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Kinetic Analysis of the Bisubstrate Cysteine Desulfurase SufS from *Bacillus subtilis*[†]

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ABSTRACT: Cysteine is the major sulfur donor for thio cofactors in bacterial and eukaryotic systems. The first step in sulfur mobilization involves a PLP-dependent enzymatic mechanism. During catalysis, free cysteine is converted into alanine with the concomitant formation of a persulfide bond with the catalytic cysteine residue, thus forming a covalent enzyme intermediate. Cysteine desulfurases in their persulfurated forms serve as donors at the intersection of various cellular sulfur-requiring pathways. Most Gram-positive bacteria, including *Bacillus subtilis*, contain a cysteine desulfurase gene *sufS* located adjacent to the gene encoding the proposed Fe−S cluster scaffold SufU. In this work, we identified the participation of SufU as a substrate in the SufS catalytic mechanism. Development of a sensitive method for detection of alanine formed in the SufS reaction enabled the identification of its associated mechanistic features. Steady-state kinetic analysis of alanine formation provided evidence of a double-displacement mechanism (ping-pong) of the cysteine:SufU sulfurtransferase reaction catalyzed by SufS. Results from site-directed mutagenesis of the catalytic cysteine ($SufS^{C361A}$) and iodoacetamide alkylation of SufU support the occurrence of persulfide sulfur transfer steps in the mechanism of SufS.

Sulfur-containing cofactors are widely distributed in nature and participate in essential biochemical reactions (*1*, *2*). While the biological significance of sulfur's versatile chemistry is widely recognized, the mechanisms responsible for its mobilization and incorporation into protein cofactors are far from understood. However, cysteine is well-established as the major source of sulfur for thio cofactors (*3*), and the initial step in sulfur mobilization uses a pyridoxal 5-phosphate (PLP)[1] enzymatic mechanism. Free cysteine binds to the cysteine desulfurase PLP cofactor, to form a PLP−cysteine adduct. The sulfur atom of the substrate cysteine is attacked by the thiolate group of a cysteine residue at the enzyme's active site. This event results in the activation of the sulfur through formation of a persulfide bond (R-S-SH) and the release of alanine. Cysteine desulfurases in their persulfurated forms serve as sulfur carriers and donors to various cellular targets. This sulfur activation process is an elegant metabolic strategy for trafficking sulfur in nontoxic forms and for the provision of SH^- or S^o at the interface of several metabolic pathways, including the synthesis of several thio cofactors such as Fe−S clusters, molybdenum cofactor, thiamin, biotin, lipoic acid, and thionucleosides (*3*, *4*).

Sequence analysis of cysteine desulfurases has identified signature motifs and conserved residues involved in catalysis. To date, all cysteine desulfurases contain a conserved lysine that forms a Schiff base with the PLP cofactor in the resting state and a conserved catalytic cysteine involved in transient persulfide formation. On the basis of sequence alignments, Mihara and Esaki identified two distinct groups of cysteine desulfurases: group I (NifS and IscS) and group II (SufS and CsdA) (*5*). Group-specific signature motifs in their tertiary structural contexts provide a rationale for their catalytic differences and for their specific interactions between cysteine desulfurases and sulfur acceptors. For example, the IscS includes a conserved sequence motif that forms a flexible loop containing the catalytic cysteine unique to only group I (*6*). This structural feature allows for the molecular movement of the persulfide sulfur away from the PLP active site to favor interactions with various sulfur partners. Alternatively, group II cysteine desulfurases have been shown to contain a shorter, more structurally defined loop as found in the *Escherichia coli* and *Synechocystis* SufS enzymes (*7*, *8*).

The rate-limiting step in the group II cysteine desulfurase reaction has been proposed to be the cleavage of the C−S bond of the cysteine substrate (*8*). This step involves concomitant formation of the persufide bond with the catalytic cysteine thiol group (*9*). The shorter and structured loop present in group II cysteine desulfurases may potentially hinder the nucleophilic attack of the catalytic cysteine onto the cysteine substrate thiol. Additionally, the lack of loop flexibility is hypothesized to perturb the subsequent delivery of the sulfur to acceptor molecules. The low specific activity of *E. coli* SufS (15−25 nmol of S^{2-} min^{-1} mg^{-1}) (*10*, *11*) may be explained by the stiffness of this structural loop during cleavage of the C−S bond and/or the transfer of sulfur to acceptor molecules.

Interestingly, in most members of group II of cysteine desulfurases, the activity is enhanced in the presence of a specific E-type sulfur acceptor [e.g., SufE (*10*) and CsdE (*12*) enhanced 8- and 2.5-fold the activity of SufS and CsdA, respectively]. The mechanism by which E-type acceptors enhance the activity of group II enzymes is not fully understood. This feature appears to be enzyme-specific as cross-reactivity among sulfur donors and acceptors has not been observed. That is, SufE was unable to stimulate the activity of IscS or NifS (*10*), nor was CsdE able to stimulate SufS or IscS (*12*). Nevertheless, sulfur acceptor activity enhancement does not appear to be limited to group II of cysteine desulfurases, because it has been shown that 1 equiv of IscU can stimulate the activity of the group I IscS 6-fold (∼100−600 nmol of S^{2-} min^{-1} mg^{-1}) (*13*).

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[1]Abbreviations: DTT, dithiothreitol; NDA, naphthalene dialdehyde; S^{2-}, sulfide; Fe−S, iron−sulfur; PLP, pyridoxal 5-phosphate; BSA, bovine serum albumin.

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Despite the similarities in structure and in general chemistry involving the cysteine-PLP-dependent formation of a persulfide bond, cysteine desulfurases are markedly distinct in their subsequent sulfur transfer reactions. Substantial progress has been made in the identification of immediate sulfur acceptors and other pathway components involved in the synthesis of sulfur-containing cofactors. However, the unique features of cysteine desulfurases that dictate acceptor specificity and their distinct sulfurtransferase mechanisms have not been fully explored.

Structural and functional studies of group I and group II enzymes have identified key residues that participate in sulfur transfer. Insights into specific sites of interaction between cysteine desulfurases and sulfur acceptors have been identified through an amino acid substitution approach with IscS (*14*−*16*). These studies targeted the identification of specific residues that interact with sulfur acceptors as well as the effects of substitutions on tRNA thiolation and Fe−S cluster formation. Because of the involvement of IscS in many sulfur transfer reactions, its inactivation causes pleiotropic metabolic defects that pose a technical challenge for the study of intracellular IscS−protein interactions (*17*−*19*).

The Gram-positive model organism *Bacillus subtilis* does not contain the canonical ISC system, composed of IscS and other Isc partners (IscU, IscA, HscBA, and Fdx). Instead, its genome encodes four group I cysteine desulfurases (YrvO, NifS, NifZ, and YcbU) and one group II cysteine desulfurase (SufS). Their genomic locations suggest their functional participation in specific sulfur-dependent biochemical pathways. For example, the only group II cysteine desulfurase gene is located in the essential *sufCDSUB* transcriptional unit. Antibiotic insertional disruption of *B. subtilis sufU* dramatically affects the activity of the Fe−S enzymes aconitase and succinate dehydrogenase (*20*). Because of its ability to accumulate transient labile Fe−S species and to activate a eukaryotic Fe−S enzyme (Leu1), SufU has been proposed to be an Fe−S cluster scaffold. The involvement of SufU in Fe−S metabolism and the apparent absence of genes encoding an additional Fe−S biosynthetic system led to the proposal that SufU is involved, along with SufC, SufD, SufS, and SufB, in the housekeeping formation of Fe−S clusters. In this system, SufC, SufD, and SufB show high levels of sequence identity to the SufBCD complex of *E. coli*, the function of which has been implicated in sulfur mobilization via interaction with SufE (*21*), and in Fe−S cluster assembly (*22*). Lastly, it has been shown that SufS is a cysteine desulfurase and that its activity is enhanced in the presence of SufU (*20*).

