

A Mutant Human IscU Protein Contains a Stable [2Fe–2S]²⁺ Center of Possible Functional Significance

Matthew W. Foster,[†] Sheref S. Mansy,[†] Jungwon Hwang,[‡] James E. Penner-Hahn,[‡] Kristene K. Surerus,[§] and J. A. Cowan^{*,†}

Evans Laboratory of Chemistry, The Ohio State University
100 West 18th Avenue, Columbus, Ohio 43210
Department of Chemistry, University of Wisconsin
Milwaukee, Milwaukee, Wisconsin 53201
Department of Chemistry, University of Michigan
Ann Arbor, Michigan 48109-1055

Received March 7, 2000

The mechanism by which iron–sulfur clusters are assembled in vivo is poorly understood.¹ The inherent toxicity of free iron and sulfide has led to the proposal that certain proteins participate in the delivery of Fe and S^{2–} during cluster assembly. In *Azotobacter vinelandii* (A.v.) and other diazotrophic bacteria, cotranscribed *nifS* and *nifU* genes of the *nif*-specific gene cluster are essential for the maturation of the Fe–S clusters of nitrogenase component proteins.² NifS catalyzes the desulfurization of cysteine to provide S^{2–} for incorporation into Fe–S clusters.³ NifU is a cysteine-rich, modular protein, containing a [2Fe–2S]²⁺ cluster coordinated to four cysteine residues in the protein's central domain.⁴ The N-terminal domain of NifU contains three cysteine residues in the sequence CX₂₅CX₄₄C that do not have any similarity to known Fe–S containing proteins; however, recent studies have demonstrated a role for these cysteines in cluster binding. Yuvaniyama et al. have shown by UV–vis and resonance Raman characterization that the N-terminal domain of A.v. NifU (NifU-1) is dimeric and can bind a transient [2Fe–2S]²⁺ cluster upon reconstitution with Fe³⁺, cysteine, and NifS.⁵ The same phenomenon has been observed for the homologous A.v. IscU.⁶ It has been suggested that this labile cluster may provide iron and sulfide equivalents for assembly of nitrogenase component proteins,⁵ or for general Fe–S cluster assembly in the case of the IscU.⁶ Interestingly, a D37A mutant of the A.v. NifU-1 has a stable [2Fe–2S]²⁺ cluster with presumably the same coordinating ligands as the transient cluster.⁵ Aspartate37 is conserved among NifUs and IscUs.

IscU proteins from prokarya and eukarya are homologous to the N-terminus of NifUs, and like NifUs have three conserved cysteine residues.⁷ Excluding mitochondrial targeting sequences of the eukaryotic proteins, IscUs show ~65–70% sequence identity to each other, and ~40–45% sequence identity to the N-terminal domain of NifUs. Eukaryotic IscUs are basic proteins (pI ≈ 8–9), whereas prokaryotic IscUs are acidic (pI ≈ 4–5).

Nonetheless, the high degree of conservation among IscUs suggests that they have an essential and conserved function.^{7,8} *S. cerevisiae* has two IscUs (Isu1p and Isu2p)⁹ and a NifS homologue (Nfs1p),¹⁰ and genetic studies have suggested roles for these proteins in iron metabolism and Fe–S cluster assembly.^{9,10} In humans, an IscU (hIscU) and a NifS homologue have been identified.^{8,11} Herein we report the first characterization of an Fe–S center bound to a eukaryotic IscU protein. Native hIscU has been expressed,¹² and Mössbauer and EXAFS spectroscopies on D37A hIscU show that the D37A mutant contains a stable [2Fe–2S]²⁺ cluster, whereas native hIscU has no cluster as isolated.¹³ In contrast to the dimeric A. *vinelandii* NifU-1 and IscU, holo D37A hIscU is monomeric and the cluster appears to be ligated by the four cysteine residues contained in each protein monomer.

