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Changes in Protein Dynamics in *Escherichia coli* SufS Reveal a Possible Conserved Regulatory Mechanism in Type II Cysteine Desulfurase Systems

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Abstract

In the Suf Fe-S cluster assembly pathway, the activity of the cysteine desulfurase, SufS, is regulated by interactions with the accessory sulfotransferase protein, SufE. SufE has been shown to stimulate SufS activity, likely through inducing conformational changes in the SufS active site that promote the desulfurase step and by acting as an efficient persulfide acceptor in the transpersulfuration step. Previous results point toward an additional level of regulation through a ‘half-sites’ mechanism that affects the stoichiometry and affinity for SufE as the dimeric SufS shifts between desulfurase and transpersulfuration activities. Investigation of the covalent persulfide intermediate of SufS by backbone amide hydrogen/deuterium exchange mass spectrometry identified two active site peptides (residues 225–236 and 356–366) and two peptides at the dimer interface of SufS (residues 89–100 and 243–255) that exhibit changes in deuterium uptake upon formation of the intermediate. Residues in these peptides are organized to form a conduit between the two active sites upon persulfide formation and include key cross-monomer interactions, suggesting they may play a role in the half-sites regulation. Three evolutionarily conserved residues at the dimer interface (R92, E96, and E250) were investigated by alanine scanning mutagenesis. Two of the substituted enzymes (E96A and E250A SufS) resulted in 6-fold increases in the value of K_{SufE} , confirming a functional role. Re-examination of the dimer interface in reported crystal structures of SufS and the SufS-homolog CsdA identified previously unnoticed residue mobility at the dimer interface. The identification of conformational changes at the dimer interface by hydrogen/deuterium exchange confirmed by mutagenesis and structural reports provides a physical mechanism for active site communication in the half-sites regulation of SufS activity. Given the conservation of the interface interactions, this mechanism may be broadly applicable to type II cysteine desulfurase systems.

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Supporting Information

Table of fitting parameters for HDX-MS results and PLP incorporation. Figures for deuterium uptake for all peptides and analytical gel filtration results.

Graphical abstract

Keywords

Iron-sulfur cluster biogenesis; cysteine desulfurase; hydrogen/deuterium exchange mass spectrometry (HDX-MS); protein dynamics

Iron-sulfur clusters are essential cofactors found in all forms of life. These cofactors play key roles in electron transfer reactions, radical-based reactions, and in sensing iron and oxygen levels.¹ Due to their essential nature, proteins involved in the assembly pathways for Fe-S clusters serve as targets for the development of novel antibacterial and antifungal agents. Assembly pathways for Fe-S clusters consist of multi-protein scaffolds where clusters are made prior to transfer to a target metalloprotein. Iron and sulfur atoms used to construct the clusters are delivered to the scaffold in a highly choreographed manner from additional proteins. The assembly process appears to be regulated through protein-protein interactions, as illustrated by the recent structure of the human ISC Fe-S assembly subcomplex², but many of these mechanisms remain unclear. Currently, three different pathways for Fe-S cluster assembly have been identified. The pathways utilize unique scaffold complexes and support specific and distinct physiological Fe-S cluster needs. However, a common first step is the liberation of sulfur from the amino acid cysteine by a cysteine desulfurase enzyme.³

Cysteine desulfurases are 5'-pyridoxal phosphate (PLP) dependent enzymes that catalyze the breakage of the C-S bond in cysteine to generate sulfur and alanine.³ These enzymes share a dimeric aminotransferase V fold that consists of two domains (Figure 1A). The larger domain contains much of the dimer interface and is constructed of a parallel β -sheet produced by repeating $\alpha\beta$ motifs. The smaller domain contains an anti-parallel β -sheet surrounded by α -helices. The active site lies at the interface of the two domains with the PLP-bound lysine residing in the large domain and the nucleophilic cysteine residing in the small domain. Additionally, one wall of the active site is comprised of residues from the neighboring monomer suggesting the dimeric nature of the fold is essential for function. Cysteine desulfurase enzymes can be characterized as type I and type II on the basis of distinctive features, including the length of the active site cysteine-containing loop.³