Genome scanning of 100 Gram-positive bacterial species has revealed that 81 species contain gene products that have sequence similar to that of *sufU* (P. C. Dos Santos, unpublished results). In all these cases, *sufS* was the preceding gene. For *Bacilli* (*sufCDSUB*), *Actinobacteria* [*sufRBD*(*fdx*)*CSU*], and *Clostridia* species (*sufRSU-mnmA*), the *sufSU* gene pair is located adjacent to other Fe−S or sulfur cofactor biosynthetic genes. Although several members of group II of cysteine desulfurases work in a specific and concerted manner with E-type proteins, none of these Gram-positive species contain a locus encoding either SufE or CsdE. Conversely, the genes encoding this subtype of group II cysteine desulfurases found in Gram-positive organisms show a pattern of coevolution with *sufU*. This observation led to the hypothesis that SufU is the specific sulfur acceptor of SufS in Gram-positive bacteria. In this study, we investigate the interactions and reactivity of *B. subtilis* SufS and the participation of SufU as a substrate in the catalytic ping-pong mechanism of the SufU:cysteine sulfurtransferase reaction of SufS. Additionally, a sensitive and affordable method for detection of enzymatically produced alanine was developed.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were purchased from New England Biolabs. Reagents were purchased from Fisher, unless specified. *B. subtilis* PS832 (*23*) was a gift from D. Popham, and *E. coli* CL100 (*24*) was a gift from T. J. Larson.

Plasmid Construction and Site-Directed Mutagenesis. All genes were amplified from *B. subtilis* PS832 chromosomal DNA. The PCR products were all previously cloned into the TopoTA vector (Invitrogen) for subcloning purposes. The correct sequences of all plasmids used in this study were confirmed by DNA sequencing (Wake Forest DNA Sequencing Laboratory). The 1690 bp *sufSU* fragment engineered with 5′ NdeI and 3′ BamHI was cloned into the 5′ NdeI and 3′ BglII sites of pDB1588 (*25*), previously digested with the same enzymes, yielding pDS2. Plasmid pDS2 was digested with 5′ NcoI and 3′ KpnI, and the isolated fragment was cloned into the NcoI and KpnI sites of pBad, placing *sufSU* under arabinose control, and yielding pDS34. Plasmid pDS63 is the 5′ NcoI and 3′ XhoI engineered 444 bp fragment of *sufU* ligated into pET28A(+) (Novagen) in frame for histidine tag expression at the 3′ end and puts *sufU* under lactose control. Plasmid pDS69 contains a codon substitution on pDS34 at position 361 of the *suf*S gene, Cys (TGT) → Ala (GTC). Plasmid pDS72 was obtained by a codon substitution on pDS34 at position 128 of the *sufU* gene, Cys (TGT) → Ala (GTC). The amino acid substitutions were performed with a QuickChange site-directed mutagenesis kit (Stratagene) as specified by the manufacturer.

Protein Expression and Purification. We expressed constructs pDS34 (SufSU), pDS69 ($SufS^{C361A}U$), and pDS72 ($SufSU^{C128A}$) by transforming each plasmid into *E. coli* CL100 competent cells and selecting them on LB agar plates containing 50 μg/μL ampicillin. Single colonies were used to inoculate 500 mL of LB medium with the same antibiotic concentration as the solid medium and outgrown overnight (16 h) at 30 °C in the presence of L-arabinose (0.2%). The cells were then harvested by centrifugation at 6000*g* for 10 min and stored at −20 °C.

Cells were resuspended (3 mL/g of cell wet weight) in 25 mM Tris-HCl (pH 8) (buffer A), then lysed by a single passage through a high-pressure cell disrupter (French Press) at 12000 psi, and centrifuged at 12800*g* for 20 min to remove cell debris. The supernatant containing soluble protein was incubated with 1% (w/v) streptomycin sulfate for 30 min and centrifuged at 12800*g* for 20 min. The treated supernatant was then loaded onto a fast protein liquid chromatography (FPLC) system (GE Healthcare) equipped with a Q-Sepharose fast flow column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 5 column volumes of buffer A, and the bound material was eluted with a linear gradient (from 0 to 70%) of 20 column volumes of 25 mM Tris-HCl (pH 8) and 1 M NaCl (buffer B). The required amount of buffer B to displace the protein from the column was 30% for SufSU and $SufS^{C361A}$-SufU and 45% for SufS. The fractions containing the desired protein(s) were pooled and concentrated prior to gel filtration using Sephacryl S-200 (GE Healthcare). For concentration purposes, the sample was diluted 5-fold with buffer A and then passed through a Q-Sepharose fast flow column pre-equilibrated with the same buffer and quickly eluted from the column with 50% buffer B, which removed all bound sample at once. The sample was instantly loaded

onto a gel-filtration column, pre-equilibrated with buffer A. Pure sample fractions were pooled, frozen in liquid nitrogen, and stored at −80 °C. SufSU (pDS34) and SufS^{C361A}U (pDS69) were both isolated as a complex. SufS was isolated and purified from SufU when expressed via the modified plasmid pDS72 (SufSU^{C128A}).

The pDS63 (SufU$_{His6}$) plasmid was transformed into *E. coli* BL21(DE3) cells and selected on LB agar plates containing 40 μg/μL kanamycin. Single colonies were used to inoculate 500 mL of LB medium with the same antibiotic concentration as the solid medium and grown at 37 °C until the OD_{600} reached 0.7. Lactose (0.2%) was added for further growth at 30 °C for 4 h. Cells were harvested by centrifugation at 6000*g* for 10 min and stored at −20 °C. The cell pellets were resuspended in 25 mM Tris-HCl (pH 8) and 0.2 M NaCl (buffer C) and disrupted as described above, followed by centrifugation at 12800*g* for 20 min to remove the cell debris. The supernatant was loaded onto a FPLC IMAC-Ni^{2+} (GE Healthcare) column and washed with 5 column volumes of buffer C. The bound proteins were eluted through a step gradient (5 and 15%) of 25 mM Tris-HCl (pH 8), 0.2 M NaCl, and 500 mM imidazole (buffer D). SufU was displaced from the column at 15% buffer D. Fractions containing SufU were pooled, diluted 5-fold, and then loaded onto a FPLC Q-Sepharose fast flow column, which was pre-equilibrated with buffer A. The column was washed with 5 column volumes of buffer A, and the bound sample was eluted via a linear gradient (from 0 to 70%) of 20 column volumes of buffer B. SufU was displaced from the column when the concentration of B reached 45%. The pure sample fractions were pooled, frozen in liquid nitrogen, and stored at −80 °C. Elution profiles were followed at 280 nm. All of the protein purifications were monitored by SDS−PAGE (*26*), and the protein concentrations were determined by the method of Bradford et al. (*27*), using the Bio-Rad protein assay kit and bovine serum albumin as the standard. Pyridoxal 5′-phosphate bound to SufS was quantified as described previously (*28*).

Determination of the Native Molecular Mass. The molecular masses of the native homogeneous proteins were estimated by gel-filtration chromatography on a Superose 12 10/300 GL column (GE Healthcare). Samples were eluted from the column with 25 mM Tris-HCl containing 0.2 M NaCl (pH 7.4) at a flow rate of 0.4 mL/min. The eluted samples were monitored at 280 nm, and the column was calibrated with the following protein standards (GE Healthcare): aprotinin (6500 Da), ribonuclease A (13700 Da), carbonic anhydrase (29000 Da), ovalbumin (43000 Da), and conalbumin (75000 Da). Blue Dextran 2000 was used to determine the void volume (V_0). The K_{av} value was calculated for each protein using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for the protein and V_t is the total bed volume, and K_{av} was plotted against the logarithm of standard molecular masses.

