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Transfer of Sulfur from IscS to IscU during Fe/S Cluster Assembly*

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The cysteine desulfurase enzymes NifS and IscS provide sulfur for the biosynthesis of Fe/S proteins. NifU and IscU have been proposed to serve as template or scaffold proteins in the initial Fe/S cluster assembly events, but the mechanism of sulfur transfer from NifS or IscS to NifU or IscU has not been elucidated. We have employed [^{35}S]cysteine radiotracer studies to monitor sulfur transfer between IscS and IscU from *Escherichia coli* and have used direct binding measurements to investigate interactions between the proteins. IscS catalyzed transfer of ^{35}S from [^{35}S]cysteine to IscU in the absence of additional thiol reagents, suggesting that transfer can occur directly and without involvement of an intermediate carrier. Surface plasmon resonance studies and isothermal titration calorimetry measurements further revealed that IscU binds to IscS with high affinity (K_d ~2 μM) in support of a direct transfer mechanism. Transfer was inhibited by treatment of IscU with iodoacetamide, and ^{35}S was released by reducing reagents, suggesting that transfer of persulfide sulfur occurs to cysteinyl groups of IscU. A deletion mutant of IscS lacking C-terminal residues 376–413 (IscSΔ376–413) displayed cysteine desulfurase activity similar to the full-length protein but exhibited lower binding affinity for IscU, decreased ability to transfer ^{35}S to IscU, and reduced activity in assays of Fe/S cluster assembly on IscU. The findings with IscSΔ376–413 provide additional support for a mechanism of sulfur transfer involving a direct interaction between IscS and IscU and suggest that the C-terminal region of IscS may be important for binding IscU.

Cysteine desulfurase enzymes play important roles in biological sulfur mobilization and provide inorganic sulfur for incorporation into Fe/S proteins. NifS, which functions in metallocluster formation of the nitrogenase system of *Azotobacter vinelandii*, was the first enzyme discovered to catalyze desulfuration of cysteine (1). The reaction catalyzed was found to yield alanine and either sulfane (S^0) or, in the presence of a reducing agent, sulfide (S^{2-}). Subsequent studies showed that NifS, cysteine, and iron could be used *in vitro* to reconstitute Fe/S clusters of several different Fe/S proteins, including the nitrogenase apo-Fe protein (2), regulatory proteins apo-FNR (3) and apo-SoxR (4) from *Escherichia coli*, apo-biotin synthase from *Bacillus sphaericus* (5), and the Fe/S cluster assembly protein apo-NifU from *A. vinelandii* (6). Similar *in vitro* studies have shown that IscS, a homolog of NifS found in nondiazotrophic as well as nitrogen-fixing organisms, is also effective in Fe/S cluster formation. IscS from *E. coli* could be used to reconstitute the Fe/S cluster of dihydroxyacid dehydratase (7), and IscS from *A. vinelandii* could be used to reconstitute Fe/S clusters on IscU (8, 9). IscS is also able to transfer sulfur to ThiI (10), proposed to function as a sulfur transferase in thiamine (11) and thionucleoside biosynthesis (10, 12–14).

The molecular mechanism by which sulfur is transferred from NifS or IscS to apo-Fe/S proteins is not known. Studies on NifS have provided evidence for formation of a persulfide intermediate on a cysteine residue presumed to be located near the active site (15), but whether this sulfur is donated directly to acceptor proteins, is transferred via an intermediate carrier, or is released as HS^- is not known. Recently, the crystal structure of a NifS-like protein from the thermophilic bacterium *Thermotoga maritima* was determined (16). The peptide region containing the cysteine residue presumed to be involved in sulfur transfer was disordered and could not be resolved in the crystal structure, and it was suggested that this may reflect the requirement for this segment to be flexible in order to both accept sulfur at the active site and transfer it to acceptor proteins (16). It has not been determined, however, whether sulfur transfer involves direct interactions between NifS or IscS and acceptor proteins. In the *in vitro* studies in which sulfur transfer from NifS or IscS to various acceptors has been reported, reaction mixtures contained levels of reducing agent (1–5 mM dithiothreitol or β-mercaptoethanol) sufficient to release persulfide-bound sulfur from NifS or IscS as free sulfide. Thus, the activities observed may reflect the ability of NifS/IscS to provide reactive HS^- in solution rather than a direct interaction between NifS or IscS and the acceptor proteins under investigation. In studies involving transfer from NifS to NifU (6) and from IscS to IscU (8), a weak interaction between NifS/IscS and NifU/IscU was indicated by size exclusion chromatography, but the nature of the interaction and its role in sulfur transfer were not investigated.

NifU and IscU proteins have been proposed to function as template/scaffold proteins for assembly of intermediate Fe/S clusters prior to delivery to apo-Fe/S proteins (6, 8, 9, 17, 18) and may serve as the immediate acceptors of sulfur provided by NifS and IscS. The mechanism of sulfur transfer between NifS/IscS and NifU/IscU, however, has not been studied in detail. We present evidence herein that sulfur transfer between *E. coli* IscS and IscU is direct, involves high affinity complex formation, and involves cysteine residues of IscU. Evidence is also presented that complex formation is mediated in part by a region of IscS not required for cysteine desulfurase activity and that this region is required for efficient Fe/S cluster formation.