SufS from *E. coli* is a type II cysteine desulfurase that belongs to the Suf Fe-S cluster pathway. This pathway is the most ancient of the Fe-S cluster assembly systems and is proposed to operate under conditions of iron starvation or oxidative stress in *E. coli*.⁴ SufS is proposed to use a ping-pong mechanism.⁵ The overall reaction, shown in figure 1B, occurs in two half reactions. In the resting state, PLP forms a Schiff base with a conserved active site lysine residue (K226 in *E. coli* SufS). The first half reaction involves binding of L-cysteine to the lysine-bound PLP to form an external aldimine. Upon abstraction of the α -

proton and formation of a ketamine intermediate, a conserved active site cysteine (C364 in *E. coli* SufS) carries out a nucleophilic attack on the cysteine sulfur, breaking the C-S bond and generating the SufS persulfide covalent intermediate (SufS_{per}) and L-alanine. In the group I desulfurases, the persulfide intermediate can be directly transferred to Fe-S cluster biosynthesis machinery. In the group II enzymes, this intermediate is initially shuttled to a small transfer protein en route to the Fe-S cluster assembly scaffold. In the Suf pathway, SufE acts as the cognate persulfide transfer protein for SufS. Addition of SufE to the SufS reaction increases the cysteine desulfurase rate at least 8-fold, likely by increasing the rate of the transpersulfuration reaction.^{6, 7}

Biophysical characterization of the SufS/SufE complex suggests that in addition to simply increasing the rate of persulfide transfer, SufS activity is also regulated through a ‘half-sites’ mechanism. ITC binding studies show that SufE_{apo} binds to SufS_{apo} in a two-sites model with negative cooperativity and K_d values of ~ 4 μ M and ~300 μ M for the first and second binding site, respectively.⁸ However the activated intermediates SufE_{alk} and SufE_{D74R} bind with a one-site model and K_d values 10-fold lower than SufE_{apo} (0.3 and 0.5 μ M for SufE_{alk} and SufE_{D74R}, respectively). These modified forms of SufE were shown to be locked in an “activated” conformation for interacting with SufS.^{8, 9} Importantly, SufE_{alk} and SufE_{D74R} do not fully occupy the two active sites as they exhibit between 0.56–0.73 sites bound/dimer SufS consistent with a half-sites mechanism.

On the basis of the proposed mechanism, a key catalytic event for the two component sulfotransferase systems is the transition from a conformation that catalyzes formation of the persulfide intermediate in the first half reaction to one catalyzing the subsequent transpersulfuration reaction with increased affinity for SufE. The structures of SufS in the absence (SufS_{apo}) and presence (SufS_{per}) of the C364 persulfide intermediate have been reported, and comparison of the two structures reveals conformational changes at the active site.¹⁰ First, the persulfurated C364 side chain swings upwards so that the γ -sulfur is stabilized by new interactions with H124, H362, and the backbone amide of S254' (from the adjacent SufS monomer). This conformational change disrupts the interaction of C364 with R359, causing R359 to form a new interaction with D28. No significant structural changes are seen outside of the active site. However, the structures are static representations of the two conformational states (or ensembles of states) and do not reveal the dynamic motions that accompany formation of the persulfide reaction intermediate and subsequent sulfur transfer to SufE, which are crucial to enzyme function. These motions can be characterized using backbone amide hydrogen-deuterium exchange mass spectrometry (HDX-MS). This technique allows for structural information to be collected in solution and does not have the mass limitations associated with NMR. We have recently reported results of HDX-MS experiments aimed at identifying changes in the protein dynamics of SufS when in complex with native SufE⁸ as well as the activated variant D74R SufE⁹. Here, HDX-MS is utilized to investigate dynamic changes that occur upon persulfuration of SufS. The current results, in concert with those previously reported, suggest a conduit for active site communication in the half-sites mechanism of SufS regulation.

Materials and Methods

Chemicals.

Only HPLC grade reagents were used and all buffers were prepared in ultrapure water (18 MΩ at 25 °C). For deuterium labeling, 99.5% D₂O was obtained from Acros Organics. Porcine pepsin (3200–4500 u/mg), DTNB (5,5'-dithio-bis-2-nitrobenzoic acid), and DMPD (N,N-dimethyl-p-phenylenediamine sulfate) were obtained from Sigma Aldrich.

SufS persulfide intermediate formation.

A 1.5 mM SufS_{apo} stock in 25 mM Tris-HCl, 150 mM NaCl, 10 mM β-mercaptoethanol, pH 7.4 was buffer exchanged into 25 mM Tris-HCl, 150 mM NaCl, pH 7.4 in a Vacuum Atmospheres anaerobic glove box using the centrifugal filters. Twenty five microliters of 900 μM SufS_{apo} was incubated with 5 μL of 200 mM cysteine for 30 minutes and the solution was desalted using spin columns. The concentration of the desalted SufS_{per} was measured at 280 nm. Two microliters of SufS_{per} were aliquoted into thin-walled PCR tubes under nitrogen atmosphere and used immediately for analysis.