Cysteine Desulfurase Assay. Cysteine desulfurase activity was determined by quantifying the amount of sulfide and alanine produced in the assay. Sulfide production was determined with *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (DMPD) and $FeCl_3$ as previously described (*29*, *30*). Assays (800 μL) were conducted in the presence of 0.01 mg of SufS (final concentration of 0.27 μM) in 50 mM Mops (pH 7.4). Substrate saturation curves were determined by varying concentrations of cysteine (0.0125−0.5 mM) against a fixed concentration of SufU (1.3 μM) and varying concentrations of SufU (0.0013−0.020 mM) against a fixed concentration of cysteine (0.05 mM). The initial velocity was derived from the slope of a plot of product concentration versus time using four time points (0, 3, 6, and 9 min).

Alanine production was assessed through high-performance liquid chromatography (HPLC) (Waters) coupled with a fluorescence detector, using the assay mixture described above. For every time point, 100 μL aliquots from the assay were mixed with 20 μL of 10% trichloroacetic acid (TCA), and then 1 mL of a fresh mix that contained 0.1 mM naphthalene 2,3-dicarboxaldehyde (NDA) (AnaSpec Inc.) and 1 mM KCN in 100 mM borate buffer (pH 9) was added. After a 1 h incubation, the samples were analyzed by HPLC and the alanine was detected by fluorescence. Ten microliters of sample was injected onto a Zorbax SB C18 column (Agilent) at a flow rate of 0.5 mL/min with an isocratic solvent system of 55% methanol with 45% ammonium acetate (10 mM, pH 6) for 8 min, with the alanine−NDA peak eluting at 6 min. The Ala−NDA fluorescence peak was integrated, and a standard curve was used to convert the area under the peak to the concentration of alanine produced. In these assays, at least three time points (1, 2, and 4 min) were used to calculate the cysteine desulfurase activity given by slopes of nanomoles of alanine produced over time. The steady-state kinetic parameters were determined by varying concentrations of cysteine (0.0125−0.5 mM) against several fixed concentrations of SufU (0.0013−0.020 mM).

Assays in the absence of the second substrate (SufU) were also performed. The experiment consisted of individual reaction mixes that contained 2.7 μM SufS and 0.5 mM L-cysteine, conducted in the presence and absence of 5 mM DTT. Time points were measured throughout the experiment (10−1500 s), and time zero was determined via addition of 20 μL of 10% TCA prior to 0.5 mM cysteine.

Pyruvate formation was quantified through a reaction catalyzed by lactate dehydrogenase in the presence of NADH (*31*). The reaction was monitored at 340 nm (ε_{340} = 6220 M^{-1} cm^{-1}), and the amount of pyruvate present was determined by the amount of consumed NADH.

Alkylated SufU Assays. SufU or BSA was incubated with 5 mM iodoacetamide for 1 h in the dark. Five hundred microliters of the reaction was then dialyzed for 2 h against 1.5 L of 25 mM Tris-HCl (pH 8) to stop the reaction and to remove excess reagent. Alkylated SufU or alkylated BSA was added to the standard assay (800 μL) at different concentrations (0−5.5 μM), in the presence of 0.27 μM SufS, 0.5 mM cysteine, and 13.75 μM SufU. The activity was calculated and plotted versus the concentration of alkylated SufU.

RESULTS

Interactions between SufS and SufU. One-step copurification of SufS and SufU was accomplished when pBAD-*sufCDSUB* (pDS40) or pBAD-*sufSU* (pDS34) was expressed in *E. coli* CL100 cells (Figure 1A). Considerably higher levels of expression were observed using the CL100 host strain compared to *E. coli* TBI, BL21(DE3)-C41, or JM109 strains. Although the basis for higher expression levels was not determined, it is worth noting that CL100 has a Δ*iscS*, Ara$^-$ genotype. Soluble extracts containing the SufS−SufU fraction or SufCDSUB from expressing cultures were subject to anion-exchange chromatography, resulting in coelution of SufS and SufU. Because of the sequence similarities between *B. subtilis* SufS and *E. coli* SufS, control experiments included parallel purification from crude extracts of *E. coli* CL100 cells containing pBad (empty vector) or the inactive SufS^{C361A}-SufU (pDS69). Using the same purification protocol as described for isolation of the SufS−SufU fraction, the corresponding fractions showed no cysteine desulfurase activity, indicating the absence of

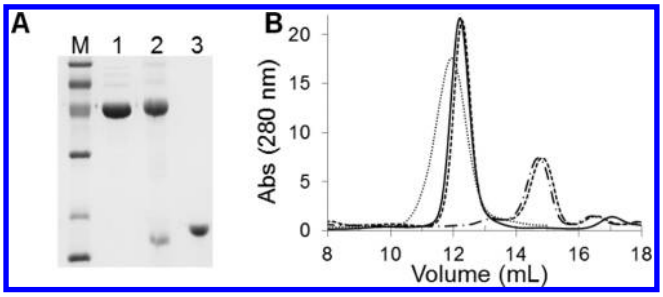


FIGURE 1: Protein purification and native size determination. Panel A shows the SDS−PAGE gel: lane 1, purified SufS from crude extracts containing SufS and $SufU^{128CA}$; lane 2, purified SufS−SufU fraction from crude extracts containing SufS and SufU; lane 3, purified $SufU_{his6}$. Lane M contained low-molecular mass standards (Bio-Rad): phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14.4 kDa). Panel B shows the gel-filtration profile for SufS (—), $SufU_{his6}$ (−·−), the SufSU complex (···), and $SufU_{his6}$ premixed with SufS (---).

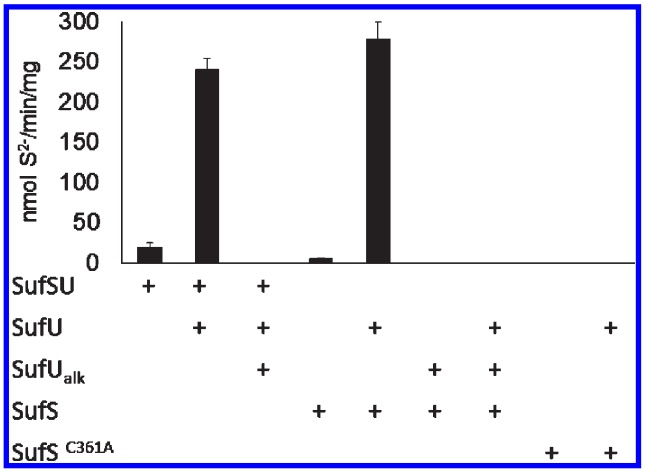


FIGURE 2: SufS cysteine desulfurase activity. The cysteine desulfurase activity was measured via the methylene blue assay. The assay contained 0.5 mM cysteine, 0.27 μM SufS or $SufS^{C361A}$, and 2 mM DTT. A plus indicates the presence of the corresponding component. SufU and $SufU_{alk}$ at 1.3 and 20.6 μM, respectively, were added to the assay.

significant amounts of *E. coli* SufS contaminating the purified sample (data not shown).

Subsequent gel-filtration chromatography resulted in a single broad peak with a corresponding molecular mass of 108 kDa (Figure 1A, lane 2, and Figure 1B). Because of the very similar elution volumes of the SufS and SufS−SufU fractions, it is possible that the SufS−SufU fraction contains excess free SufS. No peak associated with free SufU was observed, indicating that in the SufS−SufU fraction, all SufU was associated with SufS. SDS−PAGE analysis using purified SufS, SufU, and SufS−SufU fraction suggested an apparent stoichiometry of 2:1 for the SufS−SufU fraction.