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These findings provide support for a cellular mechanism of sulfur transfer that involves a direct interaction between IscS and IscU.

EXPERIMENTAL PROCEDURES

Materials—E. coli DH5αF′IQ cells were obtained from Life Technologies, Inc. Enzymes for DNA manipulation were obtained from Roche Molecular Biochemicals, New England Biolabs, Inc., or U.S. Biochemical Corp. Synthetic nucleotides were obtained from Genosys. ^{35}S-Labeled L-cysteine was obtained from Amersham Pharmacia Biotech. Bacterial growth medium components were from Difco, and other reagents were from Sigma.

Overexpression and Purification of Proteins—Recombinant IscU and Hsc20 were prepared as described previously (17, 19). For expression of recombinant IscS, the coding region of *iscS* was amplified from genomic DNA isolated from *E. coli* K-12 strain by polymerase chain reaction with *Taq* DNA polymerase and primers designed to introduce a *Nco*I restriction site at Met-1 and a *Pst*I site following the termination codon. Introduction of the *Nco*I site resulted in a change of residue 2 from lysine to glutamic acid; this recombinant form is referred to as "wild-type" IscS throughout.[1] The IscS polymerase chain reaction product was digested with *Nco*I and *Pst*I and ligated into pTrc99a (Amersham Pharmacia Biotech) to yield pTrcIscS. DH5αF′IQ cells transformed with pTrcIscS were grown in Terrific Broth at 37 °C, induced with 0.5 mM isopropyl-D-thiogalactoside at A_{600} ~0.5, and grown for 16 h to allow expression. Cells were harvested by centrifugation, frozen, thawed, and lysed by French press in TED buffer (50 mM Tris·HCl, pH 8, 0.5 mM EDTA, 1 mM DTT).[2] The soluble supernatant fluid after centrifugation at 35,000 × *g* was used for purification, and all subsequent steps were carried out in TED buffer at 4 °C. IscS protein was monitored by SDS-PAGE. Anion exchange chromatography was carried out using DE52 (Whatman) and eluted with a linear gradient of 0–250 mM NaCl. IscS fractions were loaded on a DEAE-Sepharose column (Whatman), washed with 150 mM TED, and eluted using a linear gradient of 150–250 mM sodium chloride. Final purification was achieved on a phenyl-Sepharose column equilibrated with 20% ammonium sulfate and elution with a decreasing linear gradient of 20 to 5% ammonium sulfate. Fractions appearing homogeneous by gel electrophoresis were combined, treated with 150 μM pyridoxal phosphate to restore cofactor lost during purification, dialyzed to remove ammonium sulfate and unbound pyridoxal phosphate, concentrated by ultrafiltration, and stored at −70 °C. This method yielded ~50 mg of IscS/liter at >90% purity (Fig. 1*A*) and a specific activity of 156 mol of sulfide/min/mg.

To prepare the C-terminal IscS truncation mutant IscSΔ376–413, a stop codon was substituted for Ser-376 of pTrcIscS using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to generate pTrcΔIscS. IscSΔ376–413 was expressed in an *iscS*$^-$ strain PK4331 (31) and prepared using the same methods as used for full-length IscS. As with the full-length protein, purified IscSΔ376–413 behaved as a dimer, and no differences in stability of the mutant were apparent during incubations used to determine enzyme activity or Fe/S cluster assembly assays.

^{35}S Transfer Assay—DTT was removed from samples of IscU and IscS by extensive dialysis immediately before all sulfur transfer assays. Samples subjected to multiple cycles of concentration and buffer exchange using a Centricon YM-10 centrifuge filter unit to further remove low molecular weight solutes gave similar results consistent with complete removal of reducing reagent by dialysis. Sulfur transfer reactions were carried out at 23 °C for 30 s and contained 1 μM IscS, 20 μM IscU (or other acceptor protein), and HKM buffer (50 mM Hepes, pH 7.3, 150 mM KCl, 10 mM $MgCl_2$) in a final volume of 35 μl. Reactions were initiated by the addition of ^{35}S-labeled L-cysteine (0.19 Ci/mmol; Amersham Pharmacia Biotech) to a final concentration of 15 μM and were terminated by centrifugation at 2000 × *g* through a size exclusion column (Microspin G-50 column; Amersham Pharmacia Biotech). The spin column eluant was mixed with SDS-PAGE sample loading buffer to give final concentrations of 1% glycerol, 5 mM Tris, pH 8.0, and 0.001% bromphenol blue. Samples were immediately analyzed by gel electrophoresis using a 10% polyacrylamide gel containing 0.1% SDS (20) but in the absence of added reducing agent. ^{35}S was visualized by exposure on a phosphor screen and analyzed using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA), and relative intensities were calculated using ImageQuant software supplied with the instrument. Reactions were found to be essentially complete even at 0 °C within the time required for reagent separation on spin columns, and it was not possible to obtain rapid kinetic measurements.

Chemical Modification of IscU and IscS—Cysteine residues of IscS were alkylated in 0.1 M Tris buffer, pH 7, using *N*-ethylmaleimide (21), and cysteine residues of IscU were carboxymethylated in 0.2 M potassium phosphate buffer, pH 7, using iodoacetic acid (22). For each reaction, ~0.1 mM IscS or IscU was incubated with 1 mM labeling reagent for 1 h. Excess *N*-ethylmaleimide and iodoacetic acid were removed by repeated cycles of ultrafiltration and dilution with 50 mM Tris, pH 7.0.

