccharomyces cerevisiae result in accumulation of iron in the mitochondria and drastically decreases biosynthesis of Fe-S clusters [24]. Frataxin can bind iron and interact with the cysteine desulfurase as well as the Fe-S scaffold protein. These observations led to the suggestion that frataxin could act as an iron chaperone [25]. The latter hypothesis was challenged by the finding that deletion of the E. coli gene encoding frataxin does not lead to iron accumulation or a decrease in Fe-S clusters as observed in S. cerevisiae, even though E. coli frataxin also forms a complex with SufS/SufU homologs IscS/IscU [26,27]

In Bacillus subtilis (B), a frataxin homolog Fra (formerly YdhG) shares only little sequence identity with other frataxins. However, structural analysis of BsFra showed a conserved α/β sandwich fold with a cluster of acidic residues on the N-terminal α1 and α2 helices, forming an “acidic ridge” [28]. BsFra was found to bind two equivalents of iron with a moderate K_d and to interact with the Thermotoga maritima (Tm) iron-sulfur cluster scaffold protein TmlSu [28]. Based on deletion mutants, it was suggested that BsFra is a global iron regulator involved in the distribution of iron in B. subtilis [29]. Previously, the cysteine desulfurase BsSufS and putative scaffold protein BsSufU were characterized in vivo and in vitro, and it was shown that BsFra can be utilized as an iron source [28–31]. We recently demonstrated that BsFra interacts with the ferrochelatase HemH and is crucial for the incorporation of iron into protoporphyrin [32]. However, the role of frataxin in delivery of iron to SufS/SufU is still poorly understood; in particular, in Fe-S biosynthesis, no interaction between BsFra and the BsSufS/BsSufU complex has been observed so far. Herein, we demonstrate that BsFra can indeed interact with BsSufS and BsSufU, as characterized by hydrogen/deuterium exchange (H/DX) experiments. Furthermore, we present the crystal structure of BsSufS and suggest a model for the protein complex consisting of BsSufS/BsSufU/BsFra.

**Material and Methods**

**Protein expression and purification**

Plasmids for heterologous expression were previously prepared [30,33]. The heterologous expression of fra [29,32], sufU [30] and sufS [31] was carried out in E. coli BL21(DE) cells for 20 h at 22°C in LB (lysogeny broth) medium with 50 μg/mL kanamycin and 0.2 mM IPTG. Cells were collected and washed in HEPES buffer A (50 mM HEPES, pH 8, 300 mM NaCl, 5 mM imidazole), treated with DNaseI, and lysed with a French press. The crude extract was cleared by centrifugation (17000 rpm, 4°C, 30 min) and the supernatant was filtered (0.25 μm). The target protein was purified by Ni-affinity chromatography on an FPLC system (NGC

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Quest, Biorad) using a gradient of 5–100% HEPES buffer B (50 mM HEPES, pH 8, 300 mM NaCl, 250 mM imidazole) over 20 min. The elution fractions were concentrated and further purified by size exclusion chromatography (HiLoad 26/60 Superdex 200, GE Healthcare Life Sciences) in HEPES buffer C (50 mM HEPES, pH 8, 300 mM NaCl). Fractions containing the target protein were concentrated, flash frozen in liquid nitrogen and stored at -80°C in HEPES buffer C supplemented with 10% glycerol.

**Crystallization, data collection, and structure determination**

Crystallization was performed by the sitting-drop method at 20°C in 0.6-μl drops consisting of equal parts protein and crystallization solutions. BsSufS crystallized at 20 mg/ml within one week in 0.1 M HEPES, pH 7.5, 50% (v/v) PEG 400. Prior to data collection, crystals were cryo-protected in a solution consisting of the well solution supplemented with 20% glycerol and then flash-frozen in liquid nitrogen. Data were collected under cryogenic conditions at the European Synchrotron Radiation Facility at Beamline ID29. Data were processed with XDS [34] and scaled with CCP4-implemented SCALA [35]. The structure was determined by molecular replacement with PHASER [36], manually built in COOT [37], and refined with PHENIX [38]. The SufS homolog from *Brucella suis* (PDB ID 4W91) was employed as a search model. Figures were prepared with Pymol (www.pymol.org). Coordinates and structure factors were deposited in the Protein Data Bank with the accession code 5J8Q.