The interaction between SufS and SufU was not disturbed by various treatments of crude extracts prior to purification. Anaerobic preincubation of SufS−SufU crude extracts with 1 mM DTT, 5 mM cysteine, and 5 mM ferrous ammonium sulfate in various combinations affected the overall Q-Sepharose chromatography profile, but it did not change the elution or purity of the SufS−SufU fractions after purification (Figure S1 of the Supporting Information). Under anoxic conditions, incubation with all three ingredients yielded dark blackish extracts prior to anion-exchange chromatography, with no Fe−S cluster species being trapped in the purified samples (data not shown). Albrecht and collaborators showed the isolation of Fe−S cluster-loaded SufU upon incubation with iron, cysteine, and catalytic amounts of SufS, followed by isolation through a desalting column (*20*). In our experiments, it is possible that the presence of crude extract components or the excess SufS in the reaction mixture causes dissociation of Fe−S species from SufU during purification through a positively charged resin.

Although we observed coelution of SufS and SufU in several independent purifications, the stoichiometry of this apparent complex seemed to vary slightly among preparations, indicating the dynamic nature of SufS−SufU interactions. When SufS and $SufU_{his6}$ were expressed individually, they eluted as two distinct peaks in the gel-filtration chromatogram corresponding to mass values of 88 and 24 kDa, respectively. While the calculated mass of SufS corresponded to a dimer (90 kDa), the estimated mass of SufU was between the expected size of a dimer (32 kDa) and that of a monomer (16 kDa). The gel-filtration profile did not change when the proteins were mixed prior to gel filtration (Figure 1B). Whether the coexpression and copurification of SufS and SufU was necessary for trapping the SufS−SufU complex or if the His tag on SufU disturbed these interactions has not been determined. Nevertheless, cysteine desulfurase assays of the SufS−SufU complex and SufS incubated with similar amounts of $SufU_{his6}$ showed comparable activity (described below), indicating that SufU and $SufU_{his6}$ are both equally competent for participation in the cysteine desulfurase reaction (Figure 2).

While coexpression of the wild-type SufS−SufU proteins resulted in their copurification, coexpression of SufS with the variant $SufU^{C128A}$ (*E. coli* CL100 transformed with pDS72) resulted in two distinct fractions for $SufU^{C128A}$ and SufS (lane 1 of Figure 1A shows the SufS fraction). The complex dissociation caused by the substitution at the conserved Cys 128 led to the hypothesis that the complex may be held together through a reductively protected disulfide bond between $SufU^{C128}$ and $SufS^{C361}$. However, this proposal was ruled out by an experiment that involved the coexpression and copurification of the $SufS^{C361A}$−SufU "complex", indicating that the dominant complex interaction does not involve the SufS active site C361 residue.

SufU as a Substrate of SufS. The catalytic rate of cysteine desulfurization by the SufS−SufU complex in the presence of cysteine and DTT was determined to be 18 nmol of S^{2-} min^{-1} mg^{-1}. The addition of 5 equiv of SufU to the assay containing the SufS−SufU complex showed a more than 10-fold increase in activity (from 18 to 240 nmol min^{-1} mg^{-1}) (Figure 2). This initial observation prompted us to determine the activity levels of SufS in the absence of SufU. In agreement with a previous report (*20*), SufS exhibited very modest activity (7 nmol min^{-1} mg^{-1}) when incubated in the presence of cysteine and DTT. Addition of 0.5 equiv of SufU (approximately equivalent to the amount of SufU in the SufS−SufU fraction) increased the SufS activity to 25 nmol of S^{2-} min^{-1} mg^{-1}, indicating comparable activation of SufS by SufU isolated with SufS (SufU−SufS fraction) or purified separately ($SufU_{his6}$). The stimulation of SufS activity by SufU suggests that SufU is an active participant in the catalytic mechanism of SufS.

The increase in cysteine desulfurase activity upon incubation with SufU may be explained by at least three mechanisms: (1) the formation of a heteromeric catalytic complex, (2) the involvement of SufU as an allosteric regulator of SufS, and (3) the involvement of SufU as a substrate of SufS in a bisubstrate reaction. If the SufS−SufU complex forms a catalytic two-component system (first model), titration of SufU would display first-order

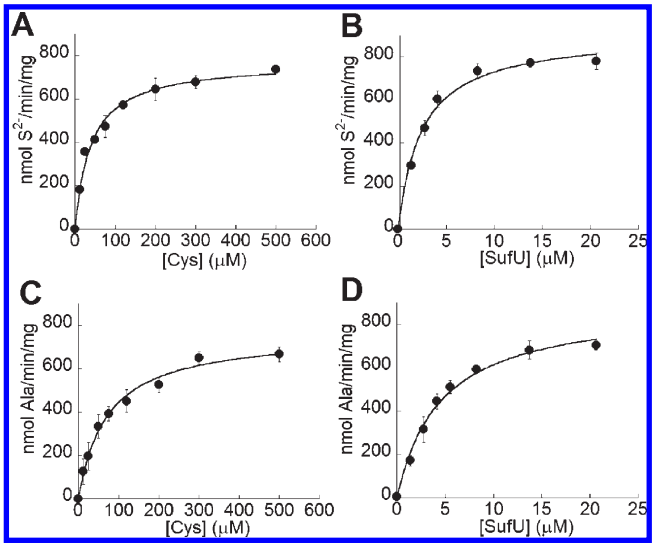


FIGURE 3: Substrate saturation curves characterizing a bisubstrate reaction catalyzed by SufS. The bisubstrate characterization was determined through quantification of sulfide (A and B) and alanine (C and D). Unless indicated, assays (800 μL) were conducted in the presence of 0.01 mg of SufS, 500 μM cysteine (A and C) or 13 μM SufU (B and D), and 2 mM DTT. Catalytic rates were calculated by the slopes of at least three time point reactions. The lines are the best fits to the Michaelis−Menten equation using Kaleidagraph.

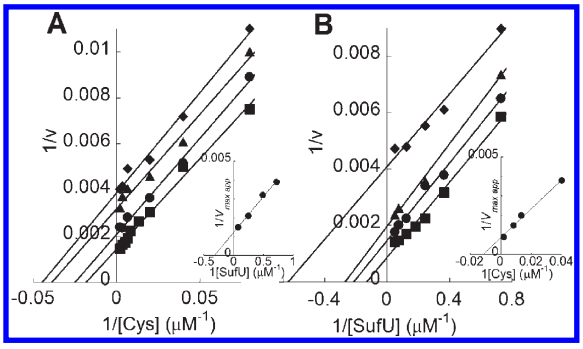


FIGURE 4: Double-reciprocal plots of alanine formation. Steady-state rates of alanine formation were determined with varying concentrations of cysteine (0.0125−0.5 mM) against several fixed concentrations of SufU (0.0013−0.020 mM). (A) Variable concentrations of cysteine in the presence of 1.37 (◆), 2.06 (▲), 4.12 (●), and 13.7 μM SufU (■). (B) Variable concentrations of SufU in the presence of 25 (◆), 75 (▲), 120 (●), and 500 μM cysteine (■). The insets show replots of $1/V_{max,app}$ vs 1/[Cys]. The lines in the plots are the linear fits to the Lineweaver−Burk equation. The inset in panel A shows the replot of the *y*-intercept of panel A [$V_{max,app} = 1/V_{max}(1 + K_{mSufU})/[SufU]]$ vs 1/[SufU] fitted to a linear equation. The inset in panel B shows the replot of the *y*-intercept of panel B [$V_{max,app} = 1/V_{max}(1 + K_{mCys})/[Cys]]$ vs 1/[Cys] fitted to a linear equation. The *x*-intercepts of the insets give $-1/K_{mSufU}$ and $-1/K_{mCys}$, respectively. The *y*-intercepts of the insets give the V_{max} values. Kinetic constants are listed in Table 1.

kinetic behavior up to the optimal complex stoichiometry, followed by a zero-order kinetic profile. Alternatively, if SufU acts as an allosteric regulator (second model), titration of SufU would yield sigmoidal behavior. In the third model, if SufU is a substrate of SufS, increasing concentrations of SufU could yield a hyperbolic curve following the Michaelis−Menten steady-state kinetic behavior. The SufU substrate saturation curve exhibited a hyperbolic response (Figure 3) and a straight line in the double-reciprocal Lineweaver−Burk plot (Figure 4). The apparent V_{max} of sulfide release is comparable to maximal activity levels for group II cysteine desulfurases (*10*, *32*) when assayed in the presence of their respective sulfur acceptor E-type proteins. As previously described for IscS, apparent substrate inhibition kinetics are observed with SufS when the cysteine concentration is held above 1 mM (data not shown).

