Characterization of an Iron−Sulfur Cluster Assembly Protein (ISU1) from *Schizosaccharomyces pombe*[†]

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ABSTRACT: Genetic studies of bacteria and eukaryotes have led to identification of several gene products that are involved in the biosynthesis of protein-bound iron−sulfur clusters. One of these proteins, ISU, is homologous to the N-terminus of bacterial NifU. The mature forms of His-tagged wild-type and D37A *Schizosaccharomyces pombe* ISU1 were cloned and overexpressed as inclusion bodies in *Escherichia coli*. The recombinant D37A protein was purified under denaturing conditions and subsequently reconstituted in vitro. By use of a 5-fold excess of iron and sulfide the reconstituted product was found to be red-brown in color, forming a homodimer of 17 kDa per subunit with approximately two iron atoms per monomer determined by protein and iron quantitation. UV−vis absorption and Mössbauer spectroscopies ($\delta = 0.29 \pm 0.05$ mm/s; $\Delta E_Q = 0.59 \pm 0.05$ mm/s) were used to characterize D37A ISU1 and show the presence of $[2Fe-2S]^{2+}$ clusters in each subunit. Formation of the holo form of wild-type ISU1 was significantly less efficient using the same reconstitution conditions and is consistent with prior observations that the D37A substitution can stabilize protein-bound clusters. Relative to the human homologue, the yeast ISU is significantly less soluble at ambient temperatures. In both cases the native ISU1 is more sensitive to proton-mediated degradation relative to the D37A derivative. The lability of this family of proteins relative to [2Fe-2S] bearing ferredoxins most likely is of functional relevance for cluster transfer chemistry. Mössbauer parameters obtained for wild-type ISU1 ($\delta = 0.31 \pm 0.05$ mm/s; $\Delta E_Q = 0.64 \pm 0.05$ mm/s) were similar to those obtained for the D37A derivative. Cluster transfer from ISU1 to apo Fd is demonstrated: the first example of transfer from an ISU-type protein. A lower limit for k_2 of 80 M^{-1} min^{-1} was established for WT cluster transfer and a value of 18 M^{-1} min^{-1} for the D37A derivative. Finally, we have demonstrated through cross-linking studies that ferredoxin, an electron-transport protein, forms a complex with ISU1 in both apo and holo states. Cross-linking of holo ISU1 with holo Fd is consistent with a role for redox chemistry in cluster assembly and may mimic the intramolecular complex already defined in NifU.

Iron−sulfur-containing proteins are widely distributed in prokaryotes and eukaryotes and exhibit diverse biological roles, including electron transfer, sites of substrate binding and catalysis, sensors of environmental stimuli, and the regulation of gene expression (*1−4*). Recent genetic studies in *Azotobacter vinelandii*, *Escherichia coli*, and *Saccharomyces cerevisiae* have identified a suite of proteins that appear to play a role in general iron−sulfur cluster assembly and repair (*5−9*). From earlier studies it was found that the nitrogen fixation (*nif*) gene cluster products were required for the full activation of the metalloenzyme nitrogenase in the facultative anaerobic nitrogen-fixing bacterium *A. vinelandii* (*10*). Subsequently, a related gene cluster, the *isc* (iron sulfur cluster) operon, was identified in a wide range of

nitrogen-fixing and non-nitrogen-fixing organisms (*11*). The products of *nif* and *isc* genes exhibit a high degree of homology to each other; however, the latter appear to play a more general role in the formation or repair of Fe-S clusters, rather than exclusively participating in nitrogenase activation. NifS and IscS, pyridoxal phosphate- (PLP-) dependent cysteine desulfurases, provide sulfide equivalents for incorporation into the Fe-S centers (*1, 11−13*). Additionally, the *isc* operon contains genes encoding ferredoxin and two chaperones (Hsc66, Hsc22) (*9, 14, 15*); however, studies of the functions of these and other conserved proteins such as ORF6 (IscA) (*5, 16, 17*) have only just begun.

