Iron sulfur cluster biosynthesis. Human NFU mediates sulfide delivery to ISU in the final step of [2Fe–2S] cluster assembly†

Yushi Liu and J. A. Cowan*

Received (in Cambridge, UK) 3rd April 2007, Accepted 10th May 2007
First published as an Advance Article on the web 6th June 2007
DOI: 10.1039/b704928e

Human NFU forms a complex with NifS-like proteins and is a functionally competent reducing agent for cysteinyl persulfide bond cleavage, releasing inorganic sulfide for incorporation into the ISU-bound [2Fe–2S] cluster.

Iron sulfur cluster proteins are ubiquitous in all life forms,[1] and have been implicated in electron transfer, gene regulation, environmental sensing and substrate activation.[1,2] Consequently, there is substantial interest in understanding the biosynthetic paths that result in protein-bound cluster formation.[3–5] Prior studies of Fe–S cluster assembly in *Azotobacter vinelandii* nitrogenase, and investigations of the *nif* operon, demonstrated a requirement for the NifS (S-donor) and NifU (cluster assembly template) proteins for full activation of nitrogenase.[6] NifS or NifU knockouts resulted in the loss of iron sulfur cluster binding to nitrogenase.[6–8] Later research demonstrated NifS to be a cysteine desulfurase that catalyses the removal of sulfur from L-cysteine, with subsequent incorporation into protein-bound iron sulfur cluster.[9] *Av* NifU contains three domains: an amino-terminal domain that binds a transient [2Fe–2S] cluster, a central domain that binds a permanent stable [2Fe–2S] cluster, and a functionally ill-defined carboxyl domain.[7] Related to the NIF (nitrogen fixation) machinery, gene products from the ISC (iron sulfur cluster)-assembly operon are responsible for cluster biosynthesis in most organisms.[10] The operon encodes homologues for NifU and NifS (IscU and IscS respectively).[10] While no homologue of the NifU C-terminal domain has been identified within the *isc* operon, a gene that encodes a protein with a similar sequence is located elsewhere in the genome.[7] Bacterial IscU and eukaryotic ISU proteins serve as a scaffold for iron sulfur cluster assembly.[5,11] The IscU-bound iron sulfur cluster is subsequently transferred to other proteins that require Fe–S centers, such as ferridoxins.[12,13]

Recent work has laid the foundation for a mechanistic understanding of iron sulfur cluster assembly that includes frataxin-mediated iron delivery to ISU/IscU,[14,15] and subsequent delivery of sulfide *via* the cysteinyl persulfide bond of IscS/NifS.[16,17] The resulting cluster is then transferred to other cluster-dependent proteins.[12,18,19] An alternative pathway,[20] involving initial transfer of sulfur with subsequent uptake of iron, is precluded by the weak binding of iron to the persulfide labeled IscU/ISU.[14,15] While iron-bound ISU has been converted to cluster-bound protein following delivery of sulfide (chemically or enzymatically),[14] reconstitution of sulfur-labeled ISU, following addition of iron ion, has not been demonstrated.[20]

The chemistry underlying reductive cleavage of the cysteinyl persulfide bonds of NifS-like proteins is poorly understood and is the subject of this paper. The C-terminal domain of *Av* NifU was earlier described as thioredoxin-like, and a gene encoding the C-terminal homologue has been located in human chromosome 22q (and named NFU).[7,21] This domain has highly conserved homologues in both prokaryotes and eukaryotes.[22–24] Human NFU has been implicated in several physiological pathways and may be multifunctional.[22,25,26] A role in transcriptional regulation through interaction with the HIRA (histone cell cycle regulation homologue A) protein has been suggested through modulation of chromatin structure.[22,24] Recent studies have also shown that human NFU could interact with laforin, mutations of which lead to lafora disease,[25] while genetic evidence has implicated NFU in iron homeostasis with a potential role in iron sulfur cluster assembly.[21] Specifically, recent reports have suggested NFU to serve as an alternative scaffold protein on the Fe–S cluster assembly pathway;[26] however, the evidence in support of such a role is relatively weak and is discussed in the context of the findings reported here.