A DTNB assay was used to calculate the number of cysteine thiolate anions in SufS_{per}.¹¹ For the buffer control, 12 μL of the final dialysis buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.4) was mixed with 75 μL of the reaction buffer (200 mM Tris HCl, pH 7.4, 150 mM NaCl, and 8 M urea) and 10 μL of 5 mM DTNB. For the protein assay, 12 μL of 30 μM SufS_{per} was mixed with 10 μL of 5 mM DTNB and 75 μL of reaction buffer. Ultrapure water was used to bring the total volume to 150 μL for all the samples. The samples were incubated for 30 minutes in an anaerobic glove box. The absorbance at 412 nm was measured to calculate the concentration of thiolate anions in the persulfurated SufS sample using the molar extinction coefficient for DTNB at 412 nm of 14.15 mM⁻¹ cm⁻¹.

For quantification of the formation of persulfide, a *N,N*-dimethyl-*p*-phenylenediamine sulfate (DMPD) colorimetric assay was performed. All the reactions were done under an anaerobic atmosphere in a glove box. A 10 mM stock of sodium sulfide (Na₂S) was prepared in Tris-HCl buffer (25 mM Tris HCl, 150 mM NaCl, pH 7.4). A standard curve was generated using 0–50 μM Na₂S, 100 μL of 10 mM DTT, 100 μL of 30 mM FeCl₃ in 1.2 M HCl, and 100 μL of 20 mM DMPD in 7.2 M HCl to a final volume of 1 mL. SufS_{per} (30 μM) was incubated with 100 μL DTT, 100 μL FeCl₃ and 100 μL DMPD to a total volume of 1 mL in Tris-HCl buffer. The reactions were incubated in the dark for 30 minutes and the absorbance at 670 nm was measured to quantify the formation of methylene blue. The standard curve for sulfide ion concentration (absorbance at 670 nm against the Na₂S concentration) was used to calculate the concentration of SufS persulfide.

Tandem MS/MS sequencing of persulfurated SufS.

The SufS_{per} and tris(2-carboxyethyl)phosphine (TCEP)-reduced SufS_{per} pepsin-generated peptides were analyzed by tandem mass spectrometry to create the peptide maps. The SufS_{apo} map has already been reported.⁸ For the persulfurated sample, 25 μL of 20 μM SufS_{per} was mixed with 25 μL of quench buffer (0.1 M KH₂PO₄, pH 2.3 at 0 °C) on ice then digested for 5 minutes with 10 μg of porcine pepsin (5 mg/mL in 0.01 M KH₂PO₄, pH 7.4).

For the TCEP reduced SufS_{per}, the quenched protein was incubated with 1 μ L of 100 mM TCEP followed by digestion as above. The generated peptides were separated by a Phenomenex 50 $\times$ 2 mm C-18 reversed phase HPLC column using a 0.1 mL/min with a linear gradient of 0–50% of 98% ACN, 2% H₂O and 0.4% formic acid over 26 minutes. Peptides were sequenced using a HCT Ultra PTM Discovery ion trap mass spectrometer in positive ion mode by data dependent MS/MS CID. The scanning range for the mass spectrometer was set at 50–1500 m/z in positive ion mode with maximum accumulation time of 200 ms and an average of three spectra. The nebulizer gas pressure was 28 psi and the temperature was set to 250 °C. The number of precursor ions fragmented per MS scan was set to six. The amino acid sequences of the peptides were determined using PEAKS with several potential sulfur states (persulfide, SO₂H, SO₃H, and S₃H) set as cysteine modifications for quenched samples without TCEP. The reduced form was analyzed without post-translational modification of cysteine. The digest results were found to be highly reproducible under these conditions.

Hydrogen-deuterium exchange mass spectrometry (HDX-MS).

HDX mass spectrometry was used to compare the persulfurated form of SufS with and without SufE essentially as previously described.⁸ Stock solutions of SufS_{apo} and SufS_{per} (125 μ M) were prepared in 25 mM Tris-HCl, 150 mM NaCl, 5 mM DTT at pH 7.4. Two microliters of 125 μ M SufS_{per} was incubated with 23 μ L of 99.5% pure D₂O at 25 °C for 15 seconds to 1 hour. The reaction was quenched by adding 25 μ L of ice cold quench buffer with 100 μ M TCEP and digested on ice for 5 min using 10 μ g of pepsin. The controls for the natural isotope distribution ($m_{0\%}$) and the amount of deuterium back exchange ($m_{100\%}$) for SufS were performed as previously described.¹² The peptides were loaded onto a C-18 reversed phase HPLC column (50 mm $\times$ 2.00 mm) and eluted over 15 minutes using a linear gradient of 0–50% HPLC grade acetonitrile solution (98% acetonitrile, 2% H₂O and 0.4% formic acid). The mass spectrometer was in positive ion mode with a scan range of 300–1500 m/z . The nebulizer gas pressure was kept at 28 psi with dry gas flow rate and temperature at 7 L/min and 250 °C, respectively. All samples for the HDX-MS were prepared individually and ran on the same day, and the overall results are the average of three separate experiments. Deuterium incorporation into individual peptides was analyzed by HDExaminer. The percent deuterium incorporation was plotted as a function of time and fit to a sum of first order rate equations,

$$D = N_{\%} - \sum_{i=1}^N \exp^{(-k_i t)}$$

where $N_{\%}$ is the percentage of amides exchanging at a rate k_i for the isotopic exchange time t .¹³

Cysteine desulfurase assay.