Cysteine Desulfurase Assay—IscS desulfurase activity was determined using the sulfide detection assay described by Siegel (23). Reactions were carried out at 23 °C in glass culture tubes capped with rubber septa stoppers. Samples contained 100 nM IscS, 10 μM pyridoxal phosphate, and 10 mM magnesium chloride in 0.8 ml of 50 mM Tris, pH 8.0 (7). Reactions were initiated by the addition of L-cysteine (Sigma) in water containing 5 mM dithiothreitol and were terminated by the addition of 100 μl of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine and 100 μl of 30 mM ferric chloride. Product was determined by measuring methylene blue formation at 670 nm (23). The rate of sulfide production (V_o) was determined for each concentration of L-cysteine by linear regression analysis, and K_m and V_{max} values were determined by fitting the Michaelis-Menten equation to a plot of V_o *versus* the concentration of L-cysteine using Kaleidagraph (Synergy Software). The rates reported represent an average of three independent experiments with error bars corresponding to ±1 S.D. Maximal turnover numbers for different preparations, 8–9 min^{-1}, were slightly greater than the specific activities previously reported for IscS from *E. coli* (7, 12), *A. vinelandii* (24), and *Synechocystis* (25), which ranged from 3 to 6 mol of sulfide/mol of IscS/min^{-1} under similar conditions. K_m values could not be determined accurately due to limitations in sulfide determination, but a value of 2.7 ± 0.4 μM was estimated from data obtained using cysteine concentrations ranging from 2 to 60 μM.

Surface Plasmon Resonance (SPR) Analysis—SPR studies were carried out at 25 °C with a Biacore 3000 instrument (Piscataway, NJ) as described previously (17, 27). Proteins were randomly cross-linked to the surface of the sensor chip by amine coupling as recommended by the manufacturer. Experiments were conducted in HKM buffer containing 5 mM DTT at a flow rate of 20 μl/min. Prior to injections, all proteins were dialyzed against running buffer. Experiments were repeated three or four times to verify that changes in the sensor chip did not occur during the course of the measurements.

Isothermal Titration Calorimetry Analysis—A Microcal Omega titration calorimeter (Amherst, MA) was used to investigate the binding of IscU to IscS and IscSΔ376–413 in HKM buffer containing 5 mM DTT using procedures previously described (17, 26, 27).

Reconstitution of Fe/S Clusters on IscU—Reaction mixtures were prepared under anaerobic conditions (Controlled Atmosphere Chamber; Plas-Labs, Lansing, MI) and contained 1 μM IscS or IscSΔ376–413, 50 μM IscU, and 2 mM ferric citrate in HKM buffer. Following a preincubation period of 5 min, reactions were initiated by the addition of 2.5 mM L-cysteine and 5 mM DTT. Fe/S cluster formation was monitored by circular dichroism. The visible region absorption spectrum of IscU-Fe/S is given in Ref. 17.

Analytical Methods—Antiserum to purified IscU and immunoblotting methods have been described (17). Antiserum to IscS was prepared from a single rabbit by Bethyl Laboratories (Montgomery, TX) and was used without further purification. Western immunoblotting was carried out using enhanced chemiluminescence detection (Amersham Pharmacia Biotech). A 1:750 dilution of rabbit anti-IscS serum was followed by a 1:10,000 dilution of an anti-rabbit horseradish peroxidase conjugate. Concentrations of IscS were determined at 280 nm using an extinction coefficient of 40,600 (M·cm)$^{-1}$ calculated from its amino acid sequence (28–30). Absorption spectra were recorded with a Cary 1 spectrophotometer (Varian Instruments), and circular dichroism measurements were recorded using a Jasco J720 spectropolarimeter.

RESULTS

Transfer of ^{35}S from IscS to IscU—We used ^{35}S-labeled cysteine to monitor sulfur transfer catalyzed by IscS. To preclude possible involvement of reducing agents as sulfur carriers, all protein samples were extensively dialyzed and diluted to remove dithiothreitol that had been included in the purification procedures. In a typical experiment, IscS (1 μM) was incubated

[1] A preliminary crystal structure of *E. coli* IscS reveals that residue 2 is located at the protein surface and appears unlikely to make significant contributions to the IscS structure or stability (J. Cupp-Vickery, H. Urbina and L. Vickery, unpublished observations).

[2] The abbreviations used are: DTT, dithiothreitol; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis.