Native hIscU and D37A hIscU were each overexpressed in *Escherichia coli* and purified aerobically to homogeneity.¹⁴ Native hIscU is colorless, but the D37A mutant is pink, and analysis for labile iron and sulfide showed the presence of an Fe–S center only in the mutant.¹⁵ When D37A hIscU was purified by gel filtration chromatography, multiple protein peaks were observed, but only one peak was colored. Comparison of the elution volume of this peak, relative to known standards, revealed that the holo-D37A hIscU is a monomer with an apparent molecular mass of 18 kDa (vs 15 kDa from SDS-PAGE.) Significant amounts of apo-D37A hIscU were also present that eluted as dimeric, hexameric, or higher MW species. When the hexameric fraction was chromatographed in buffer containing 10 mM 2-mercaptoethanol, ~one-third of the sample was monomeric, suggesting that aggregation of the apoprotein is due in part to disulfide bond formation. The UV–vis spectrum of the monomeric fraction shows absorbance bands at 328 and 460 nm, and a shoulder around 540 nm (Figure 1), typical of [2Fe–2S]²⁺ proteins,¹⁶ and in comparison to the 325, 420, 465, and 510 nm bands for D37A A.v. NifU-1.⁵ Iron quantitation of purified holoprotein gave 2.0 ± 0.3 mol Fe/mol protein,⁶ and the extinction coefficient of the cluster at 460 nm is ~8500 M^{–1} cm^{–1}, within the typical range for [2Fe–2S]²⁺ clusters.¹⁶ The holoprotein is EPR silent, while reduction by dithionite facilitated cluster degradation rather than generating an EPR-active species.

Ni–NTA purified D37A hIscU was also examined by Mössbauer¹⁷ and EXAFS¹⁸ spectroscopies. Mössbauer analysis of the

(8) Hwang, D. M.; Dempsey, A.; Tan, K.-T.; Liew, C.-C. *J. Mol. Evol.* **1996**, *43*, 536–540.

(9) (a) Schilke, B.; Voisine, C.; Beinert, H.; Craig, E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10206–10211. (b) Garland, S. A.; Hoff, K.; Vickery, L. E.; Culotta, V. C. *J. Mol. Biol.* **1999**, *294*, 897–907.

(10) (a) Strain, J.; Lorenz, C. R.; Bode, J.; Garland, S.; Smolen, G. A.; Ya, D. T.; Vickery, L. E.; Culotta, V. C. *J. Biol. Chem.* **1998**, *273*, 31138–31144. (b) Kispal, G.; Csere, P.; Prohl, C.; Lill, R. *EMBO J.* **1999**, *18*, 3981–3989. (c) Li, J.; Kogan, M.; Knight, S. A. B.; Pain, D.; Dancis, A. *J. Biol. Chem.* **1999**, *274*, 33025–33034.

(11) Land, T.; Rouault, T. A. *Mol. Cell* **1998**, *2*, 807–815.

(12) The partial hIscU gene was a generous gift from Dr. C.-C. Liew (U. Toronto). Excluding a mitochondrial targeting sequence, the mature protein has a predicted N-terminal sequence YHKKYVDHYENDPR, where R is the first residue of the partial hIscU. The numbering for hIscU in this work assumes predicted N-terminal Tyr.

(13) hIscU was cloned into pET-28b (Novagen) and expressed with an N-terminal 6xHis-tag. D37A hIscU as well as C35A-D37A, C61A-D37A, C96A-D37A, and C104A-D37A hIscUs were made using QuickChange Mutagenesis (Stratagene). All mutations were confirmed by sequencing.

(14) Native hIscU was purified by Ni–NTA chromatography. D37A hIscU was purified by Ni–NTA, P-11, and Superose-12 chromatography.

(15) (a) Moulis, J.-M.; Meyer, J. *Biochemistry* **1982**, *21*, 4762–4771. (b) Siegel, L. M. *Anal. Biochem.* **1965**, *11*, 126–132.

(16) Dailey, H. A.; Finnegan, M. G.; Johnson, M. K. *Biochemistry* **1994**, *33*, 403–407.

(17) Mössbauer spectra were recorded on a constant acceleration spectrometer, model MS-1200D from Ranger Scientific, using a Janis Super-Vari-temp cryostat (model 8DT), a Lakeshore temperature controller (model 340), and a ⁵⁷Co source from Isotope Products Laboratory.

* To whom correspondence should be addressed.

[†] The Ohio State University.

[‡] University of Michigan.

[§] University of Wisconsin, Milwaukee.

(1) Bian, S.; Cowan, J. A. *Coord. Chem. Rev.* **1999**, *190–192*, 1049–1066.

(2) Dean, D. R.; Bolin, J. T.; Zheng, L. *J. Bacteriol.* **1993**, *175*, 6737–6744.