**Hydrogen/deuterium exchange experiments**

H/DX mass spectrometric analysis of the samples was performed using an automated H/DX setup (Waters) including a two-arm robotic autosampler (LEAP Technologies), an ACQUITY UPLC M-Class System and HDX Manager (Waters). For the exchange reaction, BsFra, BsSufU, BsSufS, BsFra/BsSufU, BsFra/BsSufS, BsFra/BsSufU/BsSufS, and BsSufU/BsSufS (60 μM final concentration of each component) were individually prepared in H2O buffer (25 mM Tris-Cl, pH 7.5, 100 mM NaCl) [32] and pre-cooled to 1°C. For each LC-MS run, 7.5 μL of protein solution was pipetted into a fresh vial on an exchange plate at 25°C and diluted with 61.8 μL of either H2O buffer (t0 runs) or D2O buffer (exchange runs). After incubation for 15, 30, 60, or 600 s, 55 μL of the reaction solution was transferred to a fresh vial containing 55 μL of quenching solution (400 mM H3PO4/KH2PO4, pH 2.2, pre-dispensed and pre-cooled to 1°C for 10 min before the first run). After quenching, 95 μL of the resulting solution was immediately injected into the HDX Manager.

Digestion was done online using an Enzymate BEH Pepsin Column (Waters) at 20°C with water/0.1% formic acid at a flow rate of 100 μL/min. Subsequently, peptic peptides were trapped at 0.5°C using a C18 trap column. Separation of peptides was achieved at 0.5°C utilizing a 1 x 100 mm ACQUITY UPLC BEH C18 1.7 μm column (Waters) at a flow rate of 30 μL/min with the following gradient of solvents A (water/0.1% formic acid) and B (acetonitrile/0.1% formic acid): Linear increase from 5–35% B within 7 min, followed by a ramp to 85% B within 1 min and isocratic 85% B for additional 2 min. Finally, the column was washed with 95% B for 1 min and re-equilibrated with 5% B for 5 min.

During separation of peptides using the chromatographic column, the pepsin column was washed by injecting 3 x 80 μL 4% acetonitrile and 0.5 M guanidinium chloride. HDMSe was used for t0 peptide detection and HDMS for exchanged peptides. Lock mass spectra were measured every 45 s using Glu-fibrinopeptide B as a standard ([M+H]2+ = 785.8427 m/z). t0 peptide identification was performed using ProteinLynx Global SERVER (Waters) with custom databases and the setting "no enzyme". Final assignment of deuterium incorporation was done with DynamX 3.0 (Waters).
Microscale thermophoresis

The determination of binding constants was done by microscale thermophoresis using a Monolith NT.115 instrument (NanoTemper). 100 μM BsFra was labeled at 8°C overnight using the Monolith NT.115 Protein Labeling Kit RED MALEIMID (cysteine-reactive; NanoTemper). Labeled BsFra was buffer-exchanged into a binding buffer (25 mM Tris-Cl, 100 mM NaCl, 10 mM 2-mercaptoethanol, pH 7.5) and the concentration was adjusted to 0.25 μM. A serial dilution series of BsSufS, BsSufU, and BsSufS/BsSufU was prepared in the same buffer in a range from 560 μM to 0.030 μM and then mixed 1:1 with labeled BsFra. The titration was transferred into NT.115 MST Premium Coated capillaries (NanoTemper). Measurements were performed at 25°C, 20% LED power and 20% MST power with a heating time of 30 s and cooling time of 5 s. The binding constant for each interaction was calculated from the average of three measurements using the NT Analysis software (NanoTemper).

Cysteine Desulfurase Activity Assay

The activity of BsSufS was measured by the amount of sulfide released during conversion of cysteine to alanine. Free sulfide was quantified using N,N-dimethyl-p-phenylenediamine sulfate (DMPD) and FeCl₃ as described previously [30,31,39]. We incubated 0.5 μM BsSufS with 10 μM BsSufU and 50 μM BsFra in 25 mM Tris-Cl, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol (DTT) for 5 min at room temperature. The 200 μL reaction was started by addition of l-cysteine (2 mM) and quenched after 10 min by addition of 25 μL of DMPD (20 mM in 7.2 M HCl) and 25 μL FeCl₃ (30 mM in 1.2 M HCl). After 30 min of incubation in the dark, the absorbance was measured at 670 nm. All reactions were carried out in triplicate.

Fe-S Biosynthesis Assays

The BsSufS-dependent biosynthesis of Fe-S clusters on BsSufU was assayed in an anaerobic chamber (Coy Laboratories) with forming gas (2% H₂/98% N₂) as previously described [30,33]. Briefly, 0.5 μM BsSufS was incubated with 10 μM BsSufU in 25 mM Tris-Cl, pH 7.5, 100 mM NaCl, 5 mM DTT for 5 min at 15°C. Next, 100 μM ammonium iron(II) sulfate was added to the reaction mixture with or without 50 μM BsFra. The reaction was started by addition of 2 mM cysteine, and Fe-S cluster formation on BsSufU was monitored by UV-Vis at 465 nm (ε₄₅₆ = 5.8 mM⁻¹cm⁻¹) [40].

