Sulfur Transfer from IscS to IscU: The First Step in Iron−Sulfur Cluster Biosynthesis

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The process of Fe−S cluster biogenesis is highly conserved in nature and centers around two key proteins, termed IscS and IscU in prokaryotes, which are found in almost all organisms.[1] IscS is a homodimeric, pyridoxal phosphate-dependent L-cysteine desulfurase that catalyzes the conversion of cysteine to alanine and elemental sulfur via the formation of a persulfide intermediate on a conserved cysteine residue.[2] The ubiquitous role of IscS-like enzymes in sulfur trafficking, in general,[3] and Fe−S cluster biosynthesis, in particular,[4] is now firmly established. IscU is a homodimeric protein that forms a 1:1 complex with IscS,[5] and gene knockout studies in yeast[6] and bacteria[4d] have demonstrated a crucial role for IscU in general Fe−S cluster biosynthesis. Moreover, IscU contains three conserved cysteines and has been shown to provide a scaffold for IscS-directed sequential assembly of $[Fe_2S_2]^{2+}$ and $[Fe_4S_4]^{2+}$ clusters.[7] These clusters are likely to be inserted, intact, into apo Fe−S proteins in a process that has yet to be fully characterized. In this communication, we address the mechanism of IscS-directed Fe−S cluster assembly on the IscU scaffold. Mass spectrometry has been used to demonstrate direct transfer of sulfane sulfur, S^0, from the cysteine persulfide on IscS to the cysteine residues on IscU. The available evidence indicates that this reaction constitutes the first step in Fe−S cluster biosynthesis on an IscU scaffold.

A comparison of the mass spectra of *Azotobacter vinelandii* IscU obtained under a variety of reaction conditions is shown in Figure 1. Reaction mixtures were purified by reverse-phase HPLC, and discrete fractions were introduced by an electrospray ionization (ESI) source for analysis using a single quadrupole mass spectometer (see Supporting Information for experimental details).[8] The conditions used for repurification are denaturing, and the observed charged-state distributions, centered around $m/z \approx 1000$ amu, are characteristic of highly denatured proteins. Hence, all observed molecular ions must be covalently associated.

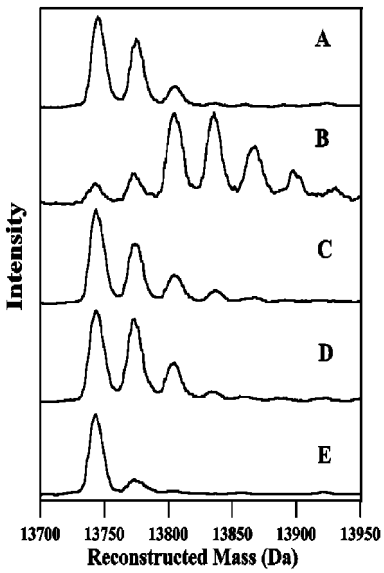

Figure 1. Reconstructed ESI-quadrupole mass spectra of *A. vinelandii* IscU. (A) IscU, 0.16 mM. (B) Reaction mixture containing 0.16 mM IscU, 0.16 mM IscS, and 3 mM cysteine. (C) Reaction mixture as in (B) after addition of 10 mM DTT. (D) IscU, 0.16 mM, with 3 mM sodium sulfide. (E) IscU, 0.16 mM with 10 mM potassium cyanide.

The mass spectrum of IscU after DTT treatment (Figure 1A) comprises the monomer molecular ion peak at 13 744 Da (theoretical mass based on primary sequence, 13 875 Da, minus 131 Da, corresponding to cleavage of the N-terminal methionine), and features at 13 774 and 13 805 Da. Since the two higher mass components occur at +30 and +61 Da, respectively, and are dramatically decreased in intensity on addition of excess potassium cyanide (Figure 1E), they are attributed to the addition of one and two sulfur atoms, respectively, in DTT-inaccessible polysulfides or persulfides. Direct evidence for multiple sulfur transfers from IscS to IscU is provided by the mass spectrum of IscU in a reaction mixture involving stoichiometric IscS and IscU with an excess of the substrate, cysteine (Figure 1B). The major peaks at 13 805 and 13 836 Da correspond to the addition of two and three sulfur atoms, respectively, and peaks corresponding to the addition of one sulfur (13 774 Da), four sulfurs (13 868 Da), five sulfurs (13 898 Da), and six sulfurs (13 930 Da) are also clearly observed. These peaks are attributed to IscU with covalently attached polysulfides or a mixture of polysulfides and persulfides, since the addition of DTT to the reaction mixture converts IscU back to the as prepared form (Figure 1C). Moreover, since covalently attached polysulfides or persulfides are not generated on IscU by the addition of excess sulfide (Figure 1D), we conclude that they are generated by transfer of S^0 from the cysteine persulfide intermediate located on IscS. In summary, the mass spectrometry results demonstrate IscS-mediated assembly

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(8) *A. vinelandii* IscU and IscS were overexpressed, purified, and assayed as previously described.[7] All samples were prepared in a glovebox under argon (<1 ppm O_2), and IscU was treated with 10 mM dithiothreitol (DTT) and then repurified by gel filtration prior to use.

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of cysteine-ligated polysulfides or persulfides on IscU via multiple transfers of S^0 from the cysteine persulfide intermediate located on IscS.