The presence of a highly conserved thioredoxin-like Cys–X–X–Cys motif (Fig. 1),[7,27,28] the absence of any other viable candidate for cysteinyl persulfide reduction, and the prevalence of a homologous, though functionally ill-defined motif at the C-terminus of NifU, led us to hypothesize a role for NFU as a reductase that mediates cysteinyl persulfide bond cleavage.[7,8] A similar role for a CXXC motif has previously been elucidated in many other protein disulfide isomerases (PDI).[29] Sequence alignment analysis shows that the C-terminal domain of NFU-type proteins is highly conserved from prokaryotes to eukaryotes, suggesting that the phylogenetically conserved NFU domain might be of functional or structural importance. Herein we report the results of experiments that validate this hypothesis. Specifically, we

```
Hs     KLQGSCTSCPSSII
Mm     KLQGSCTSCPSSII
Dm     KMQGSCSSCPSSIV
Ce     KMQGSCTGCPSSGV
Av     KLTGACTGCQMASM
Sys    RLQGACGSCPSSTM
```

Fig. 1 Sequences of Cys–X–X–Cys domains in NFU-type proteins. Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Av, *Azotobacter vinelandii*; Sys, *Synechocystis* PCC6803.

Evans Laboratory of Chemistry, Ohio State University, 100 West, 18th Ave, Columbus, OH 43210, USA.
E-mail: cowan@chemistry.ohio-state.edu

† Electronic supporting information (ESI) available: Experimental details for cloning and/or expression of key proteins, protein purification, reconstitution and sulfide production assays, circular dichroism and sequence alignments. See DOI: 10.1039/b704928e

demonstrate binding to the sulfur delivery protein NifS, reductive cleavage of NifS persulfide bonds, and the facility to mediate formation of the [2Fe–2S] center in ISU-type proteins.

The putative mitochondrial precursor of NFU contains 254 amino acids with a predicted mass of 28.4 kD (see ESI†). The mature form of NFU has been cloned into a pET 28b expression vector and the isolated purified protein characterized using mass spectrometry, with one major peak at 23831 Da, corresponding to the mature protein lacking the amino-terminal Met (23833.7 Da). Human NFU was purified under aerobic conditions and intramolecular disulfide bond formation most likely accounts for the 2 Da difference between the measured and calculated masses. Secondary structure content was evaluated by circular dichroism and fitting yielded a secondary structure composition of 31% α-helix, 12% β-sheet, and 57% random coil, consistent with sequence predictions using the program PSIPRED protein structure prediction (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html), which yielded a composition of 32% α-helix, 18% β-sheet, and 50% random coil.

By use of the methylene blue assay (see ESI†), NFU was demonstrated to mediate the release of inorganic sulfide from persulfide labeled *Tm* NifS. The *Tm* NifS homologue was used as a substitute for the human sulfur-donor protein (NFS), which is not readily available in quantity. Proteins belonging to this family exhibit similar overall folding, while structures are often superimposable.[17,30,31] The homology of human and *Tm* IscS homologues was further demonstrated through sequence alignment (see ESI†) and the functional chemistry of *Tm* NifS with human ISU and NFU. Formation of a complex between NFU and NifS was demonstrated by isothermal titration calorimetry, with $K_D \approx 10.1$ μM (Fig. 2). Binding is both entropically and enthalpically favorable. However, no direct interaction was detected between NFU and either *Hs* ISU or *Tm* IscU. Previously, NifS was shown to mediate reconstitution of the ISU-bound iron sulfur cluster by serving as the sulfur donor. The ability of NFU to promote reductive cleavage of the NifS persulfide bond, and physiologically relevant binding to NifS, suggests a role for NFU in cluster biogenesis as a reductase that promotes formation and delivery of inorganic sulfide to the ISU scaffold protein through direct interaction with NifS. Such a role is supported by the reconstitution data shown in Fig. 3, which demonstrates that NFU not only cleaves the persulfide bond formed on NifS, but facilitates delivery of the sulfide atom to reconstitute the [2Fe–2S] center in both human and *Tm* ISU-type proteins, yielding the characteristic absorption spectrum reported in earlier work.[19,32]

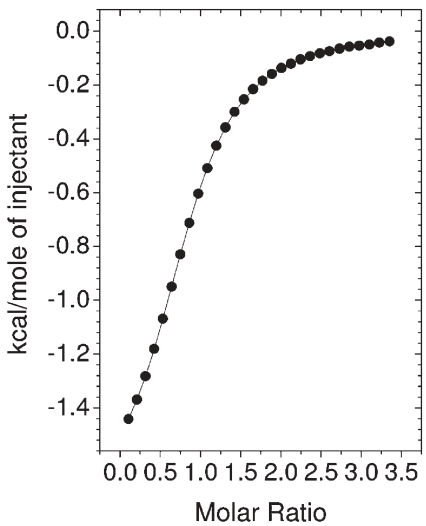

Fig. 2 ITC measurements of human NFU binding to NifS at 25 °C using a MicroCal VP-isothermal calorimeter. All buffers were degassed 20 mM HEPES buffer (pH = 7.5). Fitting yielded a stoichiometry $n \approx 0.95$, $\Delta H \approx -1.8$ kcal mol^{-1}, $\Delta S \approx 16.7$ cal K^{-1} mol^{-1}, $K_D \approx 10.1$ μM.