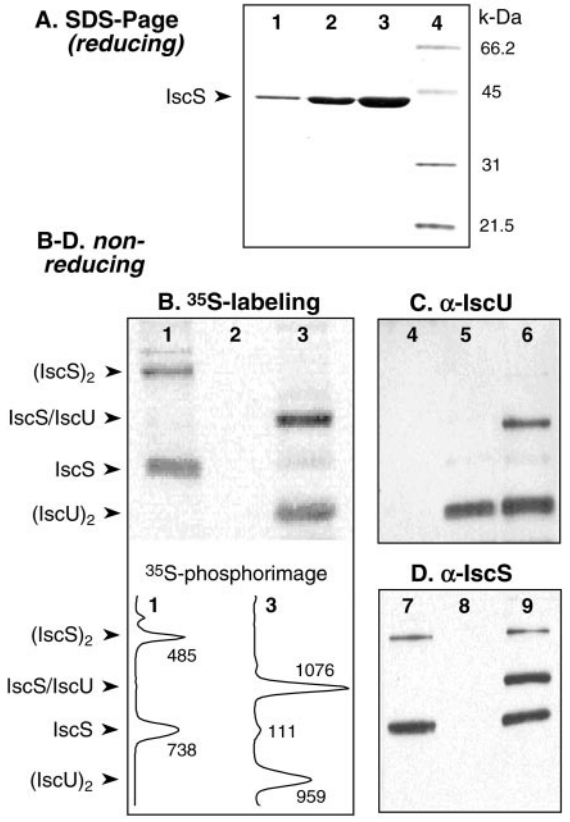

FIG. 1. **SDS-PAGE analysis of sulfur transfer from IscS to IscU.** *A*, SDS-PAGE of purified IscS denatured in the presence of 2.5% 2-mercaptoethanol (20). *Lanes 1*, *2*, and *3* contained 1, 3, and 10 μg of protein, respectively, and *lane 4* shows molecular weight standards. The gel was stained with Coomassie Blue. *B–D*, analysis of sulfur transfer reactions under nonreducing conditions. Samples were incubated for 30 s with 15 μM ^{35}S-labeled L-cysteine, resolved by SDS-PAGE without the use of 2-mercaptoethanol, and analyzed with a PhosphorImager as described under "Experimental Procedures." The *upper portion* of *B* shows the phosphor image obtained, and the lower portion shows the corresponding integration curves and relative peak intensities. *Lane 1*, 1 μM IscS; *lane 2*, 20 μM IscU; *lane 3*, 1 μM IscS plus 20 μM IscU. Migration positions of the components are indicated on the *left*. *C* and *D*, immunoblots using antisera to IscU and IscS, respectively, obtained from different regions of the same gel. *Lanes 4* and *7*, 1 μM IscS; *lanes 5* and *8*, 20 μM IscU; *lanes 6* and *9*, 1 μM IscS plus 20 μM IscU.

with [^{35}S]cysteine (15 μM) in the absence or presence of acceptor protein (20 μM), separated from residual [^{35}S]cysteine on a size exclusion spin column, and subjected to SDS-PAGE electrophoresis under nonreducing conditions. Sulfur transfer was found to be essentially complete within the time required for reagent separation on spin columns, and reaction labeling profiles therefore represent end points. The locations of proteins on gels were determined by Western immunoblotting, and the pattern and extent of ^{35}S labeling were determined by phosphorimaging.

Fig. 1*B* shows an experiment to test ^{35}S transfer from IscS to IscU, and the migration positions of IscU and IscS are revealed in the immunoblots shown in Fig. 1, *C* and *D*, respectively. *Lane 1* shows that in the absence of acceptor, ^{35}S label is incorporated into IscS. Radioactivity is observed in bands corresponding to monomeric (~45 kDa) and dimeric forms of IscS with the majority of the protein migrating as a monomer under the conditions used. *Lanes 2* and *3* show the labeling pattern in the presence of IscU. In the absence of IscS, no significant labeling of IscU is observed (*lane 2*), but two new bands of ^{35}S radioactivity are observed when a mixture of IscS and IscU is used (*lane 3*). The lower band corresponds to a dimer of IscU (~28 kDa), establishing that ^{35}S label is transferred from IscS

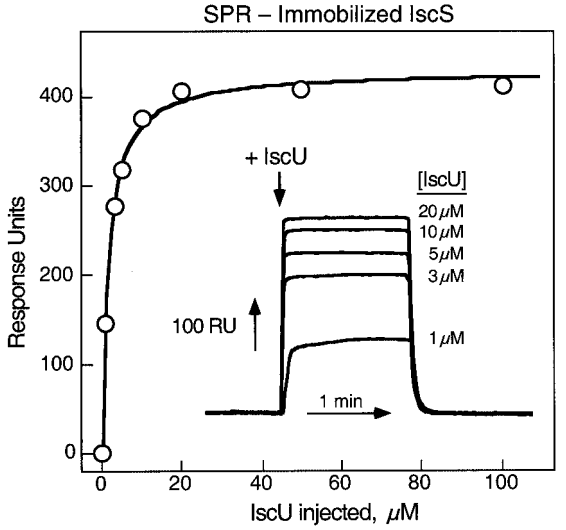

FIG. 2. **SPR analysis of IscU binding to IscS.** IscU was injected into a solution passing over a sensor chip containing immobilized IscS (~3700 response units). The data plotted represent the average of two measurements; individual values fell within the *symbols* used. The *curve* shown represents a least-squares fit of the data to a hyperbolic saturation function with K_d = 2.0 μM and a maximal signal of 426 response units. The *inset* shows representative sensorgrams for the indicated concentrations of IscU.

to IscU. The upper band corresponds to a complex formed between IscS and IscU. Based on its migration position, this complex appears to be a heterodimer of IscS and IscU, and it is not possible to determine whether ^{35}S label is present on IscS, IscU, or both proteins. In addition, the amount of label present in the IscS monomer is reduced ~7-fold compared with that observed in the absence of IscU, and no label appears associated with the IscS dimer (compare *lane 1*). We assume that the decrease in labeling of IscS reflects a transfer of ^{35}S to IscU because the immunoblot presented in Fig. 1*C*, *lane 9*, shows that approximately half of the IscS remains present as the free monomer under these conditions. The finding that the IscS dimer is not labeled to the same extent as in the absence of IscU (Fig. 1*A*, compare *lanes 1* and *3*) also suggests efficient transfer of ^{35}S to IscU.