Results

Crystal structure of BsSufS in the product-bound state

As no structure of B. subtilis SufS was available at the start of our study, we sought to fill this gap. BsSufS was thus crystallized and its structure was determined to 1.7 Å resolution by molecular replacement using the B. suis homolog (PDB ID 4W91) [41] as a search model (Table 1). One molecule of SufS was found within the asymmetric unit (AU) and could be built to completeness. The crystal structure of BsSufS is highly similar to that of E. coli SufS (PDB ID 1I29) with a root mean square deviation (r.m.s.d.) of 1.28 Å over 407 Ca atoms (S1 Fig). The BsSufS monomer forms a tightly intertwined homodimer with another monomer across the crystallographic symmetry axis (Fig 1A). The interface and architecture of the BsSufS homodimer closely resemble those of E. coli SufS [42].

BsSufS assumes a type I fold of the aminotransferase class V family, consisting of an overall α/β fold that is highly similar to that of E. coli SufS, IscS, NifS, and CsdA [41,43–46]. The enzyme consists of two domains: A large, N-terminal domain (residues 1–294) and a smaller, C-terminal domain (residues 295–406). In the N-terminal domain, a 7-stranded, parallel β-sheet is sandwiched between several α-helices to form a tightly packed core. This domain
harbors the active site pocket, where the cofactor PLP is bound to Lys224 as an aldimine. Furthermore, residues 253–265 (herein referred to as the ‘β-hook’) form a hairpin-like structure that latches on to the other monomer. The small, C-terminal domain consists of a 4-stranded, parallel β-sheet and four α-helices containing the flexible ‘Cys361-loop’ with the nucleophile Cys361 (Fig 1B). The dimer interface covers much of the active site pocket and protects PLP and Cys361. We found BsSufS in the product-bound state, with alanine bound near Cys361-persulfide, suggesting that one reaction cycle had occurred.

BsSufS forms a homodimer in solution

We performed time-resolved H/DX experiments in order to understand the dynamics of BsSufS in solution. We identified 176 peptides between 5 and 20 amino acids in length, resulting in 98.6% sequence coverage and a redundancy of 4.4 (Fig 2). BsSufS was incubated in D2O buffer for 15, 30, 60, and 600 s, and the exchange of backbone amide hydrogen to deuterium was measured for each peptide. In good agreement with the expectations from our crystallographic analysis, the solvent-exposed N- and C-termini of BsSufS exchanged readily, whereas most of the core structure was strongly protected (compare to Fig 1). Strong protection was
observed for BsSUfS regions involved in formation of the homodimer interface, validating the existence of the enzyme as a homodimer in solution [47] (Fig 2B).

**BsSUfU binds to the BsSUfS homodimer**

Next, we sought to investigate the interaction interface and conformational dynamics of SUfS and SUfU. Therefore, SUfS was incubated with SUfU in deuterated buffer. Upon completion of the H/DX reaction and peptic digest, we observed that several peptides of SUfS showed a change in deuterium uptake when compared to their counterparts from H/DX with SUfS alone (see above). Most notably, the C-terminal α-helix of SUfS showed a high degree of protection in the presence of SUfU (Fig 3). This observation agrees with recent findings that the C-terminus of *E. coli* IscS is required for interaction with IscU [48,49]. In addition, residues 50–63 of SUfS (herein referred to as the ‘α-hinge’) were protected against deuterium incorporation, suggesting the presence of a second SUfU interaction site at the SUfS homodimer interface.

We then utilized our H/DX approach to analyze changes in SUfU upon binding SUfS. We identified 87 peptides, which covered 93% of the sequence with a redundancy of 6.0 (Fig 4). The NMR solution structure of apo-SUfU (PDB ID 2AZH) reveals a compact, α/β fold. Our H/DX analysis supports this structure in solution, as medium to low H/D exchange was observed for the core of SUfU, whereas high H/D exchange was observed for an extended loop region between the N-terminal helix α1 and strand β1 (the ‘α/β linker’) as expected for a solvent-
exposed, unstructured region. In the presence of SufS, the ‘α/β-linker’ of SufU is strongly protected from deuterium uptake, which indicates that SufS interacts with SufU at this location (Fig 4C). Residues 117–130, which form a loop carrying Cys128 (the ‘Cys128-loop’), are also protected from deuterium uptake, suggesting a second SufS binding site. Therefore, we conclude that the ‘α/β-linker’ and ‘Cys128-loop’ of SufU bind to the C-terminus and ‘α-hinge’ of SufS, respectively, bringing the active site of SufS and SufU into close proximity. A similar scenario has been proposed for the interaction of IscU and IscS in E. coli [49].