NifU from *A. vinelandii* is a homodimeric modular protein containing two distinct types of iron binding sites (*18*). Each subunit has one permanent $[2Fe-2S]^{2+}$ binding site within its central domain, one labile cluster binding site at the N-terminus, and a less characterized C-terminal domain. Cysteine residues 35, 62, and 106 are necessary for binding iron at the N-terminal site, whereas Cys137, Cys139, Cys172, and Cys175 provide ligands to the stable [2Fe-2S] cluster. *Schizosaccharomyces pombe* ISU1 is homologous to the N-terminal domain of NifU and includes the three conserved

cysteine residues necessary for the coordination of the labile cluster and an additional cysteine at position 96. Dean, Johnson, and co-workers have advanced the theory that NifU/IscU proteins serve as scaffolds for NifS/IscS-directed assembly of Fe-S intermediates that are transferred to target iron−sulfur apoproteins (*13*). This model is analogous to the NifEN complex (*19, 20*), which serves as a scaffold for assembly of the FeMo cofactor of the nitrogenase MoFe component protein. The involvement of chaperone proteins to aid the assembly and transfer of clusters is likely (*14*), and the demonstrated instability of the "transient" [2Fe-2S] clusters of NifU and IscU toward reduction has led some to suggest that the transfer of Fe-S equivalents may be redox mediated. It has further been proposed that the stable [2Fe-2S]$^{2+}$ cluster in the central domain of *A. vinelandii* NifU may catalyze the release of iron and sulfur bound to the N-terminus of the protein. IscU's lack the same stable [2Fe-2S]$^{2+}$ domain. However, a gene encoding a ferredoxin is found within the *isc* operon, and homologues are found in eukaryotes. Although the primary sequence of ferredoxins share little homology to the central domain of NifU, their spectroscopic and electronic properties share many common features and thus may play similar roles in Fe-S biosynthesis (*21*).

Currently, there is insufficient data to propose a working model for iron−sulfur cluster assembly that describes the precise functions of these proteins and how they interact with each other and with the iron−sulfur apoprotein into which the Fe-S cofactor is to be assembled. The field requires functional characterization of these gene products, and in this paper we report the first example of a cloned, expressed, and characterized yeast ISU protein (*S. pombe* ISU1). Factors influencing cluster stability in this family of proteins have also been investigated, and the first direct evidence has been obtained for complex formation with a protein target and possible likely redox partner, apo- and holoferredoxin, respectively. Cluster transfer to apo Fd is also demonstrated: the first characterization of a cluster transfer reaction for this ISU class of protein.

MATERIALS AND METHODS

General Chemicals. Solutions were argon purged and handled under positive Ar(g) pressure using standard Schlenk line techniques. Iron-57 was obtained from Pennwood Chemicals. All restriction enzymes and buffers were from Life Technologies (Rockville, MD). Pfu polymerase was obtained from Stratagene (La Jolla, CA), and gel extraction kits and PCR[1] purification kits were purchased from QIAGEN (Valencia, CA). The *S. pombe* MATCHMAKER cDNA library was from Clonetech (Palo Alto, CA). The protein expression vector pET-28b(+) and *E. coli* strain BL21 (DE3) were purchased from Novagen (Madison, WI). A plasmid for expression of human ferredoxin was kindly provided by J. Markley (University of Wisconsin). Cloning and expres-

sion of the yeast homologue have been achieved in this laboratory, and details will be reported elsewhere.

Cloning and Expression of S. pombe ISU1 and the D37A Derivative. The DNA sequence corresponding to the mature ISU1 protein, beginning with tyrosine (Figure 1) as predicted by sequence alignment of prokaryotic and eukaryotic IscU and ISU's, respectively, was amplified by the polymerase chain reaction (PCR) from the *S. pombe* MATCHMAKER cDNA library. The following primers were used: 5′ primer, 5′- GGCG CAT ATG TAC CAT AAG AAT GTT TTA GA-3′; 3′ primer, 5′- CG GGA TCC CTA AGC GGT AGC AGA CTC AAT A-3′. These primers were based on the published sequence of *ISU1* and incorporated *Nde*I and *Bam*HI restriction sites (underlined) at the 5′ and 3′ ends, respectively. The reaction mixture contained 2.5 μL of cDNA template (200 ng), 2 μL of primers (5 μM each primer), 2.5 μL of 10× Pfu polymerase buffer, 0.5 μL of NTP mix (10 mM each NTP), 0.5 μL of Pfu polymerase (2.5 units/μL; hot start), and sterile ddH$_2$O to 25 μL. PCR amplification conditions followed a standard protocol with denaturation at 94 °C for 2 min and subsequent addition of 0.5 μL of Pfu polymerase, followed by an amplification sequence of 94 °C (30 s), 58 °C (30 s), and 68 °C (30 s) over 30 cycles. A final extension was achieved at 68 °C for 2 min. The amplified *ISU1* gene was digested with restriction enzymes *Nde*I and *Bam*HI and cloned into a similarly treated expression vector pET-28b(+) to produce plasmid pET-28b(+)-(ISU1). The DNA sequence of the cloned *ISU1* gene product was confirmed by DNA sequencing.