SufS cysteine desulfurase activity was determined by quantification of product, alanine by labeling reaction with naphthalene 2,3-dicarboxaldehyde (NDA) (AnaSpec Inc.)⁵. A reaction mixture contained 0.12 μ M SufS or variants, 2 mM TCEP, 50 mM MOPS (pH7.5), 150 mM

NaCl, and non-varied substrate (cysteine or SufE_{wt}) in a 1 mL reaction at 27 °C. The reactions were initiated by the addition of cysteine. Substrate saturation curves were determined by varying the concentration of cysteine (5 – 500 μM) at 4 μM SufE and varying concentration of SufE (0.1 – 6 μM) at 500 μM cysteine. The initial velocity was derived from the slope of a plot of alanine concentration versus time points (0, 2, 4, and 6 min).

Alanine production was measured through HPLC (Shimadzu) coupled with a fluorescence detector. At each time point, 50 μL aliquots from the assay were mixed with 20 μL of 10% trichloroacetic acid (TCA) to quench the reaction and 1 mL of freshly prepared NDA mix solution (0.1 mM NDA and 1 mM KCN in 100 mM borate buffer, pH 9.0) was added to each aliquot. The sample mixtures were incubated in dark for 1 hr for ala-NDA adducts formation and 10 μL of sample was injected onto a Zorbax C18 column (Agilent) at a flow rate of 0.4 mL/min with an isocratic gradient of 50% 10 mM ammonium acetate (pH 6.0) with 50% methanol for 10 min, alanine-NDA was detected by fluorescence at excitation 420 nm and emission 490 nm. The alanine-NDA fluorescence peak was integrated and the peak area of alanine-NDA was converted into nanomoles of alanine using the 0 to 50 μM alanine standard curve prepared with same concentration of cysteine added in the assay. Initial velocities were fit to the Michaelis-Menten equation to determine kinetic parameters for each enzyme.

Results

Generation of the SufS C364 persulfide intermediate.

A DTNB assay prior to the formation of the persulfide intermediate of SufS showed that SufS_{apo} contains 3.2 ± 0.3 free cysteine thiolates (~80%) per monomer.¹¹ The persulfide intermediate was formed by allowing SufS_{apo} to turn over in the presence of excess L-cysteine substrate.¹⁴ A DMPD assay to measure labile sulfide released from the desulfurase reaction^{15, 16} determined that $78 \pm 5\%$ of SufS_{per} contained a persulfide moiety suggesting successful generation of the intermediate. Tandem MS/MS sequencing was used to identify the location of the generated persulfurated intermediate. The MS/MS data was analyzed against the SufS amino acid sequence using the program PEAKS. The input for the modification of cysteine was set to detect several possible sulfur states including a persulfide. In the SufS_{apo} protein, pepsin digestion results in peptides covering all four cysteine residues (Figure 2A). However, in the persulfurated protein no coverage was seen for either a modified or unmodified C364, while the other three cysteine residues were found in their native state. To determine if a persulfide on C364 interfered with MS/MS analysis, SufS_{per} was reduced with TCEP after pepsin digestion to liberate the sulfide. The mass for peptide 356–366 with a free C364 thiolate was then detected, suggesting the presence of the persulfurated species before reduction (Figure 2B). One possibility for the lack of detection would be an intramolecular disulfide formed between two cysteine residues on SufS. However, identification of peptides containing the remaining unmodified cysteine residues in the persulfurated enzyme rules out this possibility. Thus, the appearance of the 356–366 peptide after treatment with TCEP and the quantification of sulfide released in the DMPD assay are consistent with a species containing one persulfide moiety on C364. In HDX-MS

experiments described below the C364 persulfide is present during the deuterium exchange portion of the experiment and is reduced with TCEP during digestion for analysis purposes.

Conformational analysis of the SufS persulfide catalytic intermediate state.