**Binding of BsSufU to BsSufS induces conformational changes in both proteins**

Next, we analyzed the H/DX data for changes in the dynamic behavior of SufS in the presence of SufU. Significantly higher H/D exchange rates were observed for the active site pocket of SufS when SufU was present, particularly at the ‘Cys361-loop’ and ‘β-hook’ of SufS (Fig 3).
These observations strongly suggest that the SufS homodimer opens in the presence of SufU, allowing the ‘Cys361-loop’ to move freely. This is in agreement with findings in *E. coli*, where the ‘Cys361-loop’ of IscS undergoes a major, 14-Å movement during transfer of sulfur to IscU [48,49].

Our data also show that, in the presence of SufS, SufU undergoes significant structural rearrangements at the ‘α-helical bundle’ (i.e., helices α2, α3, and α5) and ‘β-sheet surface’ (i.e., residues 32–47). The active site of SufU contains four residues (i.e., Cys41, Asp43, Cys66, and Cys128) that coordinate a structurally important zinc ion [30,50]. In particular, the ‘Cys41-loop’ showed a significantly increased deuterium uptake. To summarize, the interaction of *B. subtilis* SufS with SufU induces structural rearrangements near the active sites of both proteins, potentially facilitating persulfide transfer. This notion supports the 40-fold increase in *B. subtilis* desulfurase activity previously observed upon interaction with SufU [30,31,47,50].

**BsFra binds to BsSufU and BsSufS**

After demonstrating the dynamic behavior and interaction of the BsSufS/BsSufU complex, we sought to integrate *B. subtilis* frataxin into the picture. We conducted microscale thermophoresis (MST) experiments between fluorophore-labelled BsFra and BsSufU, BsSufS, and BsSufS/BsSufU, and found that while BsFra binds its partners fairly weakly: BsSufU ($K_d = 57.4 \pm 13.8 \mu M$), BsSufS ($K_d = 50.6 \pm 17.4 \mu M$) and the BsSufS/BsSufU complex ($K_d = 32.5 \pm 3.6 \mu M$) (Fig 5). In contrast,
the interaction of $B_s$SufS with $B_s$SufU ($K_d = 2.63 \mu M$) [31] and $B_s$Fra with $B_s$HemH ($K_d = 1.63 \mu M$) [32] were found to be significantly tighter. Nevertheless, our affinity measurement for

Fig 4. $B_s$SufS alters the H/D exchange of $B_s$SufU upon binding. (A) Detected peptic peptides of $B_s$SufU with the relative fractional uptake after 15 s of incubation in deuterated buffer. (B) Changes in the relative fractional deuterium uptake of $B_s$SufU after incubation with $B_s$SufS for 15 s in D$_2$O buffer compared to $B_s$SufU alone were mapped onto the surface of $B_s$SufU (PDB ID 2AZH). The heat map represents the differences in deuterium uptake compared to $B_s$SufU alone. A decrease (blue) in deuterium uptake signals protection (i.e., a binding event), whereas an increase (red) signals a structural rearrangement. Black regions were not detected. Binding of $B_s$SufU to (C) the $\alpha/\beta$-linker and (D) the Cys$_{128}$-loop of $B_s$SufS as a function of deuterium uptake over time. Color code: $B_s$SufU alone (red), $B_s$SufU + $B_s$SufS (green), $B_s$SufU + $B_s$Fra (blue), and $B_s$SufU + $B_s$SufS/$B_s$Fra (violet). N-terminus (NT) and C-terminus (CT).

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Fig 5. Characterization of the affinity of $B_s$Fra for $B_s$SufS, $B_s$SufU, and $B_s$SufS/$B_s$SufU using microscale thermophoresis. MST binding curve from the interaction of fluorophore-labeled $B_s$Fra with (A) $B_s$SufS, (B) $B_s$SufS/$B_s$SufU, and (C) $B_s$SufU. A Hill model was applied for $K_d$ determination. Fra* indicates fluorophore-tagged frataxin.

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BsFra with BsSuF/SBsSuF is of the same order of magnitude as that for the homologous *E. coli* system comprised of CyaY and IscS (*Kₐ* = 18.5 μM) [51].