The D37A derivative was prepared using a two-step site-directed mutagenesis method (*22*) and the purified pET-28b-(+)(ISU1) as template. The following mutagenic primer was obtained from Integrated DNA Technologies Inc., 5′-C GCC TGT GGC GCT GTT ATG CGC CT-3′, and used with the two PCR primers described earlier to execute the mutagenesis protocol. The underlined codon corresponds to the substituted residue. The amplified D37A gene was subcloned into pET-28b(+) using the same method as described above.

Plasmid pET-28b(+)(ISU1) or pET-28b(+)(D37A) was transformed into *E. coli* BL21(DE3) by electroporation. For protein hyperproduction an overnight culture (40 mL) was used as an inoculum for 4 L of LB containing 35 μg/mL kanamycin. Cells were grown at 37 °C to an OD of 0.6−0.8, and expression was induced by the addition of IPTG to a final concentration of 1.0 mM. The culture was incubated for an additional 4 h, and then the cells were harvested by centrifugation. The cell pellet was washed with 50 mM Tris-HCl and 50 mM NaCl, pH 7.5, and stored at −80 °C until used.

Purification and Reconstitution of Wild-Type and D37A ISU1. Cells were thawed on ice and resuspended in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9; ~2.5 mL/g of cells) containing 20 μg/mL PMSF and 2.5 μg/mL leupeptin and lysed by sonication. Ultrapure urea was added to the cell lysate to a final concentration of 6 M, and the mixture was stirred on ice for 1 h. The cellular debris was removed by centrifugation at 15000 rpm and 4 °C for 15 min, and the supernatant was loaded onto a Ni^{2+} chelation column (Qiagen) previously equilibrated with binding buffer and 6 M urea. After being loaded, the column was washed with 10 volumes of wash buffer (20 mM imidazole, 500 mM NaCl, 6 M urea, 20 mM Tris-HCl, pH

7.9) and eluted with elution buffer (200 mM imidazole, 500 mM NaCl, 6 M urea, 20 mM Tris-HCl, pH 7.9). Fractions containing wild-type ISU1 or D37A ISU1 as judged by SDS−PAGE were pooled and concentrated by ultrafiltration using an Amicon stirred cell concentrator. Finally, the protein was diluted to approximately 0.2 mM with 50 mM Tris-HCl buffer, pH 7.5, and 3 M urea and reconstituted by addition of 50 mM DTT, 1.0 mM Fe^{3+}, and 1.0 mM S^{2-} under rigorously anaerobic conditions. After 30 min the refolded holoprotein was desalted on a Sephadex G-25 column eluted with buffer A (50 mM Tris-HCl buffer, pH 7.5). Colored fractions containing wild-type ISU1 or D37A were loaded onto a CM32 cation-exchange column equilibrated in buffer A. After being loaded, the column was washed with buffer A + 50 mM NaCl, and holo ISU1 or D37A were eluted with buffer A + 150 mM NaCl. Fractions containing holoprotein (as judged by UV−vis absorbance) were pooled and concentrated by ultrafiltration. The CM32-purified D37A derivative (1−2 mL) was subjected to a further purification step with a Superose 12 column (HR 16/50, Pharmacia, FPLC) run at 0.5 mL/min with buffer A + 50 mM NaCl. Fractions with an A_{278}/A_{324} ratio of <1.1 were pooled, concentrated, and stored at −80 °C. Protein purity at each stage of purification was monitored by SDS−PAGE (Figure 2).