(3) Zheng, L.; White, R. H.; Cash, V. L.; Jack, R. F.; Dean, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2754–8.

(4) (a) Ouzounis, C.; Bork, P.; Sander, C. *Trends Biochem. Sci.* **1994**, *19*, 199–200. (b) Fu, W.; Jack, R. F.; Morgan, T. V.; Dean, D. R.; Johnson, M. K. *Biochemistry* **1994**, *33*, 13455–63.

(5) Yuvaniyama, P.; Agar, J. N.; Cash, V. L.; Johnson, M. K.; Dean, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 599–604.

(6) Agar, J. N.; Zheng, L.; Cash, V. L.; Dean, D. R.; Johnson, M. K. *J. Am. Chem. Soc.* **2000**, *122*, 2136–2137.

(7) Zheng, L.; Cash, V. L.; Flint, D. H.; Dean, D. R. *J. Biol. Chem.* **1998**, *273*, 13264–13272.

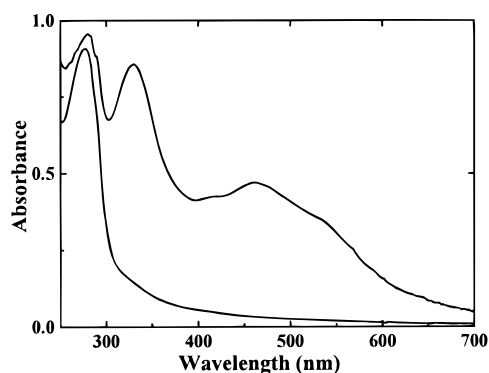


Figure 1. UV-vis spectra of hIscU proteins. (Upper) Monomeric superoxide 12 fraction of D37A hIscU ($\sim 55 \mu\text{M}$). (Lower) Native hIscU following Ni-NTA purification ($\sim 120 \mu\text{M}$). Both samples were buffered in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (buffer 1).

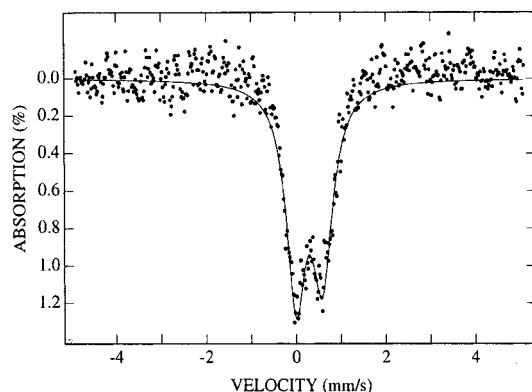


Figure 2. 100 K Mössbauer spectra in a 450 G applied field, $\sim 0.3 \text{ mM}$ holo-D37A hIscU in buffer 1 (Figure 1). The Ni-NTA purified holo- and apoprotein were not separated to minimize mechanical losses during purification of ^{57}Fe -labeled protein.

^{57}Fe -labeled protein showed a single iron-containing species (Figure 2) with an isomer shift, $\delta = 0.29 \pm 0.05 \text{ mm/s}$, and quadrupole splitting, $\Delta E_Q = 0.59 \pm 0.05 \text{ mm/s}$. No hyperfine splitting was observed at 4.2 K, consistent with a diamagnetic cluster. Fits to EXAFS data¹⁹ yielded an Fe-Fe distance of 2.70 Å and Fe-SCys distance of 2.24 Å. Best fits strongly suggest a single Fe neighbor per Fe, and the amplitude of the Fe-Fe peak in hIscU (Figure S3) is identical to that of $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-o-xy})_2]^{2-}$. Both spectroscopies give parameters that are typical for a diferric $[2\text{Fe}-2\text{S}]^{2+}$ cluster,^{21,22} although neither Mössbauer or EXAFS can exclude the possibility that the cluster has one or two noncysteinylligands.

(18) XAS data were measured at SSRL line 7-3 (3.0 GeV, $\sim 100 \text{ mA}$, Si-(220) double-crystal monochromator) using a 13-element Ge X-ray fluorescence detector (total count rates were kept $< 10^5 \text{ sec}^{-1}$). Temperature was 10 K. Data were measured in 10 eV steps in the pre-edge (6920–7080 eV), 0.25 eV steps in the edge (7080–7140 eV) and 0.05 Å⁻¹ steps above the edge (to $k = 13 \text{ Å}^{-1}$) with integration time increasing as k^3 above the edge ($t_{\text{max}} = 20 \text{ s}$) for a total of $\sim 40 \text{ min/scan}$. Eight scans were examined to confirm the absence of artifacts or photoreduction and then averaged.