To test the specificity of IscS-mediated ^{35}S transfer, we incubated IscS and [^{35}S]cysteine with several other potential acceptor proteins under similar conditions. No significant label incorporation was obtained using purified recombinant forms of other proteins present in the *isc-hsc-fdx* operon (24), including IscA, HscB (Hsc20), HscA (Hsc66), and ferredoxin (data not shown). IscA, Hsc66, and ferredoxin contain free thiol groups that might be labeled by nonspecific processes, and their failure to serve as acceptors suggests that the labeling observed with IscU reflects specific interactions with IscS.

Complex Formation between IscS and IscU—To investigate binding interactions between IscS and IscU, we first used the SPR method. Fig. 2 shows the result of a titration experiment in which IscS was randomly cross-linked to the sensor chip and exposed to different concentrations of IscU. IscU was observed to bind to and dissociate from IscS rapidly (within the mixing time of the experiment) and to exhibit stable complex formation with an apparent binding constant K_d of 2.0 μM. The maximal signal observed corresponds to ~0.37 mol of IscU bound per mol of IscS, indicating that not all of the immobilized IscS is able to bind IscU.

Because complications can arise from surface and/or immobilization effects in SPR, we also used isothermal titration calorimetry to independently determine the binding constant as well as the stoichiometry of the interaction. Fig. 3 shows the

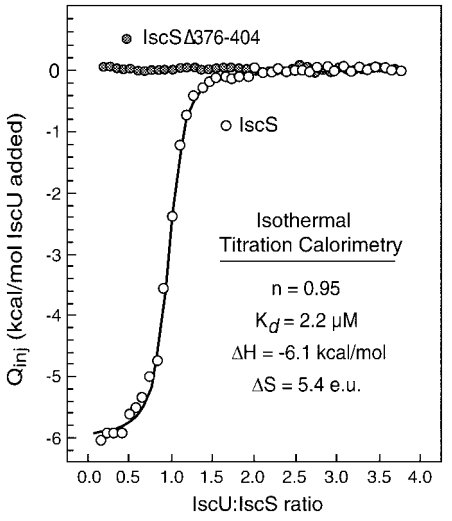

FIG. 3. **Calorimetric analysis of the binding of IscU to IscS.** Binding isotherms were generated by incremental injections of 3.2 mM IscU into a cell containing 0.2 mM IscS or the truncation mutant IscSΔ376–404. Integrated heats of binding, Q_{inj}, were corrected for heats of dilution and are plotted as a function of the molar ratio of IscU to IscS. The best fit curve shown for IscS corresponds to 0.95 binding sites having K_d = 2.2 μM, ΔH = −6.1 kcal/mol, and ΔS = 5.4 entropy units. No significant enthalpic change was observed with IscSΔ376–404.

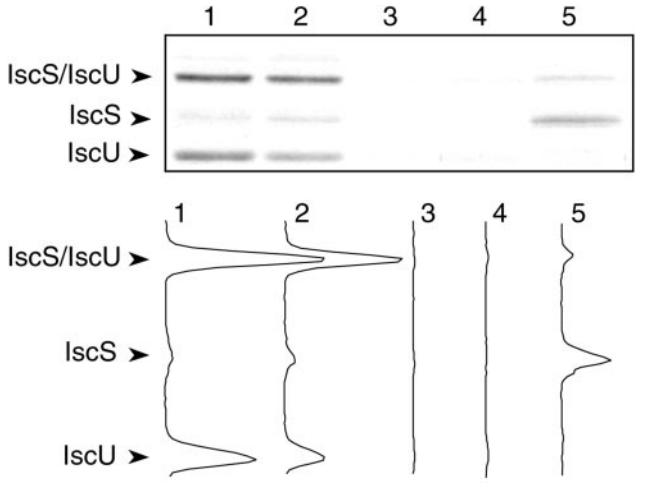

FIG. 4. **Roles of cysteine residues in sulfur transfer.** ^{35}S-Labeled L-cysteine (15 μM) was incubated for 30 s with IscS (1 μM) and IscU (20 μM) and subjected to nonreducing SDS-PAGE and PhosphorImager analysis as in Fig. 1*B*. *Lane 1*, reaction carried out in HKM buffer only. *Lane 2*, 5 mM 2-mercaptoethanol was included during the transfer reaction. *Lane 3*, 5 mM 2-mercaptoethanol was added after 30 s and incubated at 25 °C for 30 min prior to SDS-PAGE. *Lane 4*, IscS was pretreated with *N*-ethylmaleimide. *Lane 5*, IscU was pretreated with iodoacetic acid.