Determination of Molecular Weight. The relative MW of protein subunits was estimated by SDS−PAGE, using a Bio-Rad MiniProtean II apparatus, and stained with Coomassie Blue. The MW of wild-type protein was determined by FPLC using a Superose 12 column (HR 16/50, Pharmacia) at a flow rate of 0.5 mL/min with buffer A + 50 mM NaCl. The column was calibrated using a Gel Filtration Calibration Kit (Pharmacia). The standards were RNase A (13700 Da), chymotrypsinogen A (25000 Da), ovalbumin (43000 Da), and albumin (67000 Da). Blue Dextran was used to determine the dead volume. MW's were determined by plotting log MW of standards vs K_{av} where $K_{av} = (V_e - V_0)/(V_t - V_0)$ (V_e = elution volume; V_0 = dead volume; V_t = total column volume). Standards were fit to a straight line which gave log MW = $(5.41 \pm 0.13) - (2.22 \pm 0.30)K_{av}$.

UV−Vis Spectroscopy. UV−vis spectra were recorded on a Hewlett-Packard 8425A diode array spectrophotometer using On-Line Instrument Systems (OLIS) 4300S Operating System software. A 1.0 cm path-length cuvette was used for measurements, which were recorded at room temperature. The molar extinction coefficient of the D37A derivative was calculated using Beer's law ($A = \epsilon lc$), in which c was derived from the mass of completely desalted and lyophilized protein.

Iron Quantitation. Protein concentrations were quantitatively assessed from the measured extinction coefficient (above) and confirmed by calculations based on the Bio-Rad protein assay. Iron content was measured by the method of Moulis et al. (*23*). In brief, 200 μL of 0.05 mM protein was acidified by the addition of 60 μL of concentrated HCl in an Eppendorf tube. The sample was then heated to 100 °C for 15 min. The precipitated material was removed by centrifugation. Supernatant (100 μL) was diluted in 1.3 mL of 0.5 M Tris-HCl, pH 8.5. Then 0.1 mL of 5% sodium ascorbate (freshly prepared) and 0.4 mL of 0.1% bathophenanthrolinedisulfonate were subsequently added with mixing between each addition. After incubation at room temperature for 1 h, iron was quantitated by measuring the

absorbance at 535 nm and compared to a calibration curve made with 0.01−0.3 mM $FeCl_3$ solutions.

Mössbauer Spectroscopy. The $^{57}Fe(III)$ solution was prepared by dissolving ^{57}Fe metal (Pennwood Chemicals) in a 1:1 mixture of concentrated hydrochloric and nitric acids. This stock was adjusted to pH 7.5 by 1.0 M Tris base and diluted to 50 mM Fe^{3+} with 50 mM Tris-HCl buffer (pH 7.5) before use.

Samples for Mössbauer analysis were prepared from apoprotein that had been eluted from a Ni-NTA column and treated with 1.0 mM EDTA with stirring for 1 h at room temperature. The mixture was then passed through a Sephadex G-25 desalting column and ISU1 reconstituted and purified as above (see Purification and Reconstitution of Wild-Type and D37A ISU1), with use of $^{57}Fe(III)$ instead of $FeCl_3$ with natural abundance iron. Mössbauer cells were filled with 500 μL of protein solution and frozen at −80 °C. Mössbauer spectra were recorded on a constant acceleration spectrometer, model MS-1200D from Ranger Scientific, using a Janis SuperVaritemp cryostat (model 8DT), a Lakeshore temperature controller (model 340), and a ^{57}Co source in a rhodium foil purchased from Isotope Products Laboratory. All isomer shifts are quoted relative to iron metal at room temperature.

EDC Cross-Linking. A sample of 40 μM ISU1 and 40 μM ferredoxin (human or *S. pombe*) and 5 mM EDC were incubated at room temperature for 2 h. An aliquot of the reaction mix was then diluted with 4× SDS−PhastGel loading buffer (Pharmacia), boiled for 2 min, and loaded onto a Homogenous-20 gel (Pharmacia).

Determination of Reaction Rate Constants for [2Fe-2S] Cluster Transfer from Holo ISU1 to Apo Fd. Kinetic analyses of Fe-S cluster transfer from holo ISU1 to apo Fd were performed as follows. Apo Fd was incubated with 4.5 mM DTT for 30 min, followed by addition of holo ISU1. At intervals of 10 min (wild-type ISU1) or 60 min (D37A ISU1) an aliquot of the reaction mixture was withdrawn for assay by native PAGE. A greater than 10-fold excess of holo ISU1 to apo Fd was used in cluster transfer reactions, with overall concentrations of apo Fd and ISU1 of 16 and 327 μM, respectively. Following Fe-S cluster transfer from holo ISU1 to apo Fd, the concentration of holo Fd was observed to increase, as determined by both Coomassie and iron staining (*24*), following quantitative evaluation of band intensity by use of a Bio-Rad gel doc 1000. Figure 7 shows a plot of the fractional yield of holo Fd as a function of reaction time, fitted to a rate equation for a first-order decay process. Apparent rate constants (k_{obs}) for Fe-S cluster transfer from holo ISU1 to apo Fd were obtained and k_2 values determined. Control experiments show no measurable formation of holo Fd when analogous concentrations of free iron and sulfide ion were used in reconstitution mixtures under the conditions used in the protein-mediated cluster transfer reactions.