Kinetics of Formation of Alanine by SufS. The cysteine desulfurase mobilizes sulfur from cysteine, forming alanine and a persulfide sulfur. The reduction of the persulfide bond results in the release of sulfide and regeneration of the catalytic cysteine. Traditional cysteine desulfurase assays in the presence of DTT yield sulfide products that are subsequently measured through formation of methylene blue. Although this assay has been used to study cysteine desulfurases for the past two decades, it has two major limitations: low sensitivity (1 nmol of sulfide) and the need to use sealed vials because of the transient formation of gaseous hydrogen sulfide (H_2S). We therefore developed a more sensitive and safer assay for cysteine desulfurase activity.

Alternate methods of detecting alanine involving common techniques for detecting amino acids would not be suitable for quantification of the SufS reaction product because of the cross reactivity of both substrate (Cys) and product (Ala). Serendipitously, we discovered that one of the limitations of an established technique for amino acid detection involving derivatization with naphthalene dialdehyde (NDA) was a fluorescence quenching phenomenon observed in the detection of cysteine (*33*). Thus, the lack of fluorescence in the cysteine−NDA adduct and the high fluorescence intensity associated with the alanine−NDA adduct appeared to offer an ideal strategy for measuring the alanine generated in the cysteine desulfurase assay. For this reason, we have adapted and optimized the NDA derivatization method as an end point assay to quantify alanine formed in the cysteine desulfurase reaction (described in detail in Experimental Procedures).

This assay allows for the direct measurement of fluorescence intensity upon incubation of the SufS assay mixture with NDA, cyanide, and borate buffer. Although high concentrations of cysteine have an effect on the fluorescence background, cysteine does not affect the rate of alanine formation over time. Fluorescence detection adds both convenience and sensitivity in detecting the activity of enzymatic reactions for a broader spectrum of enzymes forming primary amine products not just limited to cysteine desulfurases. It is important to note that kinetic assays with varying substrate concentrations propagate small differences in baseline. Thus, to correct for the variation in background, an alanine standard curve should be generated in the presence of the same concentration of cysteine used in the assay. Ultimately, the small variations in background associated with different cysteine concentrations in the kinetic assays led us to separate the Cys−NDA adduct background from the Ala−NDA product prior to fluorescence detection. For detection of the activities shown in this study, the assay solution was mixed with the NDA cocktail and then applied to a C18 HPLC column coupled with a fluorescence detector. The fluorescence detection of this method is extremely sensitive, allowing the detection of as little as 0.1 pmol of Ala−NDA adduct during each run. This sensitivity is approximately 10000-fold higher than that of the methylene blue assay.

As a validation of this newly developed method, we conducted kinetic measurements for alanine production by SufS and compared them to those obtained with the sulfide assay (Figure 3). The rate of alanine formation was very similar to those of sulfide, suggesting that no other product is formed in this reaction; some cysteine desulfurases have been known to form pyruvate as a result of alanine deamination at the PLP site. Using a lactate dehydrogenase coupled assay, we further confirmed that no

Table 1: Kinetic Constants of the SufS Cys−SufU Sulfurtransferase Reaction

substrate	K_m (μM)	V_{max} (nmol of Ala min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Cys	86 ± 1	1157 ± 132	0.87 ± 0.09	10.1×10^3
SufU	3.0 ± 0.3			2.9×10^5

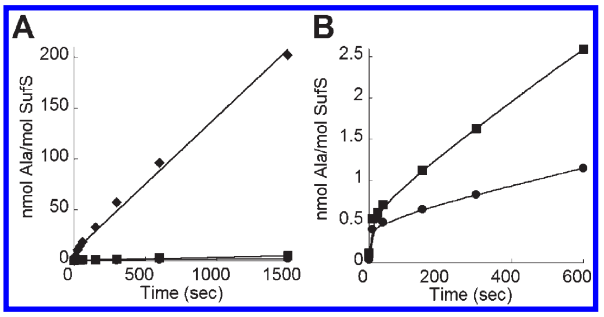


FIGURE 5: Rate of catalysis of alanine formation in the absence of SufU. SufS (2.2 nmol) was incubated with 0.5 mM cysteine in the absence (●) and presence (■) of 2 mM DTT. Panel A also shows the reaction in the presence of 0.013 mM SufU (◆). Panel B shows a zoom-in view of panel A. The amplitude of the kinetic burst is calculated from the extrapolation of the linear fit of steady-state formation of Ala from 10 to 600 s subtracted from time zero. In the absence and presence of DTT, the values were 0.44 (0.47 − 0.03) and 0.44 (0.55 − 0.11), respectively. The number of functional active sites when normalized by the PLP occupancy was 0.95 active site per SufS dimer.

pyruvate was formed in the absence or presence of SufU (data not shown).

Mechanism of Cysteine−SufU Sulfurtransferase Activity of SufS. The characterization of the transfer of sulfur from cysteine to SufU was determined through two-substrate kinetic analysis in which the concentration of one substrate was kept constant while the second one varied (Figure 4). Double-reciprocal plots generated with different SufU or Cys concentrations resulted in parallel lines, which are typical of a double-displacement mechanism (ping-pong). A replot of the double-reciprocal plots of apparent V_{max} values versus SufU concentration ($1/V_{max,app} \times 1/[SufU]$) and Cys concentrations ($1/V_{max,app} \times 1/[Cys]$) (Figure 4, inset) gave the SufS kinetic parameters K_m and V_{max} at the *x*- and *y*-intersections, respectively (Table 1).

A double-displacement mechanism is also supported by kinetic experiments in the absence of SufU (Figure 5). It has been shown that the persulfide sulfur on the SufS catalytic cysteine can be reductively cleaved in the presence of DTT (*9*); furthermore, free cysteine can promote the removal of the sulfur from the cysteine−PLP intermediate in the absence of DTT (*8*). Although DTT and free cysteine can act as "alternative substrates", their reactivities are poor compared to that of SufU (Figure 5A). Rates of alanine formation in the presence of cysteine are more than 200-fold lower (4 nmol min^{-1} mg^{-1}) than SufU-stimulated activity and are 100 times lower in the presence of cysteine and DTT (7 nmol min^{-1} mg^{-1}) (Figure 5B).

Additional kinetic experiments for monitoring alanine formation over time were conducted in the absence of SufU, but in the presence of large amounts of SufS (2.7 μM, 10-fold higher than in the standard assay). As shown in Figure 5, the steady-state formation of alanine is preceded by an apparent exponential burst phase. In the first turnover, the total amount of SufS is in its free enzyme state, therefore resulting in the rapid formation of alanine as seen in the pre-steady-state burst. During steady state, the concentration of the enzyme is partitioned into free enzyme and modified enzyme (SufS.S) and the rate of alanine formation is slower. In these experiments, the use of poor substrates results in the rapid formation of alanine followed by the slow cleavage of the persulfide. Under these conditions, the persulfide cleavage provides the rate-limiting step denoted by the slow linear steady-state formation of alanine. Further characterization of the apparent burst would require rapid chemical quench techniques, as the burst occurs prior to the first time point of the assay (10 s).