NFU-facilitated assembly of the [2Fe–2S] center on human and *Tm* ISU-type proteins was further studied by time-dependent kinetics. These experiments demonstrated no cluster to be formed in the absence of NifS, ISU or NFU (Fig. 3). A slight increase in absorbance was noted in the absence of ISU, reflecting iron binding to sulfide produced by NFU-mediated release from NifS. Kinetic rate constants are summarized in Table 1, with similar results obtained for assembly of the cluster product on human and *Tm* ISU-type proteins. The k_{obs} obtained for assembly of the *Hs* ISU (~ 0.015 min^{-1}) fits well with apparent rate constants determined for other steps in the cluster assembly/transfer pathway. A $k_{obs} \approx 0.15$ min^{-1} has been reported for frataxin-mediated assembly of ISU-bound clusters, with sulfur provided as Na_2S.[15] Moreover, the apparent rate constant for cluster transfer from *Hs* ISU to apo Fd has been estimated as ~ 0.09 min^{-1}.[12,13] An apparent $k_{cat} \approx 0.0002$ min^{-1} for a multi-turnover IscU assembly and transfer of cluster to apo Fd, using iron and sulfide ions as reagents, has been estimated for *Escherichia coli* proteins.[35] Overall, these data demonstrate the role for ancillary proteins in mediating [2Fe–2S] cluster assembly and transfer, and are suggestive of potential rate-determining steps.

The similarity in rate constants for human and *Tm* ISU-type proteins (Table 1) supports the structural homology of the ISU proteins and their partner recognition sites. Under quantitative reconstitution conditions, yields of up to 80% were readily obtained based on published extinction coefficients.[19,32]

While NFU has been earlier described as a scaffold for Fe–S cluster formation,[26,33] in our hands attempts to reconstitute an iron sulfur cluster on NFU failed. While limited reconstitution had been previously reported by use of cell extracts, the yield was low and further cluster transfer activity undocumented.[26] Efforts in our laboratory to quantitate ferric and ferrous ion binding to human

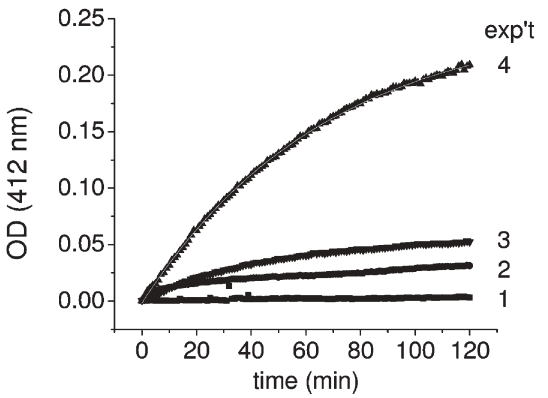

Fig. 3 Kinetics of NFU promoted *Hs* D37A ISU reconstitution. Experiments: 1, no NifS; 2, no ISU; 3, no NFU; 4, reconstitution reaction. The fit to the reconstitution reaction yielded $k_{obs} \approx 0.015$ min^{-1}.

Table 1 Observed rate constants for NFU-mediated assembly of human ISU and *Tm* IscU, respectively

	k_{obs}/min^{-1}	Cluster yield
Hs D37A ISU	0.015 ± 0.004	80%
Tm D40A IscU	0.017 ± 0.003	70%

NFU by isothermal titration calorimetry (ITC) and fluorescence measurements (see ESI†) revealed weak binding ($K_D \geqslant$ mM).

