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Published on Web 07/02/2002

Iron−Sulfur Cluster Biosynthesis: Characterization of Iron Nucleation Sites for Assembly of the [2Fe−2S]$^{2+}$ Cluster Core in IscU Proteins

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Received April 8, 2002

ISU (eukaryotes) and IscU (prokaryotes) are a homologous family of proteins that appear to provide a platform for assembly of [2Fe−2S] centers prior to delivery to a target apoprotein.[1−3] The intermediate [2Fe−2S] IscU-bound cluster is formed by delivery of iron and sulfur to the apo-IscU, with the latter delivered through an IscS-mediated reaction.[4] The identity of the iron donor is not yet established. In this report we characterize iron-binding sites on IscU that appear to nucleate [2Fe−2S] cluster assembly. This iron-bound form of IscU is shown to be viable for subsequent IscS-mediated assembly of holo-IscU. Following on recent reports,[5,6] we demonstrate the persulfide form of IscU to be a dead-end complex that is incapable of forming holoprotein following addition of ferrous or ferric ion. The latter observation reflects the low binding affinity of persulfido IscU for iron ion.

Our studies have been carried out with *Thermatoga maritima* IscU (*Tm* IscU)[8] and NifS[9] (*Tm* NifS, a member of the IscS family of enzymes). Chemical cross-linking experiments demonstrated *Tm* NifS to form a complex with *Tm* IscU.[10] Consistent with earlier reports on *Azotobacter vinelandii* and *Escherichia coli* IscUs,[6,7,11] we observed *Tm* NifS-mediated sulfur-transfer chemistry with *Tm* IscU. In fact other proteins can also be sulfur-labeled by *Tm* NifS, such as *Schizosaccharomyces pombe* ferredoxin[12] (Table 1). Sulfur transfer from NifS to IscU, presumably as persulfide adducts of Cys residues, was independently verified by both mass spectrometry[13] and a colorimetric method based on thiocyanate formation following cyanide treatment.[14,15] These sulfur adducts were degraded by treatment with DTT. Apparently the IscS family of enzymes is able to react with a variety of protein substrates, depending on the accessibility of Cys residues and the affinity of the IscS for the protein target.[6,7,16] Consistent with such an idea is the observation that the extent of sulfur labeling of IscU by IscS is protein dependent (Table 1). While labeling of IscU Cys residues by IscS is possible, we nevertheless find that this leads to a dead-end complex that cannot be converted to a final [2Fe−2S] holo form following addition of ferrous ion (with or without an electron source).

Such observations led us to consider an alternative mechanism with initial binding of iron followed by delivery of sulfur equivalents. Previous resonance Raman and MCD studies of *Av* IscU have suggested apoprotein to bind iron extremely weakly, if at all,[3,6] a result that contributed to establishing the mechanism cited earlier.[7] However, both fluorescence and calorimetric measurements that we have made for *Tm* IscU indicate significant binding of both ferric and ferrous ion (Figures 1 and 2 and Table 2). *Tm* IscU contains one Trp with $\lambda_{em}^{max} \approx 332$ nm. Iron ion quenches this emission in a concentration-dependent manner until available binding sites are populated (Figure 1). By monitoring the quenching of native Trp fluorescence as a function of iron concentration a high-affinity and a low-affinity binding site have been identified, consistent with the asymptotic approach of the binding function to $r = 2$.[17]

The high-affinity sites were further characterized by isothermal titration calorimetry (ITC) with the low-affinity sites beyond the detection limits for this technique. Good agreement in binding data was obtained for the high-affinity site. Binding experiments with

Table 1. Quantitation of IscS-mediated Delivery of S Equivalents to Cys on Target Proteins

target protein	no. of labile S	reference
Thermatoga maritima IscU	1−2	this work
Schizosaccharomyces pombe Fd	1−2	this work
Escherichia coli IscU	1	6
Azotobacter vinelandii IscU	5−6	7

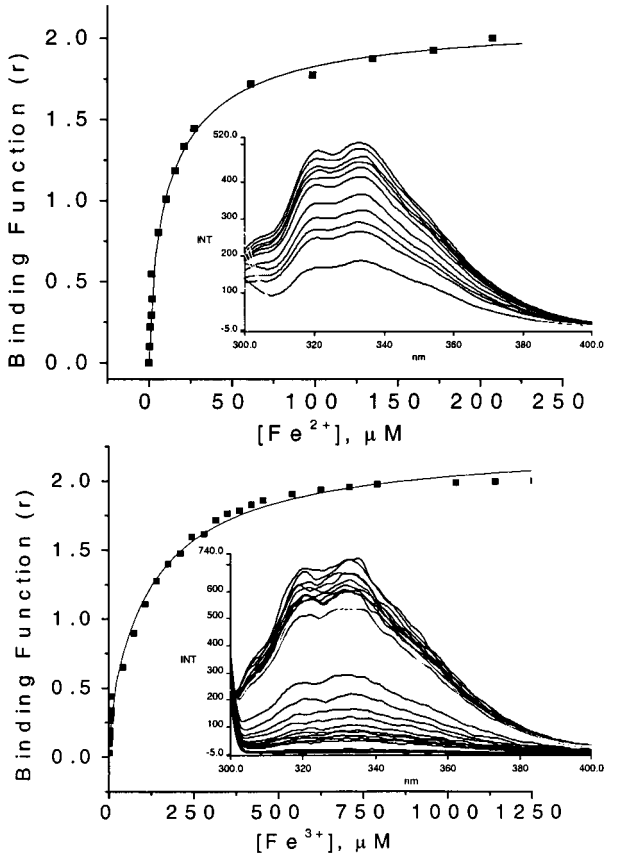

Figure 1. Binding curves generated from fluorescence measurements of ferrous- and ferric-bound *Tm* IscU. Samples of 15 and 23.9 μM *Tm* IscU were titrated with $FeSO_4 \cdot 7H_2O$ (upper) and $FeCl_3$ (lower), respectively. Protein and iron samples were prepared in argon-purged 100 mM HEPES, pH 7.5, 50 mM NaCl, with 5 mM DTT added for the Fe^{2+} titration.