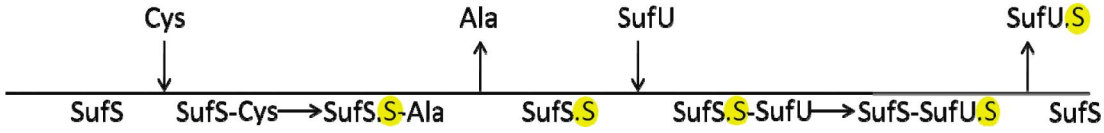
Transfer of Persulfide Sulfur between SufS and SufU. Previous studies of cysteine desulfurases described two possible routes for sulfur transfer: (1) the strict requirement of catalytic cysteine in the nucleophilic attack of substrate-cysteine thiol (*9*) or (2) the direct formation of a persulfide bond on the acceptor molecule bypassing the formation of the persulfide at the catalytic cysteine residue (*8*). To access the participation of the catalytic cysteine in sulfur transfer, a variant form of SufS containing a Cys-361 → Ala (C361A) substitution was analyzed for its cysteine desulfurase activity in the absence and presence of SufU (Figure 2). While SufS^{C361A} is able to interact with SufU (as described above) and to equally bind cysteine (data not shown), it does not generate alanine or sulfide in the presence of SufU, cysteine, and DTT.

In our proposed mechanism, the subsequent step in the route of sulfur mobilization involves the transfer of the persulfide sulfur to the acceptor molecule. It has been shown that cysteine residues in IscU (*34*), SufE (*10*, *35*), and CsdE (*12*) can receive one or more sulfur atoms from cysteine desulfurases via persulfide sulfur transfer. We thus hypothesize that SufU can also accept one or more sulfurs via the transfer of persulfide sulfur from the SufS.S intermediate (Scheme 1). To test this hypothesis, we performed iodoacetamide alkylation of SufU (SufU$_{alk}$) to eliminate its ability to perform thiol chemistry. SufS incubated with SufU$_{alk}$ is inactive even in the presence of nonalkylated SufU (Figure 2). The inhibitory effect of SufU$_{alk}$ on SufS activity in the presence of the substrate SufU was quantified by increasing concentrations of SufU$_{alk}$ in the assay mixture (Figure 6). Six equivalents of SufU$_{alk}$ was sufficient to inhibit SufS activity by 95%. No inhibition was observed upon addition of alkylated or untreated bovine serum albumin, indicating the specific inhibitory effect of SufU$_{alk}$. The concentration−response relationship of SufU$_{alk}$ with regard to the activity of SufS when fitted into the Hill equation resulted in an IC_{50} of 0.37 ± 0.01 μM with an associated Hill coefficient of 1.9 ± 0.1. The inhibition pattern of SufU$_{alk}$ in saturation plots does not show a competitive behavior as expected (Figure 6, inset). Because of the high affinity of the inhibitor SufU$_{alk}$ for SufS, we tested whether the mechanism of inhibition was reversible or dead-end. Time of incubation and dilution experiments eliminated the possibility of formation of a dead-end complex between SufS and SufU$_{alk}$. Collectively, these results are supportive of the first proposed route of sulfur transfer, in which the formation of a modified enzyme (SufS.S) is required for the subsequent transfer of persulfide sulfur to a cysteine residue at the substrate acceptor molecule SufU.

DISCUSSION

Sulfur activation is the first step in Fe−S cluster biogenesis and involves an enzymatic PLP-dependent desulfurization of cysteine with concomitant release of alanine and persulfuration of a cysteine residue at the enzyme's active site (*3*). In vivo, the persulfide sulfur is thought to be directly transferred to acceptor molecules, thus regenerating the enzyme. In vitro, the persulfide

Scheme 1: Kinetic Scheme of the SufS Cysteine−SufU Sulfurtransferase Reaction



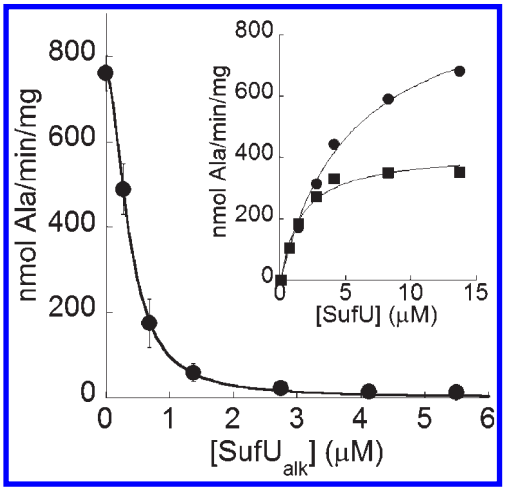


FIGURE 6: Inhibitory effect of $SufU_{alk}$ on the sulfurtransferase reaction of SufS. The assays of $SufU_{alk}$ inhibition were conducted in the presence of 0.01 mg of SufS, 0.013 mM SufU, and 0.5 and 2 mM DTT in 50 mM Mops (pH 7.4). Different concentrations of $SufU_{alk}$ (from 0 to 5.5 μM) were added to measure each point. The line is the best fit for the Hill equation $-v = V_{max}(1 + [SufUalk]/IC_{50})^{n^H}$ obtained in Kaleidagraph. The calculated v is the velocity at any given concentration of $SufU_{alk}$. V_{max} (761 ± 58 nmol min^{-1} mg^{-1}) is the maximal velocity in the assay. IC_{50} (0.37 ± 0.01 μM) is the concentration of the inhibitor that decreases the V_{max} to half. n^H (1.9 ± 0.1) is the Hill coefficient. The inset shows saturation curves in the presence (■) and absence (●) of 0.55 μM $SufU_{alk}$. The lines are the best fits for the Michaelis−Menten equation.

bond is cleaved by reducing agents, which may also be considered alternative substrates in the reaction. In some cases, anomalous kinetic behaviors of cysteine desulfurases have been observed in substrate saturation experiments (*11*, *36*, *37*). In these cases, it is possible that the absence of physiological substrates in the reaction mixture resulted in the accumulation of enzyme intermediates, thus skewing steady-state kinetic measurements.

Previous studies have showed that the presence of sulfur acceptor substrates can stimulate the rates of desulfurization in vitro. One equivalent of IscU was enough to enhance the activity of IscS 6-fold (*13*). Likewise, SufS activity was stimulated by its partner SufE, and the effect of this stimulation was amplified in the presence of the SufBCD proteins (*10*). In addition, stoichiometric amounts of CsdE resulted in a 2-fold increase in CsdA activity, which led to the proposal that CsdA and CsdE form a catalytically competent heteromeric pair (*12*). On the other hand, mechanistic studies of the chloroplastic CpNifS showed concentration-dependent CpSufE stimulation on the rates of desulfurization (*32*). While CsdE, SufE, and CpSufE are specific sulfur acceptors that mediate sulfur trafficking reactions, IscU has been shown to accept the persulfide sulfur from IscS (*34*) and to assemble transient reductively labile Fe−S clusters in the presence of Fe^{2+} (*38*). More recently, it was shown that *B. subtilis* SufS activity is increased 40-fold in the presence of SufU (*20*). Despite compelling evidence of the activation of cysteine desulfurases by sulfur acceptors, the mechanism of such stimulation has not been characterized. In this report, we describe the participation of SufU as a sulfur acceptor molecule and define its role as a substrate in the catalytic mechanism of SufS.

In the absence of SufU and in the presence of DTT, rates of sulfide formation by SufS were very modest (7 nmol min^{-1} mg^{-1}). This result is consistent with the activity of other cysteine desulfurases [13 and 19 nmol min^{-1} mg^{-1} for CpNifS (*32*) and *E. coli* SufS (*35*), respectively]. During catalysis, the *E. coli* SufS persulfide bond is protected from reducing agents; the transfer of persulfide sulfur to SufE occurred despite the presence of 10 mM DTT (*10*). The structural location of the active site cysteine in a rigid loop located away from the solvent-exposed surface may account for this lack of efficiency in reductively cleaving the persulfide bond. This structural feature is a signature of group II cysteine desulfurases and implies the critical participation of specific sulfur acceptors. In the case of *B. subtilis* SufS, our analysis showed saturating concentrations of SufU resulted in a > 100-fold increase in the rates of sulfide and alanine formation. To the best of our knowledge, SufU stimulation of SufS is the strongest stimulation effect described for a cysteine desulfurase to date. This observation perhaps denotes a sophisticated control mechanism of sulfur transfer by this group of enzymes that may protect against futile catalytic cycles and/or adventitious reactions with nonphysiological targets. It is possible that protected sulfur trafficking routes are crucial during challenging environmental conditions. In biological systems facing oxidative challenges, such as plant chloroplasts or infectious Gram-positive bacteria, group II cysteine desulfurases, along with their specific sulfur acceptors, play essential metabolic roles in these life settings. In the case of *B. subtilis*, SufS and SufU are essential for cell survival.