To further characterize the interaction of BsFra with its partners we applied H/DX experiments. Analysis of BsFra alone yielded 61 unique peptides with 98% sequence coverage and a redundancy of 4.7 (Fig 6). Human, *S. cerevisiae*, and *E. coli* frataxin (PDB ID 3S4M, 2GA5, and 2EFF, respectively) assume an α/β fold in which two α-helices are stacked against a 6-stranded β-sheet, with the N-terminal α-helix harboring several acidic residues (the ‘acidic ridge’) [21]. BsFra (PDB ID 2OC6) has an additional short, N-terminal helix (herein referred to as ‘α1-helix’) followed by the helix carrying the ‘acidic ridge’ (compare S2 Fig). The ‘α1-helix’ shows medium to high deuterium uptake in BsFra, suggesting that in solution it can move freely. Furthermore, a helix connecting β4 to β5 (the ‘EDDI-helix’) is present in BsFra but not homologous frataxin structures [19–21]. A high rate of H/D exchange was observed for this ‘EDDI-helix’, especially for residues E75, D76, D77, and I78.

We repeated the H/DX experiments of BsFra in the presence of BsSuF (S3 Fig), BsSuF (S4 Fig), and the BsSuF/BsSuF complex (Fig 7). These measurements were compared with those of BsFra alone for differences in deuterium uptake. In the presence of BsSuF, we observed a decrease of H/D exchange in β-strands 1 and 2 of BsFra (residues 37–46, herein referred to as the ‘KWN-loop’), which harbor residues K40, W41, and N42 (Fig 7C). As we found that the same residues were protected in the reaction of BsFra and BsSuF, we conclude that BsFra binds to BsSuF at the ‘KWN-loop’ area. The involvement of the ‘KWN-loop’ in the interaction with BsSuF is in agreement with previous studies in which mutations of in this region disrupted the interaction [52–55]. In the presence of both BsSuF and BsSuF, BsFra was additionally to the KWN loop protected from H/D uptake at the ‘acidic ridge’ (residues 9–25) (Fig 7 and S4 Fig), suggesting an interaction with BsSuF. This is in accordance with previously published work on *E. coli* IscS/CyaY [51,56]. Furthermore, BsSuF induces an increased rate of deuterium uptake at the N-terminal ‘α1-helix’ of BsFra (Fig 7). We propose that, upon binding of BsFra to BsSuF, the ‘α1-helix’ undergoes a structural rearrangement. Interestingly, BsFra was protected from deuterium uptake at the C-terminus and ‘EDDI helix’ (residues 74–85) compared to the protein alone. However, this was found in the BsFra/BsSuF and BsFra/BsSuF reactions (Fig 7, S3 Fig and S4 Fig), suggesting that the either protein can stabilize these highly dynamic areas (compare Fig 6). Taken together, the results indicate that BsFra binds to BsSuF and BsSuF as well as to the BsSuF/BsSuF complex. A similar binding of frataxin to a cysteine desulfurase/scaffold protein complex was described in *E. coli* [51,56].

**Binding of BsFra to BsSuF/BsSuF induces conformational changes in all three proteins**

Next, we tested whether the binding of BsFra would alter the interaction of BsSuF with BsSuF. We analyzed the H/DX data of the BsFra/BsSuF/BsSuF interaction and compared it to that of

![Peptidic peptides of BsFra detected from H/DX measurements](https://doi.org/10.1371/journal.pone.0158749.g006)
the \( \text{BsSufS}/\text{BsSufU} \) complex and proteins alone (see above). The scaffold protein \( \text{BsSufU} \) showed decreased deuterium uptake at the C-terminal helix (‘\( \alpha_5 \)-helix’), indicating binding to \( \text{BsFra} \) (Fig 8 and S3 Fig). The cysteine desulfurase \( \text{BsSufS} \) showed protection from H/D exchange at the ‘Cys361-loop’ and the ‘\( \beta \)-hook’ when \( \text{BsFra} \) was present (Fig 8), also indicating a binding event. Several positively charged residues are located in this region (e.g., R356, H359, H360, and K367) and may bind the negatively charged residues of the ‘acidic ridge’ of \( \text{BsFra} \) (see above). In contrast to the reaction of \( \text{BsSufS}/\text{BsSufU} \) with \( \text{BsFra} \), no increase in deuterium uptake was observed at the active site pocket of \( \text{BsSufS} \) when \( \text{BsFra} \) was present (compare Fig 8 to Fig 3). The \( \text{BsSufU} \) binding site on \( \text{BsSufS} \) (see above) was still observable, supporting the idea of simultaneous binding.