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10.1021/ja0264596 CCC: $22.00

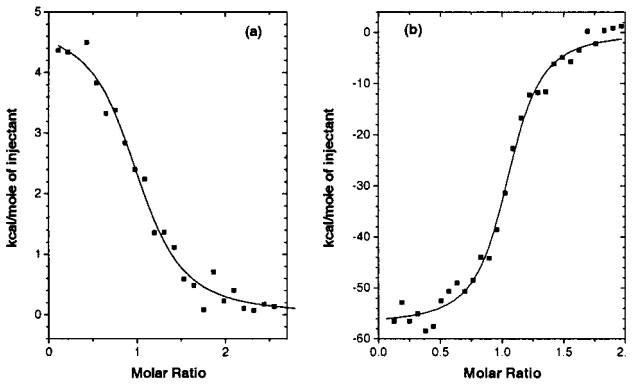

Figure 2. ITC measurements of ferrous and ferric ion binding to *Tm* IscU were carried out at 27 °C on a MicroCal OMEGA ultrasensitive titration calorimeter. All samples were in degassed 100 mM HEPES pH 7.5 buffer solution. (a) A 1 mM stock of Fe^{2+} was titrated into a solution of 20 μM *Tm* IscU. Both titrant and buffer solutions contained 5 mM DTT. (b) A 0.25 mM stock of Fe^{3+} was titrated into a solution of 20 μM *Tm* IscU. Data were collected automatically and subsequently analyzed with a Windows-based Origin software package supplied by MicroCal.

Table 2. Calorimetric and Fluorescence Quantitation[a] of Iron Binding to *Tm* IscU

isothermal titration calorimetry			fluorescence		
protein	ion	K_D (μM)	protein	ion	K_D (μM)
Tm IscU	Fe^{3+}	0.3	*Tm* IscU	Fe^{3+}	2.6, 136
Tm IscU	Fe^{2+}	2.7	*Tm* IscU	Fe^{2+}	3.0, 25

[a] Fluorescence data was analyzed following Winzor.[17] Physiological binding constants may be enhanced as a result of structural stabilization through contacts with chaperone proteins.[18]

ferrous ion were performed in the presence of 5 mM DTT, and thus the measured K_D's are apparent binding constants. That is, actual K_D's are presumably lower and of physiological relevance. The competitive mode for ferrous binding most likely underlies the change in sign of the enthalpic contribution ($\Delta H^* = -58.0$ kJ/mol for Fe^{3+} and + 4.4 kJ/mol for Fe^{2+}). Significantly, the persulfido form of IscU was found to lack significant affinity for iron binding, a fact that presumably underlies the failure of this form to yield holo-IscU, following addition of excess ferrous ion. By contrast, in the presence of sufficient ferrous ion to saturate the available iron sites on IscU, NifS/Cys treatment results in a >90% yield of IscU bound [2Fe−2S] cluster.

These data allow us to formulate a new mechanistic proposal for assembly of the [2Fe−2S] cluster in IscU-type proteins. Initial delivery of two ferrous ions is followed by delivery of sulfide. This latter step is mediated through the persulfide form of IscS (NifS) that forms a complex with IscU. Two-electron reductive cleavage of the persulfido bond of IscS yields one S^{2-}. Subsequent two-electron delivery to a second equivalent of IscS (note that such proteins are typically dimers) yields the second equivalent of sulfide. Reductive cleavage of the IscS persulfide bonds may be mediated either by the IscU-bound ferrous ions, or by an exogenous two-electron donor. In additional support of such a mechanism we note that addition of persulfido-labeled IscS (NifS) to an iron-loaded form of IscU resulted in rapid formation of holo-$[2Fe-2S]^{2+}$-loaded IscU in the presence of reducing equivalents.[19] By contrast, addition of ferrous ion to the persulfido-labeled IscU does not result in formation of holo-$[2Fe-2S]^{2+}$-loaded IscU in the presence of reducing equivalents.

In conclusion these results establish a distinct reaction pathway for assembly of the $[2Fe-2S]^{2+}$ cluster of IscU-type proteins, but readily accommodate previous observations of IscS labeling of IscU. A similar model is also likely for the assembly of the holo form of IscA-type proteins, and efforts are underway to establish such.

Acknowledgment. This work was supported by a grant from the Petroleum Research Fund, administered by the American Chemical Society (J.A.C.), the National Science Foundation, CHE-0111161 (J.A.C.).

Supporting Information Available: Mass spectrometric data quantitating persulfido formation on *Tm* IscU and *Sp* ferredoxin, and experimental methods for protein purification, cross-linking, fluorescence, microcalorimetry, and quantitation of persulfide formation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) ESI mass spectra showed an additional peak for the persulfido adduct at 18120 amu, relative to the base peak at 18088 amu.
(14) Quantitation of the persulfido adduct was also made following addition of 50 mM KCN (in 40 mM KH_2PO_4, pH 8.6) and incubation for 15 min. The reaction was stopped by the addition of 260 μL of 38% formaldehyde and the color developed by addition of 400 μL of 200 mM $FeCl_3$ dissolved in 0.5 M HCl. Formation of $Fe(SCN)_6^{3-}$ was monitored at 460 nm. The concentration of thiocyanate was determined from a calibration curve generated by use of known concentrations of sodium thiocyanate.
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JA0264596