The interactions between the sulfur donor SufS and its acceptor SufU are supported not only by stimulation of SufS catalytic properties but also by isolation of purified fractions containing both proteins. Transient interactions between SufS and SufU during the in vivo direct sulfur transfer process are assisted by protein−protein interactions and are not limited to disulfide or polysulfide linkages. The variation in the ratio of SufS and SufU in the purification preparations, along with the apparently substoichiometric amounts of SufU, supports the hypothesis of their transient interactions in physiological environments. In spite of that, gel-filtration experiments do not rule out the presence of mixed SufS species, including free SufS and SufU-bound SufS. Transient interactions between sulfur donors and acceptors have also been observed from coexpression and copurification of *E. coli* SufS (*21*) and *E. coli* and *Azotobacter vinelandii* IscU (*38*), indicating the formation of transient complexes in vivo. In the latter case, proteins expressed separately did not result in copurification of IscS and IscU (*39*). Nevertheless, the fact that the protein mixtures containing similar ratios of SufS and SufU display comparable activities indicates that in vivo preformation of the SufS−SufU protein complex is not required for the catalytic competence of the enzyme.

Initial velocity studies of SufS showed a profile consistent with a ping-pong kinetic mechanism, supporting a model for the cysteine−SufU sulfurtransferase reaction in which the formation of a modified enzyme and release of alanine precede the binding

of the substrate SufU. Rhodaneses, sulfurtransferases that do not require PLP, also use a ping-pong mechanism through formation of a persulfide−enzyme intermediate (*40*, *41*). In the case of SufS, it is possible that the catalytic cysteine dictates the ping-pong mechanism in the same fashion as rhodaneses and that the PLP cofactor plays a role in the activation of the substrate for nucleophilic attack of the catalytic cysteine. The measured K_m of 3.1 μM for SufU was more than 20 times lower than the K_m for cysteine (K_m = 83 μM), emphasizing the high specificity of SufS for its acceptor SufU, and it is consistent with the hypothesis of protected sulfur transfer reactions. Nevertheless, the assays described in this study were conducted in the presence of DTT, which regenerates the second reaction product, SufU.S (Scheme 1), eliminating any product inhibition effects.

The rates of alanine formation were also determined in the absence of SufU and DTT. Under these conditions, the enzyme turned over at a very modest rate (4 nmol of alanine min^{-1} mg^{-1}). The low specific activity may likely be due to the limited reactivity of persulfurated enzyme and/or caused by the participation of free cysteine substrate in receiving a persulfide. In the absence of the substrate SufU, the slow cleavage of the persulfide bond dictates the overall reaction rate. Under these conditions, the number of active sites can be estimated from extrapolation of the steady-state formation of alanine over time. The amplitude of the *y*-intercept (0.44 nmol of alanine/nmol of SufS) indicates the number of active sites. Unexpectedly, our results suggest that, in the first turnover, only half of the enzyme is active (44%). PLP analysis of the purified enzyme revealed the presence of 0.93 ± 0.6 PLP/SufS, indicating that the substoichiometry of active sites on SufS is not explained by low cofactor occupancy. In addition, 100 mM DTT enzyme treatment did not increase the amplitude of the *y*-intercept and suggested that the purified enzyme is likely homogeneous free SufS and does not include any mixed species containing persulfurated forms (SufS.S). It is possible that the activation of one active site elicits structural changes in the other subunit, thus providing a rationale of negative cooperative regulation between subunits of each dimer. On the basis of this proposed model, only one subunit of the enzyme would be active at a time. The occurrence of a negative cooperative behavior (flip-flop mechanism) of dimers or tetramers of other PLP enzymes has been proposed on the basis of either their patterns of inhibition in the presence of suicide inhibitors (*42*) or activity levels upon PLP titration of reconstituted enzymes (*43*). Negative cooperative behavior of PLP enzymes provides a mechanistic reason for the conservation of dimeric and tetrameric organization among enzymes of this family. This proposed enzyme behavior might result in another level of control of the sulfur-protected transferase reaction of SufS.

The essential participation of the SufS catalytic cysteine and SufU cysteine(s) in the mechanism, as evidenced by a lack of activity of $SufS^{C361A}$ or $SufU_{alk}$, is consistent with the proposed catalytic scheme that involves the persulfide sulfur transfer reaction. Interestingly, $SufU_{alk}$ had an inhibitory effect on SufS activity in the absence and presence of nonalkylated SufU. The mechanism of inhibition, although not fully characterized, did not exhibit competitive behavior. The presence of several enzyme intermediate states in the reaction scheme (Scheme 1) and the possible interaction of $SufU_{alk}$ with more than one intermediate may contribute to a noncompetitive inhibitory profile. The inhibition of $SufU_{alk}$ showed a positive cooperative behavior with a Hill coefficient of 2, which suggests that the binding of one molecule of $SufU_{alk}$ to one subunit of the SufS dimer also affects the activity of the other subunit. This observation is also consistent with the hypothesis of one functional active site at a time, at which the binding of $SufU_{alk}$ to the "open" active site would lock the dimer preventing the other subunit from being functional.

Finally, we developed a sensitive fluorescence-based assay to quantify alanine by derivatization with NDA. Detection of alanine has an additional advantage; its formation in this reaction is independent of the nature of the sulfur acceptor. That is, in the detection of sulfide, the DTT reductant might compete with the sulfur acceptor for cleavage and/or transfer of the persulfide sulfur, which could perturb the kinetic assessment of these enzymes' reactivities.

In this work, we have identified unique mechanistic features of SufS and the reaction's dependence on SufU. The development of a sensitive method for alanine detection and specific involvement of the sulfur acceptor molecule in the catalytic scheme of SufS sets the stage for future exploration of the specific mechanisms of other known cysteine desulfurases. To date, studies have concentrated on the first half of the catalytic scheme, the binding of cysteine followed by persulfide formation and alanine release. The second half of the reaction, transfer of the persulfide sulfur to acceptor molecules, has not been explored in detail. Our mechanistic studies with *B. subtilis* SufS raise the question of whether the sulfurtransferase mechanism is universal to all cysteine desulfurases or specific to this subgroup. Mechanistic studies of other known cysteine desulfurases are currently being pursued by our group.

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE of copurified SufS−SufU fractions after the treatment of crude extracts with iron, cysteine, and/or DTT (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Beinert, H. (2000) A tribute to sulfur. *Eur. J. Biochem. 267*, 5657–5664.
2. Kessler, D. (2006) Enzymatic activation of sulfur for incorporation into biomolecules in prokaryotes. *FEMS Microbiol. Rev. 30*, 825–840.
3. Mueller, E. G. (2006) Trafficking in persulfides: Delivering sulfur in biosynthetic pathways. *Nat. Chem. Biol. 2*, 185–194.
4. Ayala-Castro, C., Saini, A., and Outten, F. W. (2008) Fe-S cluster assembly pathways in bacteria. *Microbiol. Mol. Biol. Rev. 72*, 110–125.
5. Mihara, H., and Esaki, N. (2002) Bacterial cysteine desulfurases: Their function and mechanisms. *Appl. Microbiol. Biotechnol. 60*, 12–23.
6. Cupp-Vickery, J. R., Urbina, H., and Vickery, L. E. (2003) Crystal structure of IscS, a cysteine desulfurase from *Escherichia coli*. *J. Mol. Biol. 330*, 1049–1059.
7. Fujii, T., Maeda, M., Mihara, H., Kurihara, T., Esaki, N., and Hata, Y. (2000) Structure of a NifS homologue: X-ray structure analysis of CsdB, an *Escherichia coli* counterpart of mammalian selenocysteine lyase. *Biochemistry 39*, 1263–1273.
8. Tirupati, B., Vey, J. L., Drennan, C. L., and Bollinger, J. M., Jr. (2004) Kinetic and structural characterization of Slr0077/SufS, the essential cysteine desulfurase from *Synechocystis* sp. PCC 6803. *Biochemistry 43*, 12210–12219.
9. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. *Biochemistry 33*, 4714–4720.