The C-terminal domain of human NFU is highly homologous to the C-terminal domain of *Av* NifU, the functional role of which had been uncertain. While human NFU may serve several cellular roles, the data reported herein shows that NFU binds to NifS and reduces the persulfide bond on activated NifS (following formation of the persulfide bond by abstraction of S from Cys amino acid), yielding inorganic sulfide on a time frame that is compatible with Fe–S cluster assembly. Such results are consistent with the general mechanism that has been proposed for the iron sulfur cluster biosynthesis process on human ISU.[5,15,34] First, frataxin-mediated delivery of iron to ISU and formation of a nucleation site for [2Fe–2S] cluster formation.[14,15] Second, delivery of inorganic sulfide by a NifS-like donor protein,[14] where reductive cleavage of the persulfide bond is mediated by NFU. Highly conserved homologues to human NFU are found in a multitude of organisms. Consequently, the functional role(s) for NFU are most likely important and conserved, one of which appears to be involvement in iron sulfur cluster biogenesis.‡

This work was supported by the National Science Foundation CHE-0111161.

Notes and references

‡ **Quantitation of human NFU binding to NifS using isothermal titration calorimetry**. ITC measurements were carried out at 25 °C on a MicroCal VP-ITC system. The titrant and sample solutions were dialyzed against the same buffer solution (20 mM HEPES, pH 7.5), and both solutions were thoroughly degassed under vacuum and supplemented with 1 mM tris(2-carboxyethyl)phosphine (TCEP). A 10 μl volume of the titrant (750 μM human NFU) was injected into the sample cell (50 μM NifS) in 20 s intervals. The sample cell was stirred at 300 rpm to ensure the rapidness and completeness of the reaction, and a 300 s interval allowed for equilibration of the reaction prior to the next injection. Up to 3.5 equivalents of titrant were added to ensure no further NFU–NifS complex formation. A control reaction in which the titrant was injected into the cell containing only the buffer solution was performed and subtracted as the background. The data was analyzed and fitted to a one site binding model.

NFU-promoted IscU reconstitution. Human NFU and ISU were dialyzed against 50 mM Tris-HCl (pH 7.5), incubated with dithiothreitol (DTT) and excess removed by ultrafiltration (Amicon) under strict anaerobic conditions. The D37A mutant of *Hs* ISU (D40A *Tm* IscU) was routinely used since this yields a product cluster that is more stable to hydrolytic degradation. Subsequently, 400 μM NFU and 200 μM ISU were incubated with 0.8 mM Fe^{2+}, 2 mM L-cysteine and 7 μM NifS. After 2 h, the mixture was loaded onto a Sephadex G-25 gel filtration column equilibrated with argon-purged 50 mM Tris-HCl pH 7.5. For His-tagged ISU, the colored fractions of holo IscU were concentrated. If non-His tagged ISU was used, the protein fractions were loaded onto an Ni-NTA column, and washed with 3 volumes of argon-purged 50 mM Tris-HCl (pH 7.5) buffer. The washed and eluted fractions were combined and loaded onto an anion exchange column (DE-52). A 50 mM Tris-HCl–300 mM NaCl pH 7.5 buffer was used to elute the reconstituted non-His tagged ISU. The colored fractions containing holo ISU judged by SDS-PAGE were pooled and concentrated. UV-visible spectra were recorded on a Hewlett-Packard 8425A diode array spectrophotometer using On-Line Instrument Systems (OLIS) 4300S operating system software.

Kinetic analysis of IscU reconstitution promoted by human NFU. For kinetic analyses, both NFU and IscU were dialyzed into 50 mM Tris-HCl–50 mM NaCl (pH 7.5) buffer solution and incubated with excess DTT. DTT was subsequently removed by repeated ultrafiltration. These exchanges were carried out under strict anaerobic conditions and all buffers used were argon-purged. IscU (50 μM) and excess NFU were mixed anaerobically, and a 4-fold excess of ferrous iron and a 10-fold excess of L-cysteine were added. Finally, the reaction was initiated by addition of NifS (to 3.6 mM) and the time-dependence of iron sulfur cluster assembly on IscU was monitored by use of a Hewlett-Packard 8425A diode array spectrophotometer. Control experiments in the absence of NFU, IscU and NifS were also conducted. The absorbance at 412 nm was monitored over a period of 120 min and kinetic data were fit to a first-order process using Origin 6.0. Human ISU was studied in a similar fashion, with 90 μM of human reduced ISU and excess reduced human NFU.