(19) Data reduction followed standard procedures,^{20a} using $E_0 = 7130 \text{ eV}$. The k^3 weighted data were fit using ab initio amplitude and phase parameters^{20b} calibrated by fitting data for models of known structure.

(20) (a) Riggs-Gelasco, P. J.; Mei, R.; Yocum, C. F.; Penner-Hahn, J. E. *J. Am. Chem. Soc.* **1996**, *118*, 2387–2399. (b) Rehr, J. J.; Mustre, d. L. J.; Zabinsky, S. I.; Albers, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 5135–40.

The presence of an additional cysteine residue in the human IscU (resulting in a CX₂₅CX₃₄CX₇C motif) as compared to the *A. v.* NifU-1 and IscU suggested the possibility of a novel ligand environment for the $[2\text{Fe}-2\text{S}]^{2+}$ cluster. Site-directed mutagenesis of D37A hIscU was carried out to examine the role of individual cysteine residues as possible ligands to the $[2\text{Fe}-2\text{S}]^{2+}$ cluster. When any one of the four cysteines of D37A hIscU were mutated to alanine,¹³ the mutants could be overexpressed and purified, but were colorless and lacked any UV-vis absorbance bands such as observed for the D37A mutant. This suggests that cysteines 35, 61, 96, and 104 are all ligands to a single $[2\text{Fe}-2\text{S}]^{2+}$ cluster that binds per hIscU monomer, although mutation of a cysteine to alanine could influence cluster stability for other unknown reasons. This may be a common coordination environment among some eukaryotic IscUs, since *Mus musculus*, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* IscUs all have the same four conserved cysteines. No prokaryotic IscU or NifU identified from a search of protein sequences at NCBI²³ has a Cys at position 96 (human numbering). This suggests that if NifUs and IscUs lacking C96 are able to form a similar $[2\text{Fe}-2\text{S}]^{2+}$ cluster, then the cluster must either possess non-cysteinylligation, or it must form at the interface of a dimer as has been proposed for the *A. v.* proteins.^{5,6}

To date we have been unable to reconstitute a $[2\text{Fe}-2\text{S}]^{2+}$ center in native hIscU. However, by analogy to the *A. v.* NifU-1 and IscU, we expect that under appropriate physiological conditions, the native protein will transiently bind a cluster similar to that of the D37A hIscU. Possible roles for the conserved D37 in cluster formation/assembly include direct coordination to the cluster, or labilization of the cluster by general acid catalysis. Inasmuch as the hIscU possesses four cysteines that may readily bind a “typical” $2\text{Fe}-2\text{S}$ core, the first possibility seems less likely. No specific function or activity has been shown for the hIscU, or for the IscU or NifU family in general, but the similarities between the D37A hIscU and D37A *A. v.* NifU-1 in their abilities to bind a stable $[2\text{Fe}-2\text{S}]^{2+}$ cluster suggest that they share an evolutionarily conserved role in iron-sulfur cluster assembly and/or repair.

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-9706904 (J.A.C.), and the NIH, GM-38047 (J.P.-H.). M.W.F. and S.S.M. were supported by the NIH Chemistry and Biology Interface Training Program at The Ohio State University (GM08512-03). X-ray absorption data were measured at Stanford Synchrotron Radiation Laboratory which is operated by the U.S. Department of Energy (DOE) office of Basic Energy Services, with additional support from NIH, National Center for Research Resources, and the DOE Office of Biological and Environmental Research.

Supporting Information Available: Figures showing results from gel filtration chromatography and EXAFS experiments, and an alignment of IscU/NifU proteins (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA000800+

(21) Khoroshilova, N.; Popescu, C.; Munck, E.; Beinert, H.; Kiley, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6087–6092.

(22) Teo, B.-K.; Shulman, R. G.; Brown, G. S.; Meixner, A. E. *J. Am. Chem. Soc.* **1979**, *101*, 5624–5631.

(23) <http://www.ncbi.nih.gov>.