10. 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.
11. Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) Kinetic and mutational studies of three NifS homologs from *Escherichia coli*: Mechanistic difference between L-cysteine desulfurase and L-selenocysteine lyase reactions. *J. Biochem. 127*, 559–567.
12. 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.
13. Kato, S., Mihara, H., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: Implications for the mechanism of iron-sulfur cluster assembly. *Proc. Natl. Acad. Sci. U.S.A. 99*, 5948–5952.
14. Lauhon, C. T., Skovran, E., Urbina, H. D., Downs, D. M., and Vickery, L. E. (2004) Substitutions in an active site loop of *Escherichia coli* IscS result in specific defects in Fe-S cluster and thionucleoside biosynthesis in vivo. *J. Biol. Chem. 279*, 19551–19558.
15. Shi, R., Proteau, A., Villarroya, M., Moukadiri, I., Zhang, L., Trempe, J. F., Matte, A., Armengod, M. E., and Cygler, M. (2010) Structural basis for Fe-S cluster assembly and tRNA thiolation mediated by IscS protein-protein interactions. *PLoS Biol. 8*, e1000354.
16. Nilsson, K., Lundgren, H. K., Hagervall, T. G., and Bjork, G. R. (2002) The cysteine desulfurase IscS is required for synthesis of all five thiolated nucleosides present in tRNA from *Salmonella enterica* serovar *typhimurium*. *J. Bacteriol. 184*, 6830–6835.
17. Tokumoto, U., and Takahashi, Y. (2001) Genetic Analysis of the isc Operon in *Escherichia coli* Involved in the Biogenesis of Cellular Iron-Sulfur Proteins. *J. Biochem. 130*, 63–71.
18. Schwartz, C. J., Djaman, O., Imlay, J. A., and Kiley, P. J. (2000) The cysteine desulfurase, IscS, has a major role in in vivo Fe-S cluster formation in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 97*, 9009–9014.
19. Lauhon, C. T. (2002) Requirement for IscS in biosynthesis of all thionucleosides in *Escherichia coli*. *J. Bacteriol. 184*, 6820–6829.
20. Albrecht, A. G., Netz, D. J., Miethke, M., Pierik, A. J., Burghaus, O., Peuckert, F., Lill, R., and Marahiel, M. A. (2010) SufU is an essential iron-sulfur cluster scaffold protein in *Bacillus subtilis*. *J. Bacteriol. 192* (10), 2512–2524.
21. Layer, G., Gaddam, S. A., Ayala-Castro, C. N., Ollagnier-de Choudens, S., Lascoux, D., Fontecave, M., and Outten, F. W. (2007) SufE Transfers Sulfur from SufS to SufB for Iron-Sulfur Cluster Assembly. *J. Biol. Chem. 282*, 13342–13350.
22. Chahal, H. K., Dai, Y., Saini, A., Ayala-Castro, C., and Outten, F. W. (2009) The SufBCD Fe-S scaffold complex interacts with SufA for Fe-S cluster transfer. *Biochemistry 48*, 10644–10653.
23. Corfe, B. M., Moir, A., Popham, D., and Setlow, P. (1994) Analysis of the expression and regulation of the gerB spore germination operon of *Bacillus subtilis* 168. *Microbiology 140* (Patt 11), 3079–3083.
24. Lauhon, C. T., and Kambampati, R. (2000) The iscS gene in *Escherichia coli* is required for the biosynthesis of 4-thiouridine, thiamin, and NAD. *J. Biol. Chem. 275*, 20096–20103.
25. Dos Santos, P. C., Johnson, D. C., Ragle, B. E., Unciuleac, M. C., and Dean, D. R. (2007) Controlled expression of nif and isc iron-sulfur protein maturation components reveals target specificity and limited functional replacement between the two systems. *J. Bacteriol. 189*, 2854–2862.
26. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
27. 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.
28. Wada, H., and Snell, E. E. (1962) Enzymatic transamination of pyridoxamine. I. With oxaloacetate and α-ketoglutarate. *J. Biol. Chem. 237*, 127–132.
29. Siegel, L. M. (1965) A Direct Microdetermination for Sulfide. *Anal. Biochem. 11*, 126–132.
30. Chen, J. S., and Mortenson, L. E. (1977) Inhibition of methylene blue formation during determination of the acid- labile sulfide of iron-sulfur protein samples containing dithionite. *Anal. Biochem. 79*, 157–165.
31. Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. U.S.A. 90*, 2754–2758.
32. Ye, H., Abdel-Ghany, S. E., Anderson, T. D., Pilon-Smits, E. A., and Pilon, M. (2006) CpSufE activates the cysteine desulfurase CpNifS for chloroplastic Fe-S cluster formation. *J. Biol. Chem. 281*, 8958–8969.
33. Zhang, L. Y., and Sun, M. X. (2005) Selective determination of γ-aminobutyric acid, glutamate and alanine by mixed micellar electrokinetic chromatography and fluorescence detection. *J. Chromatogr., A 1095*, 185–188.
34. 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.
35. 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.
36. 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.
37. Cartini, F., Remelli, W., Dos Santos, P. C., Papenbrock, J., Pagani, S., and Forlani, F. (2010) Mobilization of sulfane sulfur from cysteine desulfurases to the *Azotobacter vinelandii* sulfurtransferase RhdA. *Amino Acids* (in press).
38. Raulfs, E. C., O'Carroll, I. P., Dos Santos, P. C., Unciuleac, M. C., and Dean, D. R. (2008) In vivo iron-sulfur cluster formation. *Proc. Natl. Acad. Sci. U.S.A. 105*, 8591–8596.
39. Unciuleac, M. C., Chandramouli, K., Naik, S., Mayer, S., Huynh, B. H., Johnson, M. K., and Dean, D. R. (2007) In vitro activation of apo-aconitase using a [4Fe-4S] cluster-loaded form of the IscU [Fe-S] cluster scaffolding protein. *Biochemistry 46*, 6812–6821.
40. Bordo, D., Deriu, D., Colnaghi, R., Carpen, A., Pagani, S., and Bolognesi, M. (2000) The crystal structure of a sulfurtransferase from *Azotobacter vinelandii* highlights the evolutionary relationship between the rhodanese and phosphatase enzyme families. *J. Mol. Biol. 298*, 691–704.
41. Cheng, H., Donahue, J. L., Battle, S. E., Ray, W. K., and Larson, T. J. (2008) Biochemical and Genetic Characterization of PspE and GlpE, Two Single-domain Sulfurtransferases of *Escherichia coli*. *Open Microbiol. J. 2*, 18–28.
42. Burnett, G., Yonaha, K., Toyama, S., Soda, K., and Walsh, C. (1980) Studies on the kinetics and stoichiometry of inactivation of *Pseudomonas* ω-amino acid:pyruvate transaminase by gabaculine. *J. Biol. Chem. 255*, 428–432.
43. Churchich, J. E., and Moses, U. (1981) 4-Aminobutyrate aminotransferase. The presence of nonequivalent binding sites. *J. Biol. Chem. 256*, 1101–1104.