Analysis of the mass spectrometry data for reaction mixtures containing 1:1 IscU:IscS with excess cysteine also provides evidence for a covalent complex between IscU and IscS monomers involving a disulfide or polysulfide linkage. This complex is distinct from the noncovalent $\alpha_2\beta_2$ complex between homodimeric IscS and IscU that has been demonstrated via gel filtration and chemical cross-linking studies,[5] and has important implications for the catalytic mechanism of S^0 transfer. Samples treated with DTT eluted from the HPLC as two resolved bands (based on both UV absorption and total ion chromatographs), corresponding to monomeric IscU (Figure 1C) and monomeric IscS with a molecular ion peak at 44 666 Da (see Supporting Information, Figure S1).[9] Samples not treated with DTT eluted as three resolved bands corresponding to monomeric IscU (Figure 1B), monomeric IscS (47 453 Da) and a covalently associated IscU/IscS dimer (58 530 Da) (see Supporting Information, Figure S2). The monomeric IscS band corresponds to the mass in the DTT-treated sample with zero to five S atoms, and the IscU/IscS dimer band corresponds to the DTT-treated IscU and IscS molecular ions with zero to six S atoms. Since the covalently associated IscU/IscS complex is cleaved by DTT, it is attributed to IscS bound to IscU via a disulfide or polysulfide linkage between the active site cysteine on IscS and one of the three cysteine residues on IscU. The observation of this complex provides evidence for direct transfer of S^0 from the cysteine persulfide intermediate on IscS to a cysteine residue on IscU.

Although no structural data is available for the IscU family of proteins, direct transfer of S^0 from IscS to the cluster assembly site on IscU is consistent with the recent crystallographic data for three IscS homologues.[10,11] The active-site cysteine residue in the IscS-like protein from *Thermotoga maritima* and the putative selenocysteine lyase from *Escherichia coli* (CsdB) are both located on flexible loops that are capable of delivering S^0 to regions remote from the protein.[10] The cystine C−S lyase from *Synechocystis* that is involved with assembly of $[Fe_2S_2]$ clusters in plant-type ferredoxins provides an interesting variation on the general cysteine desulfurase mechanism.[11] The cysteine persulfide is formed on the substrate cystine rather than a conserved active-site cysteine residue. In the context of the current work, this serves to demonstrate the importance of a mobile cysteine persulfide in Fe−S cluster biosynthesis.

Direct transfer of S^0 from IscS to partner proteins is likely to be a general method of initiating IscS-mediated sulfur trafficking. Indeed, transfer of S from IscS to the ThiI protein has already been demonstrated via radiolabeling studies using ^{35}S.[3b] ThiI is an enzyme common to the biosynthetic pathway of thiamin and 4-thiouridine, and recent studies indicate it to be a sulfurtransferase that functions via a cysteine persulfide intermediate.[3]

On the basis of the mass spectrometry results presented herein, it is proposed that direct transfer of S^0 from IscS to the cysteine residues on the IscU scaffold protein constitutes the first step in Fe−S cluster biosynthesis. As discussed below, this proposal is supported by several lines of evidence and affords the ability to synthesize Fe−S clusters without generating high cellular concentrations of free sulfide. The concept of S^0 transfer from IscS to some type of "carrier protein" as an early step in the Fe−S cluster biosynthetic pathway, was first suggested by Flint.[12] In addition, the ability of apo Fe−S proteins to accumulate S^0 on cysteine residues destined for cluster ligation has previously been demonstrated by the finding of up to three S^0 atoms bound to aconitase after oxidative cluster degradation.[13]

In principle, IscS-directed Fe−S cluster assembly on the IscU scaffold can be initiated by either Fe or S binding to the cysteines residues on IscU. However, we have been unable to elicit any evidence for Fe^{3+} or Fe^{2+} ion binding to IscU, with stoichiometric excesses up to 10-fold, using the combination of UV−visible absorption, variable temperature magnetic circular dichroism, and Mössbauer spectroscopies. Fe^{3+} binding to the IscU domain of the NifU protein in a rubredoxin-like coordination environment has been observed at low temperatures.[14] However, subsequent studies have indicated that this is associated with the ability of NifU and IscU to rapidly reduce Fe^{3+} ion and that Fe^{2+} ion rather than Fe^{3+} ion is used exclusively for cluster assembly on IscU and NifU scaffold proteins.[15] Moreover, studies of IscS-mediated cluster assembly on IscU and on IscA, an alternative scaffold protein for Fe−S cluster biosynthesis,[16] have shown that Fe^{2+} ion provides reducing equivalents for the reduction of S^0 to S^{2-}.[16b] Taken together with the results presented herein, this leads to a working hypothesis for IscS-directed assembly of the initial $[Fe_2S_2]^{2+}$ cluster product on IscU. The initial step involves transfer of S^0 from the cysteine persulfide on IscS to one or more of the cysteine residues on IscU. The resultant cysteine persulfides or polysulfides on IscU are then reduced by two Fe^{2+} ions, concomitant with the oxidation of two cysteine thiols to yield a disulfide and a bound $[Fe_2S_2]^{2+}$ cluster. Although the mechanistic details, as well as the subunit location and ligation of the initial $[Fe_2S_2]^{2+}$ cluster, remain to be elucidated, the results of this work provide strong support for the hypothesis that S^0 transfer from IscS is the initial step in Fe−S cluster biosynthesis.

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Supporting Information Available: Experimental procedure for mass spectrometry measurements and figures showing mass spectra data for reaction mixtures containing IscU, IscS, and excess cysteine before and after addition of DTT (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) The observed molecular mass of IscS, 44 666 Da, is 33 Da less than that predicted from the primary sequence and prosthetic group content (44 468 Da plus 231 Da for the lysine-bound pyridoxyl phosphate). The origin of this discrepancy is currently under investigation using tryptically cleaved protein fragments.

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