We propose that \( \text{BsFra} \) binds via the ‘acidic ridge’ to the positively charged ‘Cys361-loop’ and ‘\( \beta \)-hook’ of \( \text{BsSufS} \). \( \text{BsFra} \) may act as a clamp at the \( \text{BsSufS} \) homodimer interface, locking the ‘Cys361-loop’ and ‘\( \beta \)-hook’ and thus preventing the opening of the \( \text{BsSufS} \) active site pocket. Furthermore, \( \text{BsFra} \) binds via its ‘KWN-loop’ to the ‘\( \beta \)-sheet surface’ of \( \text{BsSufU} \). A similar binding site was suggested for \( \text{E. coli IscS/CyaY} \) [51,56].

To test whether the binding of \( \text{BsFra} \) to the \( \text{BsSufS}/\text{BsSufU} \) complex alters the activity of \( \text{BsSufS} \) we conducted cysteine desulfurase activity assays \textit{in vitro}. We found that \( \text{BsSufU} \) greatly enhances the activity of \( \text{BsSufS} \), in agreement with previous reports [30,33]. The introduction of \( \text{BsFra} \), however, does not appear to affect the activation of \( \text{BsSufS} \) by \( \text{BsSufU} \) (Fig 9). This is in agreement with the \( \text{E. coli IscS/CyaY} \) interaction, where conversion of cysteine to alanine by

Fig 7. H/DX analysis of \( \text{BsFra} \) upon binding \( \text{BsSufU}/\text{BsSufS} \). (A) Changes in the relative fractional deuterium uptake of \( \text{BsFra} \) after incubation with \( \text{BsSufS}/\text{BsSufU} \) for 15 s in D\(_2\)O buffer compared to \( \text{BsFra} \) alone were mapped onto the surface of \( \text{BsFra} \) (PDB ID 2OC6). The heat map represents the differences in deuterium uptake compared to \( \text{BsFra} \) alone. A decrease (blue) in deuterium uptake signals protection (i.e., a binding event), whereas an increase (red) signals a structural rearrangement. Black regions were not detected. Binding of \( \text{BsSufS}/\text{BsSufS} \) to (B) the ‘acidic ridge’ and (C) the KWN-loop of \( \text{BsFra} \) as a function of deuterium uptake over time. Color code: \( \text{BsFra} \) alone (blue), \( \text{BsFra} + \text{BsSufS} \) (green), \( \text{BsFra} + \text{BsSufU} \) (red), and \( \text{BsFra} + \text{BsSufU}/\text{BsSufS} \) (violet). N-terminus (NT) and C-terminus (CT).

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Fig 8. **BsFra alters the H/D exchange of BsSuS and BsSuU upon binding the BsSuS/BsSuU complex.** Changes in the relative fractional deuterium uptake of BsSuS/BsSuU after incubation with BsFra for 15 s in D_2O buffer compared to BsSuS and BsSuU alone were mapped onto the surface of the BsSuS (A) monomer and (B) homodimer as well as (C) BsSuU (PBD ID 2AZH). The heat map represents the differences in deuterium uptake compared to the solo incubation. A decrease (blue) in the uptake signals protection (i.e., a binding event), whereas an increase (red) signals a structural rearrangement. Black regions were not detected. Changes in (D) the SuS Cys361-loop and (E) the β-hook as a function of deuterium uptake over time. Color code: BsSuS alone (green), BsSuS + BsFra (blue), BsSuS + BsSuU (red), and BsSuS + BsSuU/BsFra (violet). Changes in (F) the SuU α5-helix and (G) the Cys41-loop as a function of deuterium uptake over time. Color code: BsSuU alone (red), BsSuU + BsFra (blue), BsSuU + BsSuS (green), and BsSuU + BsSuS/BsFra (violet). N-terminus (NT) and C-terminus (CT).

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IscS is not affected by CyaY [56]. We next analyzed whether frataxin has an effect on the formation of Fe-S clusters in vitro. In an assay described by Albrecht and colleagues [30], we incubated BsSufS and BsSufU in the presence of ferrous iron and cysteine and monitored the formation of Fe-S clusters by UV-Vis spectroscopy. When BsFra was present in the reaction mixture the initial rate of formation was unaffected, although we observed a very minor increase in the yield of Fe-S clusters formed on BsSufU. Such a subtle change could indicate that either BsFra does not efficiently donate iron to BsSufU under assay conditions or that its presence is redundant under the supplied concentration of iron. Additionally, other, unidentified components may be required for efficient transfer.