RESULTS AND DISCUSSION

Purification, Reconstitution, and Characterization of Wild-Type and D37A ISU1. The DNA sequence corresponding to mature ISU1 lacking the mitochondrial targeting sequence was predicted from the available gene sequence by comparison with other eukaryotic and prokaryotic homologues, and the translation start site is indicated in Figure 1. An ∼500

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                        -51                                                       -4
        Sp ISU1:  MSVFRRSVQC VGVLPSILAQ RSSLLARPAN LQFLKTNSSK FVPQVTANVS
        Hs ISU1:                                                       MVLIDMS
        Hs ISU2:  .......... ......MAAA GAGRLRRVAS ALLLR...SP RLPARELSAP
        Av IscU:  .......... .......... .......... .......... ..........
        Av NifU:  .......... .......... .......... .......... ..........


                        -3                                                        46
        Sp ISU1:  RRMYHKNVLD HYNNPRNVGT LPKGDPDVGI GLVGAPACGD VMRLAIRVN.
        Hs ISU1:  VDL-STQVVD HYENPRNVGS LDKTSKNVGT GLVGAPACGD VMKLQIQVD.
        Hs ISU2:  ARLYHKKVVD HYENPRNVGS LDKTSKNVGT GLVGAPACGD VMKLQIQVD.
        Av IscU:  .MAYSDKVID HYENPRNVGK LDAQDPDVGT GMVGAPACGD VMRLQIKVN.
        Av NifU:  MWDYSEKVKE HFYNPKNAGA VEGAN...AI GDVGSLSCGD ALRLTLKVDP
       Consensus  ...*-.**.- *-.**-*-*. -.........--- *.**---*** ---*.--*-.


                        47                                                        95
        Sp ISU1:  KDGVIEDVKF KTFGCGSAIA SSSYVTTMVK GMTLEEASKI KNTQIAKEL.
        Hs ISU1:  EKGKIVDARF KTFGCGSAIA SSSLATEWVK GKTVEEALTI KNTDIAKEL.
        Hs ISU2:  EKGKIVDARF KTFGCGSAIA SSSLATEWVK GKTVEEALTI KNTDIAKEL.
        Av IscU:  EQGIIEDAKF KTYGCGSAIA SSSLATEWMK GRTLEEAETI KNTQIAEEL.
        Av NifU:  ETDVILDAGF QTFGCGSAIA SSSALTEMVK GLTLDEALKI SNQDIADYLD
       Consensus  -.-.*.*-.* -*-******* ***..*---* *.*--**--* -*--**.-*.


                        96                                                        139
        Sp ISU1:  CLPPVKLHCS MLAEDAIKSA VKHYRSKQLT PVGTTAGAIE SATA......
        Hs ISU1:  CLPPVKLHCS MLAEDAIKAA LADYKLKQEP KKGEAEKK
        Hs ISU2:  CLPPVKLHCS MLAEDAIKAA LADYKLKQEP KKGEAEKK.. ..........
        Av IscU:  ALPPVKIHCS VLAEDAIKAA VRDYKHKKGL V......... ..........
        Av NifU:  GLPPEKMHCS VMGREALQAA VANYRGETIE DDHEEGALIC KCFAVDEVMV
       Consensus  .***-*.*** -----*---* -..*-.-... ..........