results of an experiment in which successive additions of IscU were made to a solution containing IscS. The data are plotted as the integrated heats of binding *versus* the molar ratio of IscU/IscS. The best fit curve shown corresponds to binding of 0.95 mol of IscU/mol of IscS with K_d = 2.2 μM. The binding ratio observed is consistent with the 1:1 complex observed under the partially denaturing electrophoresis conditions used in Fig. 1; in solution, however, both IscS and IscU exist as dimers (7, 17), and it seems likely that the active complex will contain a dimer of each protein. The dissociation constant is similar to that observed in the SPR measurements (Fig. 2), and because cellular levels of IscU and IscS are estimated to be >10 μM, a large fraction of IscU would be expected to be bound to IscS *in vivo*.[3]

Studies on the Mechanism of Sulfur Transfer—Additional experiments were carried out using [^{35}S]cysteine to investigate the role of cysteine residues in the mechanism of sulfur transfer between IscS and IscU (Fig. 4). A control reaction is shown in *lane 1*, and the effects of the addition of 5 mM β-mercaptoethanol to samples during or following the transfer reaction are shown in *lanes 2* and *3*, respectively. The presence of β-mercaptoethanol during the sulfur transfer reaction results in a decrease in label incorporation into both free IscU (~54% less) and the IscU-IscS complex (~31% less), but the failure to fully prevent labeling suggests that intermediates formed are at least partially protected from this reductant during the transfer process. The addition of β-mercaptoethanol for 30 min following the reaction, in contrast, leads to complete loss of ^{35}S from both IscU and IscS as well as the IscU-IscS complex. The finding that label is released by prolonged exposure to reducing agent is consistent with formation of cysteine persulfide derivatives on IscU as well as on IscS and suggests that the cysteine residues involved are solvent-accessible. To further assess the possible role of cysteine residues, we tested the effects of thiol-blocking reagents on both IscS and IscU. Based on the reactivity of the active site cysteine of NifS (15), treatment of IscS with *N*-ethylmaleimide would be expected to alkylate Cys-328 and inactivate the enzyme. Fig. 4 (*lane 4*) shows that this treatment completely inhibits ^{35}S incorporation, consistent with involvement of Cys-328 of IscS in persulfide transfer. *Lane 5* shows that treatment of IscU with the cysteine carboxymethylation reagent iodoacetic acid blocks the transfer of ^{35}S, and label remains associated with IscS. Only a small amount of ^{35}S is associated with the IscS-IscU complex, suggesting that the majority of the radioactivity in *lanes 1* and *2* associated with the IscS-IscU complex is due to labeled IscU. The findings that carboxymethylated IscU will not accept ^{35}S and that β-mercaptoethanol releases ^{35}S from IscU suggest that cysteine residue(s) of IscU serve as acceptor(s) for persulfide transferred from IscS.

An IscS Transferase Mutant—Differences in the amino acid sequences near the C termini of different NifS and IscS proteins suggest that this region may be important in determining the specificity of interactions with acceptor proteins. To test this possibility, we prepared a truncation mutant of IscS lacking 29 C-terminal residues by insertion of a stop codon at the location of Ser-376. This mutant, designated IscSΔ376–404, was expressed in an *iscS*$^-$ strain of *E. coli* (31). The *iscS*$^-$ disruption strain grows poorly on glucose minimal medium (31), but cells expressing IscSΔ376–404 exhibited normal growth rates, suggesting that IscSΔ376–404 was active. The mutant enzyme was purified, and its kinetic properties were compared with full-length wild-type IscS. As shown in Fig. 5, IscSΔ376–404 exhibited cysteine desulfurase activity (k_{cat} = 8.3 ± 0.5 min^{-1}; K_m = 2.2 ± 0.5 μM) similar to that observed for wild-type IscS (k_{cat} 8.5 ± 0.3 min^{-1} and K_m 2.7 ± 0.4 μM). We next tested the mutant in the ^{35}S transfer assay. As shown in the *inset* of Fig. 5, the IscSΔ376–404 mutant is active but exhibits reduced ^{35}S transfer activity compared with full-length IscS. With IscSΔ376–404, label incorporation into the band corresponding to the IscU dimer is only 38% of that observed using wild-type IscS. In addition, less labeling is associated with the band corresponding to the IscSΔ376–404-IscU complex compared with the IscS-IscU complex, and a greater portion of label is associated with the uncomplexed dimeric form of the mutant protein.

[3] The cellular concentration of IscU has been estimated by immunoblotting methods to be ~40 μM in cells grown in rich medium to stationary phase (17). Similar immunoblotting measurements for IscS using W3110, DH5α, and BL21 strains of *E. coli* yield a value ~45 μM (H. D. Urbina and L. E. Vickery, unpublished observations).