Discussion

*Bacillus subtilis* has five paralogous cysteine desulfurase genes, namely *nifS* [57], *yrvO* [58], *nifZ* [59] and *ycbU* [60]. However, only the product of the *sufS* gene is involved in biosynthesis of Fe-S clusters. Until now, no structural information was available on the *B. subtilis* cysteine desulfurase. We were able to determine the crystal structure of the *BsSufS* dimer to 1.7 Å resolution, revealing high structural homology to its *E. coli* counterpart. To better understand the conformational dynamics of SufS and its interaction with SufU and Fra, we conducted H/DX experiments with proteins from the Gram-positive model organism *B. subtilis*. Many insights into the SUF system were previously obtained from studies on *E. coli*, in which the SUF system only acts as a backup under stress conditions [7]. The ISC system mediates the major “housekeeping” functions for Fe-S cluster biosynthesis in *E. coli* [9]. In *B. subtilis*, we find similarities to both *E. coli* ISC and SUF systems [30,31,33]. We applied hydrogen/deuterium exchange experiments on *BsSufS* in order to investigate structural dynamics upon its interaction with the putative iron delivery protein BsFra and the scaffold protein BsSufU. H/DX detects the exchange of hydrogen on the backbone amides with deuterium in the solvent, where the exchange rate of highly dynamic or surface-exposed areas is rapid compared to residues that are buried in the protein core or otherwise protected. Investigation of *BsSufS* confirmed the homodimer interface in solution and suggested that the enzyme is tightly packed. Nevertheless, the strength of H/DX lies in its ability to detect changes in residues following a binding event. We showed that the C-terminal region and ‘α-hinge’ of *BsSufS*
interact with the long ‘α/β-linker’ and ‘Cys128-loop’ of BsSufU (Fig 10). This interaction is accompanied by an opening of the BsSufS dimer interface and rearrangement of the ‘Cys361-loop’. BsSufU, on the other hand, showed H/D exchange in the loop carrying Cys41 and Asp43. BsSufU fits on the BsSufS interaction site, which brings the ‘Cys361-loop’ of BsSufS and the ‘Cys41-loop’ of BsSufU into close proximity for persulfide transfer.

The E. coli SufS/SufE interaction was previously characterized by H/DX [61] and the crystal structure of the homologous E. coli CsdA/CsdE enzyme complex was solved recently [41]. In both cases, SufE/CsdE binds near the active site of SufS/CsdA to accept the sulfur as persulfide, which is then passed on to the scaffold protein complex SufBC2D of the E. coli SUF system. E. coli SufB has been shown to act as a scaffold and carries an FAD binding site that is possibly important for the reduction of Fe3+ to Fe2+ in the generation of Fe-S clusters [18]. In B. subtilis, the roles of SufB, SufC, and SufD are still unclear. BsSufB, though conserved, lacks the Fe-S cluster and FAD binding sites, suggesting an important role which differs from that of its E. coli homolog. We hypothesize that the BsSufBCD enzyme complex is involved in later steps of Fe-S cluster maturation, similar to the E. coli HscA/HscB proteins of the ISC system [62–64]. Taken together, our data suggest that binding of BsSufU to BsSufS activates the latter for transfer of sulfur from persulfide to the active site of BsSufU.

In the present study, we further established an interaction between BsSufS/BsSufU and the B. subtilis frataxin homolog BsFra. Previous biochemical characterization showed that BsFra can bind ferric and ferrous iron, and plays a role in Fe-S cluster biosynthesis and transfer to a target protein [28,29]. The frataxin family has been extensively studied in E. coli, S. cerevisiae, and H. sapiens. Generally, this family consists of a conserved α/β sandwich fold and harbors several acidic residues at the N-terminal α-helix and the first β-sheet (S2 Fig). In vitro enzyme assays measuring the rate at which Fe-S clusters form on the scaffold protein in the presence of a cysteine desulfurase and frataxin highlights a discrepancy: While frataxin inhibits cluster formation in the E. coli system [51,56], it enhances the rate of cluster formation in the yeast system [65]. It was shown that the cysteine desulfurase determines whether frataxin acts as an
inhibitor or activator [66]. It was further shown that a single point mutation on the scaffold protein renders E. coli as frataxin-dependent and S. cerevisiae as frataxin-independent [67–69]. We conducted analogous assays with BsFra and found that, in contrast to E. coli and S. cerevisiae homologs, it did not appear to alter the initial rate of cluster formation, but resulted in a very minor increase in cluster yield. The cluster formed on Siae homologs, it did not appear to alter the initial rate of cluster formation, but resulted in a likely degrades to an uncharacterized species upon isolation of holo- BsSufU [30,50]. That we were unable to isolate holo-BsSufU may suggest that our current model for Fe-S biosynthesis includes only the minimal number of participants.