        Av NifU:  RDTIRANKLS TVEDVTNYTK AGGGCSACHE AIERVLTEEL AARGEVFVAA
        Av NifU:  PIKAKKKVKV LAPEPAPAPV AEAPAAAPKL SNLQRIRRIE TVLAAIRPTL
        Av NifU:  QRDKGDVELI DVDGKNVYVK LTGACTGCQM ASMTLGGIQQ RLIEELGEFV
        Av NifU:  KVIPVSAAAH AQMEV
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FIGURE 1: Sequence alignment of *S. pombe* ISU1 with eukaryotic and prokaryotic homologues. Abbreviations: Sp ISU1, *S. pombe* ISU1; Av IscU, *A. vinelandii* IscU; Av NifU, *A. vinelandii* NifU; Hs ISU1 and Hs ISU2, *Homo sapiens* ISU1 and ISU2. Numbers above the sequences refer only to the *S. pombe* ISU1 sequence. A dot represents a gap in the alignment. Conserved cysteine residues are indicated in bold, sequence similarities are shown by a dash (−), and identical residues are indicated by a star (*). Alignments were performed using the MULTALIN alignment program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html). The first residue (Y) in our expressed protein structure is in bold and underlined.

bp DNA fragment was amplified from the *S. pombe* cDNA library. This fragment was then digested with *Nde*I and *Bam*HI and ligated to a similarly treated T7/*lac*-controlled expression plasmid pET-28b(+) vector, yielding pET-28b-(+)(ISU1). With the knowledge that a single point substitution in *A. vinelandii* NifU and human ISU1 allowed the formation of a more stable [2Fe-2S]$^{2+}$ cluster, the homologous substitution was made in *S. pombe* ISU1. The D37A gene was amplified with mispaired primers by a two-step PCR strategy and subcloned into pET-28b(+) to yield pET-28b(+)(D37A), with an N-terminal His tag. When the protein was expressed with or without a supplement of FeSO$_4$ (40 μM), cells appeared the same color, pale brown. The cells did not show the green or red-brown color that is characteristic of most cells that produce iron−sulfur cluster bound proteins. From SDS−PAGE (data not shown) both wild-type and D37A ISU1 were overexpressed at high levels as inclusion bodies in *E. coli*. Accordingly, the first step of the purification protocol was carried out under denaturing conditions. After Ni-NTA affinity chromatography, the protein was isolated in the apo form and could be reconstituted by the addition of DTT, Fe^{3+}, and S^{2-}. The refolded proteins were purified to homogeneity by an additional three-step procedure that included a desalting column (Sephadex G-25), a cation-exchange column (CM32), and a gel filtration column (Superose 12). The purity of the samples were confirmed by SDS−PAGE gel electrophoresis (Figure 2),
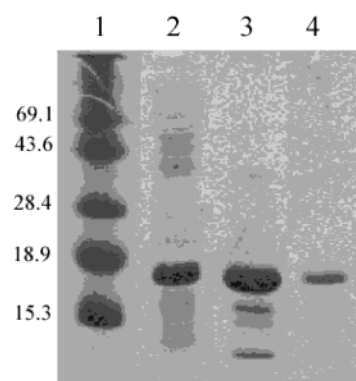

FIGURE 2: SDS−PAGE analysis of purification steps for ISU1-(D37A). Lanes: 1, high MW markers; 2, cell lysate; 3, Ni-NTA eluted with 200 mM imidazole; 4, after CM32 column. Masses of MW markers (in kDa) are indicated on the left.

and the molecular mass of the homogeneous preparation of the gene product was estimated to be ∼17 kDa, in good agreement with the value predicted from the amino acid sequence (17037 Da). The yield of reconstituted wild-type ISU1 was found to be significantly lower than that of the D37A derivative (∼10 mg/L of cells). This also confirms that the D37A derivative can stabilize the cluster.

Gel filtration chromatography was also used to determine the aggregation state of the protein. On the basis of the elution profile of standard proteins the red-brown holo form
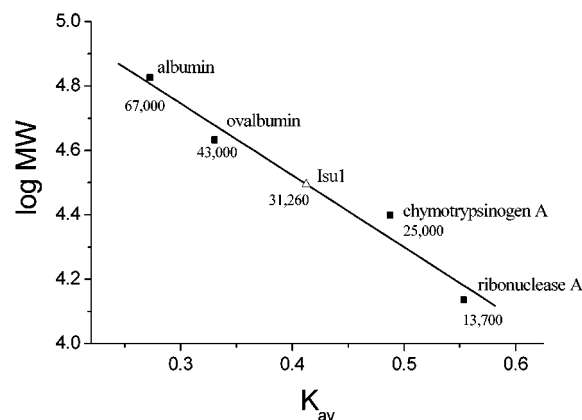
FIGURE 3: Molecular weight determination (plot of log MW vs K_{av}) of D37A ISU1 by FPLC Superose 12. $K_{av} = (V_e - V_0)/(V_t - V_0)$ (V_e, elution volume; V_0, dead volume; V_t, total volume). Filled squares indicate the elution positions of RNase A (13700 Da), chymotrypsinogen A (25000 Da), ovalbumin (43000 Da), and albumin (67000 Da), while the open triangle shows the elution position of the holo ISU1 D37A derivative.