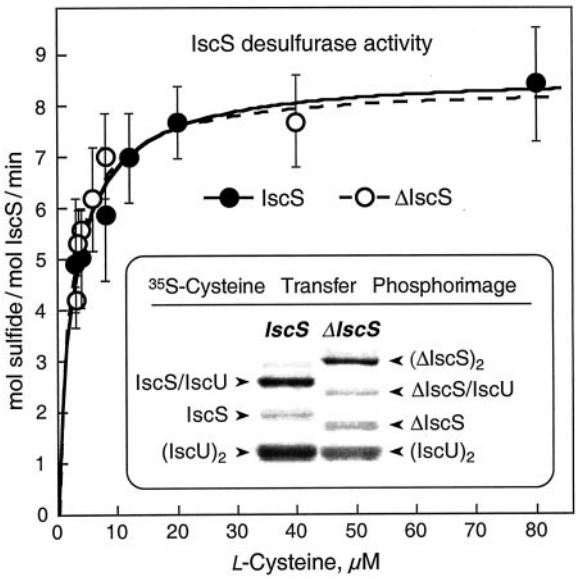

FIG. 5. **Desulfurase and sulfur transfer activity of IscSΔ376–404.** The rate of sulfide production by IscS (●) and the ΔIscS mutant (○) is shown as a function of the concentration of L-cysteine. The best fit curve shown for IscS (*solid line*) corresponds to $K_d = 2.7 \pm 0.4$ μM and $V_{max} = 8.5 \pm 0.3$ min^{-1}; the curve shown for IscSΔ376–4045 (*dashed line*) corresponds to $K_d = 2.2 \pm 0.5$ μM and $V_{max} = 8.2 \pm 0.5$ min^{-1}. The *inset* shows a phosphor image obtained for sulfur transfer reactions containing 15 μM ^{35}S-labeled L-cysteine, 1 μM IscS or IscSΔ376–404, and 20 μM IscU carried out as described in the legend to Fig. 1 and subjected to nonreducing SDS-PAGE.

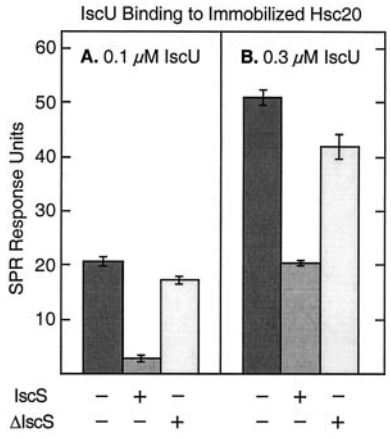

FIG. 6. **Effect of IscS and IscSΔ376–404 on the binding of IscU to Hsc20.** The relative binding affinities of IscS and IscSΔ376–404 for IscU were compared by measuring their ability to compete with IscU for binding to Hsc20 using SPR. Hsc20 was immobilized on a sensor chip (470 response units), and signals obtained from solutions containing IscU in the presence or absence of 5 μM IscS or IscSΔ376–404 were compared. *A*, 0.1 μM IscU; *B*, 0.3 μM IscU. *Error bars* represent values obtained from duplicate injections.

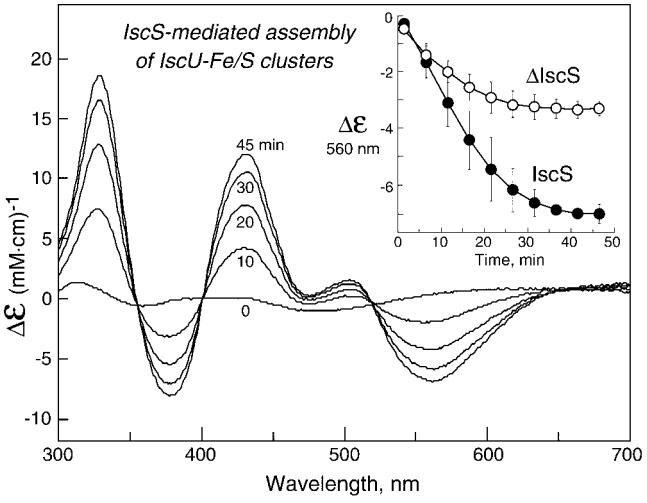

FIG. 7. **IscS-mediated assembly of Fe/S clusters on IscU.** IscS or IscSΔ376–404 (1 μM) was incubated with IscU (50 μM) and ferric citrate (200 μM), and cluster formation was initiated by the addition of L-cysteine (2.5 mM) and DTT (5 mM). Reaction progress was monitored by circular dichroism. Scans were recorded at 5-min intervals at a scan rate of 100 nm/min; complete spectra using IscS are shown for scans initiated at 0, 10, 20, 30, and 45 min. The *inset* shows a plot of the rate of cluster formation observed in two experiments (*error bars* show individual values where these fell outside the *symbol* used). CD intensities are corrected for the scan interval required to reach 560 nm.

To determine whether the reduced ability of IscSΔ376–404 to transfer sulfur to IscU might result from altered binding, we attempted to investigate the interaction of IscU with IscSΔ376–404 by isothermal titration calorimetry. As shown in Fig. 2, however, no enthalpic change was observed when IscSΔ376–404 was mixed with IscU. This result could reflect a failure of the truncation mutant to bind to IscU but could also arise from a lack of a significant net enthalpic change upon binding of the mutant to IscU. We therefore used SPR as an independent measure of the interaction. Because SPR measurements are sensitive to how substrate molecules are immobilized on the surface, we made use of a competitive binding system in which another protein is immobilized on the chip surface and interactions between IscU and IscS occur in solution. For this purpose, we used Hsc20, a co-chaperone protein shown to bind and target IscU to the molecular chaperone Hsc66 (17). Fig. 6 shows the results of experiments in which the binding of IscU to immobilized Hsc20 was determined in the absence or presence of full-length or mutant IscS. Under the conditions used, full-length IscS bound to IscU and inhibited its binding to immobilized Hsc20 (87 and 56% inhibition using 0.1 and 0.3 μM IscU, respectively). The mutant IscSΔ376–404, on the other hand, gave significantly less inhibition (~15% inhibition using 0.1 or 0.3 μM IscU). While it is difficult to determine the exact binding constant of IscSΔ376–404 for IscU by this method, the results indicate a significantly lower affinity than that of the wild-type protein. This finding, together with the reduced activity of IscSΔ376–404 in the ^{35}S transfer assay, suggests that formation of a specific complex between IscS and IscU is required for efficient sulfur transfer.