In order to complement the static structural data, HDX-MS was used to determine changes in backbone dynamics in SufS upon formation of the persulfide intermediate. While the majority of the protein was not affected by formation of the intermediate (Figure S1), several key peptides exhibited changes in deuterium incorporation kinetics (Figure 3A). Peptide 356–366 exhibits a 50% reduction in deuterium incorporation. From fits of the kinetic data, the loss of deuterium incorporation is mainly due to a loss of a fast exchange phase (exchange rate of $\sim 2 \text{ min}^{-1}$). This peptide is found in the active site of the enzyme and contains the C364, where the persulfide intermediate forms (Figure 3B and 3C). Residues H362, which forms an interaction with the newly formed persulfide, and R359, which formed an interaction with C364 prior to persulfide formation are also located on this same peptide.¹⁰ A second active site peptide (residues 225–236) also exhibits a significant decrease in deuterium incorporation. This peptide contains K226 that forms the initial Schiff base with the PLP cofactor. Overall, the behavior exhibited by these two peptides is consistent with changes predicted by structural changes in the static structures.¹⁰

The HDX kinetic traces for SufS_{per} also reveal a loss of conformational flexibility for regions at the dimer interface of protein upon persulfuration. The peptides 88–100 and 243–255 display a noticeable decrease in the rate of deuterium incorporation for SufS_{per}, compared to SufS_{apo} (Figures 3 and 4A). Peptide 88–100 was identified in our previous work examining interactions with the activated SufE variant D74R and contains residues E96 and R92. These residues form a salt bridge across the dimer interface in several reported crystal structures (including one containing the persulfide intermediate) of the SufS enzyme (Figure 4B, tan structure).^{10, 17} The peptide containing residues 243–255 forms a flexible loop region between the main beta sheet and a β -hairpin motif at the dimer interface (Figure 4C). This loop contains S254, which has been shown to interact with the persulfide intermediate through a backbone amide.¹⁰ The adjacent β -hairpin lies next to the active site opening on the neighboring monomer and has previously been shown to exhibit increased deuterium trapping in the formation of the SufS_{per} and alkylated SufE (SufE_{alk}) complex, suggesting it may be involved in the binding of SufE.⁸

Comparison of the structures of SufS_{apo} and SufS_{per} does not reveal any changes in the overall conformation of the regions covering residues 88–100 and 243–255. However, in the reported structure of SufS with a substrate analog where an external aldimine intermediate is trapped, R92 shifts away from its interaction with E96 and forms a new interaction with E250 (Figure 4B, blue structure) providing a link between persulfide-induced changes at the base of the β -hairpin and the dimer interface.¹⁷ The decrease in the rate of deuterium incorporation in these two peptides upon persulfuration of SufS suggests a conformational change occurs in this region that may not be apparent in the static SufS_{per} crystal structure. Importantly, no changes were seen in regions covering peptides 88–100 or 243–255 when HDX-MS was performed after binding of unmodified wild-type SufE to SufS_{apo}.⁸ This result suggests that these regions only exhibit altered dynamics upon formation of the

SufS_{per} intermediate in the catalytic cycle and may play a role in the proposed half-sites regulation.

Site-directed mutagenesis.

Single residue variants of SufS were expressed and purified to test the hypothesis that interactions between R92 and E96/E250 of SufS identified in the HDX-MS study play a functional role. Alanine scanning mutagenesis was used to create single site substitutions to R92, E96, and E250. All three variant enzymes were successfully expressed and purified to homogeneity similar to the wild-type enzyme. UV-Vis spectroscopic analysis of PLP liberated from wild-type and the variant enzymes show that all enzymes have 89–97% PLP incorporation (Table S2), and size-exclusion chromatography results are consistent with dimeric quaternary structure for all three variants (Figure S2). The most accurate activity assay for cysteine desulfurase enzymes is to measure alanine production after derivatization with the fluorescent label naphthalene-2,3-dicarboxaldehyde (NDA) followed by HPLC separation and quantification of the labeled alanine product.⁵ Kinetic parameters determined for the wild-type and variant enzymes are shown in Table 1.

The SufS R92A variant exhibits a 2-fold decrease in k_{cat} value for alanine production relative to that determined for the wild-type enzyme, while the E96A and E250A variants show little effect on the k_{cat} parameter. All three variants exhibit increases of ~2-fold in their Michaelis constants for cysteine (K_{cys}). Taken together, these effects result in 2–3-fold decreases in their $k_{\text{cat}}/K_{\text{cys}}$ values relative to the wild-type parameters. Modest changes are seen when SufE is added to SufS E96A and E250A substitution mutants resulting in a 6-fold increase in the Michaelis constants for SufE (K_{SufE}), while the R92A substitution does not perturb this parameter. Similar effects are seen on the $k_{\text{cat}}/K_{\text{SufE}}$ values for the E96A and E250A substitutions. These variants exhibit 6–7-fold decreases in their $k_{\text{cat}}/K_{\text{SufE}}$ values relative to those determined with the wild-type enzyme. Overall, the kinetic results suggest that the residues E96 and E250 at the dimer interface contribute to productive interactions between SufS and SufE as substitution does not affect catalysis (i.e. k_{cat}) or interactions with cysteine.