1 H. Beinert, R. H. Holm and E. Munck, *Science*, 1997, **277**, 653.
2 H. Beinert, *JBIC, J. Biol. Inorg. Chem.*, 2000, **5**, 2.
3 D. R. Dean, J. T. Bolin and L. Zheng, *J. Bacteriol.*, 1993, **175**, 6737.
4 J. Gerber and R. Lill, *Mitochondrion*, 2002, **2**, 71.
5 S. S. Mansy and J. A. Cowan, *Acc. Chem. Res.*, 2004, **27**, 719.
6 M. R. Jacobson, V. L. Cash, M. C. Weiss, N. F. Laird, W. E. Newton and D. R. Dean, *Mol. Gen. Genet.*, 1989, **219**, 49.
7 J. Frazzon, J. R. Fick and D. R. Dean, *Biochem. Soc. Trans.*, 2002, **30**, 680.
8 J. Frazzon and D. R. Dean, *Curr. Opin. Chem. Biol.*, 2003, **7**, 166.
9 L. Zheng, R. H. White, V. L. Cash, R. F. Jack and D. R. Dean, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 2754.
10 L. Zheng, V. L. Cash, D. H. Flint and D. R. Dean, *J. Biol. Chem.*, 1998, **273**, 13264.
11 D. C. Johnson, D. R. Dean, A. D. Smith and M. K. Johnson, *Annu. Rev. Biochem.*, 2005, **74**, 247.
12 S. P. Wu, G. Wu, K. K. Surerus and J. A. Cowan, *Biochemistry*, 2002, **41**, 8876.
13 S. P. Wu and J. A. Cowan, *Biochemistry*, 2003, **42**, 5784.
14 M. Nuth, T. Yoon and J. A. Cowan, *J. Am. Chem. Soc.*, 2002, **124**, 8774.
15 T. Yoon and J. A. Cowan, *J. Am. Chem. Soc.*, 2003, **125**, 6078.
16 L. Zheng, R. H. White, V. L. Cash and D. R. Dean, *Biochemistry*, 1994, **33**, 4714.
17 J. T. Kaiser, T. Clausen, G. P. Bourenkow, H. D. Bartunik, S. Steinbacher and R. Huber, *J. Mol. Biol.*, 2000, **297**, 451.
18 A. D. Smith, J. N. Agar, K. A. Johnson, J. Frazzon, I. J. Amster, D. R. Dean and M. K. Johnson, *J. Am. Chem. Soc.*, 2001, **123**, 11103.
19 G. Wu, S. S. Mansy, S. P. Wu, K. K. Surerus, M. W. Foster and J. A. Cowan, *Biochemistry*, 2002, **41**, 5024.
20 S. S. Mansy, G. Wu, K. K. Surerus and J. A. Cowan, *J. Biol. Chem.*, 2002, **277**, 21397.
21 B. Schilke, C. Voisine, H. Beinert and E. Craig, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 10206.
22 S. Lorain, Y. Lecluse, C. Scamps, M. G. Mattei and M. Lipinski, *Biochim. Biophys. Acta*, 2001, **1517**, 376.
23 S. Lorain, J. P. Quivy, F. Monier-Gavelle, C. Scamps, Y. Lecluse, G. Almouzni and M. Lipinski, *Mol. Cell. Biol.*, 1998, **18**, 5546.
24 P. Magnaghi, C. Roberts, S. Lorain, M. Lipinski and P. J. Scambler, *Nat. Genet.*, 1998, **20**, 74.
25 S. Ganesh, N. Tsurutani, T. Suzuki, K. Ueda, K. L. Agarwala, H. Osada, A. V. Delgado-Escueta and K. Yamakawa, *Hum. Mol. Genet.*, 2003, **12**, 2359.
26 W. H. Tong, G. N. Jameson, B. H. Huynh and T. A. Rouault, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 9762.
27 A. Holmgren, *J. Biol. Chem.*, 1989, **264**, 13963.
28 S. K. Hiroyuki Yano and R. B. Buchanan, *Proteomics*, 2002, **2**, 1090.
29 L. Ellgaard and L. W. Ruddock, *EMBO Rep.*, 2005, **6**, 28.
30 J. R. Cupp-Vickery, H. Urbina and L. E. Vickery, *J. Mol. Biol.*, 2003, **330**, 1049.
31 R. Percudani and A. Peracchi, *EMBO Rep.*, 2003, **4**, 850.
32 M. W. Foster, J. Whang, J. E. Penner-Hahn, K. K. Surerus and J. A. Cowan, *J. Am. Chem. Soc.*, 2000, **122**, 6805.
33 K. Nishio and M. Nakai, *J. Biol. Chem.*, 2000, **275**, 22615.
34 S. S. Mansy, S. P. Wu and J. A. Cowan, *J. Biol. Chem.*, 2004, **279**, 10469.
35 F. Bonomi, S. Iametti, D. Ta and L. E. Vickery, *J. Biol. Chem.*, 2005, **280**, 29513.