Fe/S Cluster Assembly—The decreased affinity of IscSΔ376–404 for IscU and its lower activity in ^{35}S transfer to IscU afforded the opportunity to investigate the importance of complex formation between IscS and IscU in assembly of Fe/S clusters on IscU. Fig. 7 shows the rate of formation of the IscU-Fe/S complex using either full-length IscS or the IscSΔ376–404 mutant. In these experiments, cysteine was provided as the source of sulfur, and ferric citrate was used to provide iron. DTT was included to provide reducing equivalents required for cluster generation, and incubations were carried out anaerobically (8, 9, 17). Fe/S cluster formation was monitored by circular dichroism to avoid complications due to overlapping absorption spectra of IscS and ferric citrate with the IscU-Fe/S complex. The amount of IscU-Fe/S formed using wild-type IscS was found to be similar to the maximal level obtained by the careful addition of sodium sulfide together with ferric citrate (*cf.* Ref. 17). In addition, the rate of cluster formation using the wild-type *E. coli* enzyme was similar to that reported by Agar *et al.* using IscS and IscU from *A. vinelandii* under similar conditions (8). The IscSΔ376–404 mutant, however, was less effective in cluster assembly and yielded only 46% of the amount of IscU-Fe/S complex formed using IscS. Because IscSΔ376–404 has desulfurase activity similar to that

of wild-type IscS, this result suggests that complex formation between IscS and IscU is important not only for efficient sulfur transfer but also for efficient Fe/S cluster assembly.

DISCUSSION

Previous studies have shown that the cysteine desulfurase enzymes NifS and IscS can provide sulfur for assembly of Fe/S proteins, but the mechanism of sulfur transfer to the acceptor proteins was not determined. The findings described herein provide three lines of evidence that transfer of sulfur from IscS to IscU proceeds by a direct interaction between the two proteins without the involvement of an intermediate sulfur carrier. First, by employing ^{35}S-labeled cysteine, we were able to measure sulfur transfer independent of Fe/S protein formation and avoid the use of added reducing reagents required for Fe/S cluster assembly. Sulfur transfer from IscS to IscU was found to proceed efficiently in highly purified samples treated to remove reducing agents, suggesting that intermediate carriers are not required. Second, IscS and IscU were found to form a 1:1 complex with high binding affinity. The dissociation constant observed (K_d ~2 μM) is well below the estimated cellular concentrations of both IscS and IscU,[3] suggesting that the interaction is physiologically relevant. Finally, a mutant form of IscS having lower binding affinity for IscU was found to exhibit reduced activity in both sulfur transfer and in Fe/S cluster assembly experiments. The requirement for high affinity binding between IscS and IscU for efficient sulfur delivery provides additional support for a direct mechanism of sulfur transfer between the two proteins. The finding that the mutant is less effective in Fe/S cluster assembly despite having cysteine desulfurase activity similar to the wild-type protein further suggests that specific interactions between IscS and IscU may in some way facilitate Fe/S cluster assembly as well as sulfur transfer. Specific interactions between IscS and IscU could play a role in determining the pathway of sulfur transfer or in subsequent steps involved in Fe/S cluster formation.

The mechanism of sulfur transfer between IscS and IscU is not known, but two results obtained suggest that transfer involves formation of cysteine persulfide derivatives on both IscS and IscU; incubation of reaction products with reducing agent released label as expected for formation of [^{35}S]persulfide-cysteine, and pretreatment of either IscS or IscU with the cysteine-blocking agents prevented ^{35}S label incorporation. The finding that IscS and IscU form a high affinity complex suggests that sulfur transfer between the two proteins is likely to proceed by persulfide exchange in which Cys-328 of IscS interacts directly with a specific cysteine residue of IscU. Preliminary crystallographic data obtained for *E. coli* IscS indicate that Cys-328 is in a solvent-accessible mobile loop[4] as has been proposed for NifS from *T. maritima* (16), consistent with such a direct transpersulfidation mechanism. In the case of IscU, however, the cysteine residue that serves as the sulfur acceptor has not been identified. Three cysteine residues are conserved in the sequences of IscU proteins reported to date, but the structure of IscU and solvent accessibility of these residues have not been determined. Two cysteine residues are expected to be involved in Fe/S cluster binding, and it is possible that the third may play a role in sulfur transfer from IscS to the nascent cluster. Our finding that IscU can accept sulfur in the absence of iron suggests that sulfur transfer may precede iron binding. Once iron is bound, release of persulfide sulfur (S^0) to the metal cluster as sulfide (S^{2-}) requires reduction, and thus additional electron transfer proteins are likely to be required to complete cluster formation. Studies are under way to investigate the roles of specific residues of IscU in sulfur transfer and to identify other proteins involved in Fe/S cluster assembly.

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[4] J. Cupp-Vickery, H. Urbina, and L. Vickery, unpublished observations.

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