Discussion

Protein-protein interactions appear to be critical for the regulation and proper function of Fe-S cluster biosynthesis pathways. As an initial point of control, mobilization of sulfur from cysteine by type II cysteine desulfurases is enhanced through interactions with accessory sulfur transfer proteins. In the *E. coli* Suf system, this step is governed by interactions between SufS and SufE. However, the mechanism of regulation for SufS activity remains unclear. Kinetic results with SufS/SufU (a SufE ortholog) from *B. subtilis* are consistent with a ping-pong mechanism with of two half reactions (Figure 1B).⁵ The first half reaction is the formation of the SufS_{per} intermediate and release of alanine. Rapid-reaction kinetics on the cysteine desulfurase Slr0077/SufS from *Synechocystis* demonstrated a slow step in the reaction with cysteine corresponding to breakage of the C-S bond in the absence of acceptor protein.¹⁸ In the second half reaction, the persulfide intermediate is transferred to SufE. The presence of SufE as a dedicated persulfide acceptor is expected to increase the

rate of the second half reaction.^{6, 7} Catalysis may also be regulated through a half-sites mechanism on the SufS dimer as binding studies show a negative cooperativity, two-sites model for SufE/SufS complex formation shifts to a one-site model with increased affinity for SufE when an activated version (i.e. ready to transfer the persulfide) of the complex is formed.

There is currently no high-resolution structure of the SufS/SufE complex so structural information concerning the role of complex formation in catalysis comes from HDX-MS results and comparisons with structures from homologous systems. For example, previous HDX-MS results are consistent with the SufS/SufE interaction promoting a SufE conformation capable of accepting the persulfide through extension of the C51-containing loop.⁹ Such a change is also supported by the conformational change seen in the persulfide acceptor protein CsdE in the crystal structure of the homologous CsdA/CsdE complex.¹⁹ CsdA is a cysteine desulfurase and close homologue of SufS (41% sequence identity) that liberates persulfide primarily for non-Fe-S biogenesis pathways.²⁰ CsdA also uses a SufE homologue, CsdE (35% sequence identity), to transport the liberated persulfide to its currently unidentified target protein.

In contrast to changes seen in the persulfide acceptor loop of the acceptor protein, the structure of the CsdA_{apo}/CsdE_{apo} complex shows no major structural changes to the active site of the CsdA cysteine desulfurase relative to the non-complex CsdA dimer.¹⁹ HDX-MS studies of either the SufS_{apo}/SufE_{apo} complex or the SufS_{apo}/SufE_{D74R} complex provide evidence for changes in two regions of the active site upon complex formation. These two regions cover residues 223–236 (containing K226-PLP) and 356–366 (containing C364) and suggest that SufE binding can cause changes at the active site of SufS. These two peptides have also been identified here as undergoing a change in dynamics upon formation of the C364 persulfide intermediate on SufS in the absence of SufE. Identification of these two regions is in strong agreement with reported conformational changes in the crystal structure of SufS_{per} intermediate.¹⁰ The fact that similar peptides are identified in SufS/SufE complex formation and upon formation of the persulfide intermediate suggest a complementarity role for SufE in active site remodeling of SufS to promote formation of the SufS_{per} intermediate, perhaps in concert with the half-sites reactivity.

While changes in protein dynamics at the active site are seen under numerous experimental conditions, changes at the dimer interface are only found when one of the protein partners is in an activated or intermediate state (e.g. SufS_{per}, or SufS_{apo} in complex with SufE_{alk/D74R}).⁹ The most dynamic of these regions covers residues 88–100. Solvent exchange with deuterium for this peptide exhibits unique changes depending on the state of the system (Figure 5). In this report, residues 88–100 undergo a decrease in fast exchange upon formation of the persulfide intermediate (black circles vs. red squares in Figure 5). In contrast these residues exhibit an increase in deuterium exchange upon formation of the SufS-SufE_{D74R} complex (black circles vs. blue diamonds in Figure 5).⁹ Taken together, these results suggest that the interactions at the dimer interface are sensitive to complex formation by activated SufS/SufE partners and may play a role in the proposed half sites regulatory mechanism.

Additional clues about the role of remodeling the dimer interface can be gleaned from the other interface region perturbed upon persulfide formation, residues 243–255. These residues cover a loop region that directly precedes a conserved β -hairpin structure (residues 255–271) (Figure 4C). The hairpin reaches across to the neighboring monomer and interacts with an extended lobe (residues 362–375) containing the active site C364. These structures are well conserved in type II cysteine desulfurases and have been previously implicated in deuterium trapping experiments with the SufS_{apo}/SufE_{alk} complex, suggesting they form part of the SufE binding site.⁸ It is possible that changes at the dimer interface are responsible for communicating the persulfide status of a SufS monomer through modulation of the hairpin structure.