We performed H/DX experiments to analyze the interaction of BsSufS and BsSufU with BsFra. We observed binding of BsFra to BsSufU and BsSufS, which did not change in the presence of BsSufU/BsSufS and therefore indicates formation of a BsFra/BsSufU/BsSufS complex in solution. The binding epitope of BsFra associates with the 'Cys361-loop' and 'β-hook' motifs of BsSufS (Fig 10). The interaction points between BsFra and BsSufU are the 'KWN-loop' and 'β-sheet surface', respectively. The short 'α1-helix' of BsFra appears to rearrange upon binding BsSufS, forming an extended α1-α2 helix hybrid, which then fits in the groove of BsSufS/ BsSufU. This interaction brings the putative iron binding site of BsFra into close proximity to the active site of BsSufU, and it is reasonable to propose that iron incorporation is mediated by the ‘acidic ridge’ of BsFra. Previous SAXS measurements of E. coli IscS/IscU/CyaY revealed a similar binding mode [51], and characterization of the interaction of frataxin with the ferrochelatase established that BsFra binds with its acidic residues in a similar fashion to the iron accep- tor BsHemH [32]. In B. subtilis, it is well established that frataxin is involved in incorporating iron into the nascent Fe-S cluster in vivo [33], but whether frataxin also serves as an intracellular iron carrier remains elusive.

We determined that BsSufU binds BsSufS and further showed how BsFra binds to the BsSufU/BsSufS complex as well as the individual proteins. In accordance with what is known about E. coli IscS/IscU/CyaY [51,56], the interaction of BsSufU/BsSufS is tight compared to that of BsFra with the complex. We assume that the interaction of BsFra to the complex is transient and that BsFra competes with additional participants of the Fe-S biogenesis pathway, as is known for E. coli IscS/Fdx [17]. The results presented here represent a single snapshot in a highly dynamic assembly process whose parts have not been fully identified.

The biogenesis of Fe-S clusters is a multistep process consisting of sulfur abstraction from cysteine, persulfide transfer, iron delivery and incorporation, and reductive generation of the Fe-S cluster, followed by transfer onto a target protein. Further studies will be necessary to identify any additional participants and determine how the biosynthetic steps are organized. In particular, the identity of the electron donor for Fe-S cluster biogenesis in vivo is unknown, and the role of SufB/SufC/SufD is yet to be determined.

The authors declare no financial conflict of interest.

Supporting Information

S1 Fig. Overlay of BsSufS with E. coli Cysteine Desulfurases. The structure of a B. subtilis SufS monomer (green) is superimposed with: (A) E. coli CsdA monomer (cyan; PDB ID 4LW2) with an r.m.s.d. of 3.30 Å over 401 Ca atoms; (B) E. coli SufS monomer (yellow; PDB ID 1129) with an r.m.s.d. of 1.28 Å over 407 Ca atoms; and (C) E. coli IscS monomer (magenta; PDB ID 3LVL) with an r.m.s.d. of 5.55 Å over 389 Ca atoms (TIFF)

S2 Fig. Comparison of frataxin homologs. Frataxin usually consists of two [α-helices and one 6-stranded β-sheet. Two additional helices appear in BsFra. (TIFF)
S3 Fig. Analysis of the BsFra/BsSufU interaction by H/DX. Differences in H/D uptake of the interaction complex compared to each individual protein are mapped onto the structures of (A) BsFra (PDB ID 2OC6) and (B) BsSufU (PDB ID 2AZH). The relative amount of deuterium incorporated is indicated by a color code ranging from blue (low; stable region) to red (high; flexible region). Black regions were not detected. N-terminal (NT) and C-terminal (CT).

(TIFF)

S4 Fig. Analysis of the BsFra/BsSufS interaction by H/DX. Differences in H/D uptake of the interaction complex compared to each protein alone are mapped onto the structures of (A) BsFra (PDB ID 2OC6), (B) the BsSufS monomer, and (C) the BsSufS homodimer. The relative amount of deuterium incorporated is indicated by a color code ranging from blue (low; stable region) to red (high; flexible region). Black regions were not detected. N-terminal (NT) and C-terminal (CT).

(TIFF)

S1 File. H/DX Files.

(XLS)

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Author Contributions

Conceived and designed the experiments: MAM UL. Performed the experiments: BB AM. Analyzed the data: BB FA. Contributed reagents/materials/analysis tools: UL. Wrote the paper: BB MAM GB CDF.

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