To determine if interactions at the dimer interface identified by HDX-MS are functionally relevant, site-directed mutagenesis was utilized to generate SufS variant enzymes. As described above, R92 exhibits a small shift in interactions with E250 and E96' of the adjacent monomer in the crystal structures of SufS_{apo} and SufS bound with L-propargylglycine to mimic the alanine external aldimine (Figure 4B).¹⁷ While all three substitutions create small perturbations to SufS function, the main changes are seen in the K_{SufE} value for E96A and E250A SufS. The 6- or 7-fold increase in K_{SufE} values correlates well with the magnitude of change seen in the K_d values for SufE and the activated SufE_{alk/D74R}. Thus, interactions at the dimer interface appear to be necessary for optimal interactions between SufS and SufE.

With a functional role for residues at the dimer interface of SufS established, structures of homologous type II cysteine desulfurases were scanned for similar interactions. The structure of CsdA is very similar to SufS (RMSD 0.93 Å over 329 atom pairs). Based on our results suggesting a role for localized conformational changes at the SufS dimer interface in regulation, we re-inspected the reported structure of the CsdA/CsdE complex (Figure 6A) in comparison to CsdA alone with a focus on that region.¹⁹ CsdA has residues corresponding to R96 and E96 at its dimer interface (R88 and E92), however the residue corresponding to E250 in SufS is a leucine in CsdA (L246) (Figure 6B). Closer inspection of the CsdA/CsdE complex reveals an alternate conformation for R88 in one of the CsdA monomers.¹⁹ One conformation of R88 forms an interaction with E92' of the adjacent monomer, similar to the structures of SufS described above (Figure 6B shows CsdA, Figure 4B shows SufS). However, in the alternate conformation the side chain of R88 interacts with the backbone carbonyl of A271. A271 is located at the C-terminal end of the β -hairpin (residues 250–267 in CsdA). While this is in contrast to E250 in SufS, which resides at the N-terminal end of the hairpin, both structures highlight a connection between the dimer interface and a region adjacent to the β -hairpin.

The CsdA/CsdE complex structure also provides evidence for the role of this interaction in a half-sites model described above due to a lack of symmetry. In the CsdA/CsdE complex with the dynamic R88, the β -hairpin of CsdA and the persulfide acceptor loop of CsdE are not represented in the electron density suggesting they are flexible (Figure 6A). In the adjacent complex, where R88 only interacts with E92, both structures are clearly defined. This suggests that changes in orientation of the dimer interface arginine residue can affect the flexibility of key portions of both the desulfurase and the accessory transport protein. While

there are no further reports investigating a regulatory role for residues at the dimer interface of CsdA, the dynamic nature of R88 in reported structures and the conservation of contacts between residues adjacent to the β -hairpin suggest that this regulatory mechanism is evolutionarily conserved. It should be noted, however, that the transfer proteins SufE and CsdE are not interchangeable, as neither will support activity of its non-cognate desulfurase. This suggests that interactions between the desulfurase and transferase have evolved to provide specificity but may rely on a similar mechanism to shift the desulfurase between different conformations.

Overall, combining the HDX-MS and site-directed mutagenesis data suggests that conserved interactions at the dimer interface are required for optimal SufS/SufE complex formation and may play a role in the half-sites regulatory mechanism. A potential physical mechanism for active site communication mediated by contacts at the dimer interface is shown in figure 7. After the first half-reaction, the persulfide intermediate on C364 would interact with the backbone amide of S254' at the base of the β -hairpin on the adjacent monomer, resulting in increased affinity for SufE. The persulfide-status of the active site could be communicated to the dimer interface (and the other active site) through changes in interactions between residues on either side of the β -hairpin (E250 in SufS, A271 in CsdE) and the charged residues at the dimer interface. Evolutionary conservation of the charged interface residues and analysis of non-symmetrical structures from the homologous CsdA/CsdE system suggest this mechanism is evolutionarily conserved in type II cysteine desulfurases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

β-ME	β -mercaptoethanol
DMPD	N,N-dimethyl – phenylenediamine sulfate
DNTB	5–5'-dithio-bis-2-nitrobenzoic acid
HDX-MS	hydrogen-deuterium exchange mass spectrometry
NDA	naphthalene-2,3-dicarboxyaldehyde
PLP	5'-pyridoxal phosphate
SufS_{apo}	SufS without a covalent persulfide
SufS_{per}	SufS with a covalent persulfide
TCA	trichloroacetic acid

TCEP

tris(2-carboxyethyl)phosphine

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Figure 1.

A) Structure of SufS from *E. coli* (PDB id 1jf9). The *N*-terminal domain is shown in blue and yellow for each monomer, and the *C*-terminal domains shown in tan. The key catalytic residues (C364 and K226-PLP internal aldimine) are rendered as spheres. B) Proposed ping-pong mechanism for SufS enzymes. L-Cysteine binds to the apo-SufS enzyme forming an external aldimine (3). Sulfide is transferred from cysteine to form the persulfurated enzyme (SufS_{per}) and the first product, L-alanine. In the second half-reaction, SufE binds to SufS_{per}, and the persulfide is transferred to C51 of SufE to regenerate the resting SufS enzyme.

A: Non reduced persulfurated SufS

B: TCEP reduced persulfurated SufS

Figure 2.

Peptide digest maps for SufS_{per}. Tandem MS/MS generated peptide coverage maps for (A) SufS_{per} and (B) TCEP reduced SufS_{per}. The coverage map for SufS_{per} has no peptide corresponding to the active site Cys364; however, after reduction the peptide fragment containing Cys364 is detected. The red box highlights the location of C364.

Figure 3.

HDX-MS results for SufS_{apo} versus SufS_{per}. A) Deuterium uptake curves for selected peptides. Data are shown for SufS_{apo} (black) and SufS_{per} (red). Solid lines are fits of the data to single or double exponential rate equations as described in Methods and Materials. Numerical results from the fits are found in Table S1. B) Structural representation of peptides shown in panel A. Peptides are highlighted in blue on the dimeric SufS structure (PDB ID 1I29). C) Same as panel B with one monomer shown as surface to better highlight dimer interface.

Figure 4.

Structures showing possible electrostatic interactions of R92 in SufS. A) Structural representation of SufS_{apo} dimer with monomers colored brown and blue (PDB ID, 1jff9). Area in red square contains the peptides under investigation. B) Superposition of SufS_{apo} structure (brown, PDB ID 1jff9) and SufS bound to an external aldimine analog (blue, PDB ID 1i29). C) Ribbon structure of a SufS monomer with the β -hairpin (255–271, yellow) and peptide 243–255 (blue) highlighted. The PLP cofactor is shown as a space-filling molecule. The adjacent monomer is shown with a surface rendering.

Figure 5. HDX-MS data for peptide 88–100 in various experimental conditions. The data show deuterium incorporation plots for SufS_{apo} (black circles), SufS_{per} (red squares), and the SufS/SufE_{D74R} complex (blue diamonds). Solid lines are fits to first order rate equations as described in Materials and Methods. Data from SufS_{apo} and the SufS/SufE_{D74R} complex are taken from reference 9.

Figure 6. Structures showing perturbations in the structure of the CsdA/CsdE complex. A) Structural representation of CsdA/CsdE complex (PDB ID, 4lw4) with monomers of CsdA colored brown and blue and CsdE shown in yellow. Area in red square contains dimer interface residues corresponding to those described with SufS. Differences in missing electron density between the to CsdA/CsdE structures are labeled and correspond to those described in the text. B) Structure of alternate conformations and interaction partners of R88 in CsdA. This panel uses an identical orientation to Figure 4B for comparison.

Figure 7. Map of the proposed conduit for a half-sites regulatory mechanism in SufS. A structural representation of SufS_{per} (PDB ID, 1kmj) highlighting key contacts in a potential conduit for the half-sites regulatory mechanism. Both the β -hairpin and the loop containing residues 243–255 are shown as ribbon, and key residues identified in this study are rendered as spheres. PLP moieties are shown as sticks to denote the location of the active site.

Table 1.Kinetic parameters determined for wild-type and variant SufS enzymes^a

SufS	k_{cat} (min^{-1})	K_{cys} (μM)	$k_{\text{cat}}/K_{\text{cys}}$ ($\mu\text{M}^{-1}\text{min}^{-1}$)	K_{SufE} (μM)	$k_{\text{cat}}/K_{\text{SufE}}$ ($\mu\text{M}^{-1}\text{min}^{-1}$)
WT	12.1 ± 0.4	50 ± 7	0.24 ± 0.03	0.41 ± 0.05	30 ± 4
R92A	7.4 ± 0.2	95 ± 10	0.08 ± 0.01	0.63 ± 0.09	12 ± 2
E96A	13.8 ± 0.6	95 ± 10	0.14 ± 0.02	2.6 ± 0.3	5.3 ± 0.7
E250A	10.0 ± 0.4	81 ± 9	0.12 ± 0.01	2.4 ± 0.2	4.1 ± 0.4

^aAll reactions were performed as described in Materials and Methods.