of D37A ISU1 shows one major peak and corresponds to a dimer (Figure 3). A smaller colorless peak corresponds to an aggregated state of the apoprotein. As judged by SDS–PAGE, all of the peaks observed during Superose 12 chromatography were composed of D37A ISU1.

The iron content of the more stable D37A ISU1 was determined by the method of Moulis et al. (*23*). This method utilized HCl treatment of the cluster, which facilitates degradation and release of Fe that is then quantitatively complexed by bathophenanthrolinedisulfonate. By this method, the reconstituted red-brown form of D37A ISU1 was found to have 2.0 ± 0.2 mol of Fe per mole of subunit, which is consistent with each subunit containing a [2Fe-2S] cluster.

ISU1 is one of the most conserved protein sequences in nature. By comparison of the primary sequences of the IscU protein family (Figure 1), mature *S. pombe* ISU1 exhibits a high degree of amino acid sequence identity to IscU from *A. vinelandii* (70%), *E. coli* (65%), and human (71%) (*11*). *A. vinelandii* and *E. coli* IscU contain three conserved cysteine residues (Cys35, Cys61, and Cys104, Figure 1 and *S. pombe* numbering) and have been characterized as homodimers with each subunit accommodating one [2Fe-2S]²⁺ cluster. The human homologue also has been recently cloned and hyperproduced in *E. coli* (*25*) and contains four cysteines (Cys35, Cys61, Cys96, and Cys104, *S. pombe* numbering), including a nonconserved cysteine residue at position 96, and shows UV–vis and Mössbauer spectra similar to those exhibited by bacterial IscU proteins. By contrast to other IscU proteins, the human homologue is a monomeric protein as determined by gel filtration chromatography and contains one [2Fe-2S] cluster. This observation may suggest a possible relationship between the aggregation state of IscU proteins and the number of cysteine residues. Interestingly, *S. pombe* ISU1 also has the extra nonconserved cysteine residue at position 96. However, gel filtration and SDS–PAGE results of holo *S. pombe* ISU1 (wild type and D37A) indicated that *S. pombe* ISU1 is a homodimer. The amino acid residues that mediate dimer formation are yet to be identified.

Spectroscopic Characterization of [2Fe-2S] Cluster Cofactors. The UV–visible absorption spectrum of D37A ISU1
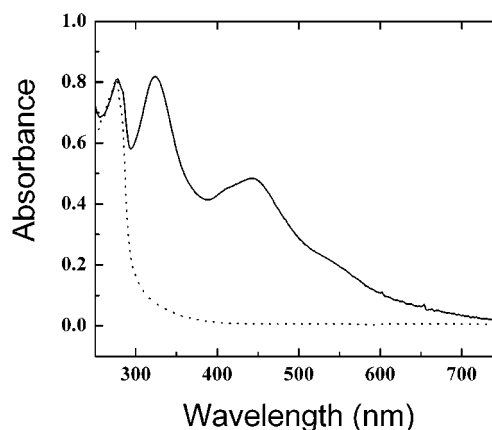

FIGURE 4: UV–visible absorption spectra of holo D37A ISU1 (solid line) and the apo D37A derivative (dashed line). Spectra were taken in 50 mM Tris-HCl buffer and 50 mM NaCl, pH 7.5.

Table 1: Mössbauer Parameters for Wild-Type and D37A *S. pombe* ISU1

protein	δ (mm/s)	ΔE_Q (mm/s)
wild-type ISU1	0.31 ± 0.05	0.64 ± 0.05
D37A ISU1	0.29 ± 0.05	0.59 ± 0.05
wild-type Isa1 (*34*)	0.27 ± 0.05	0.56 ± 0.05

is shown in Figure 4 and is consistent with the presence of a [2Fe-2S] form of D37A ISU1 with λ_{max} at 278 nm ($\epsilon = 26770$ M⁻¹ cm⁻¹), 324 nm ($\epsilon = 27040$ M⁻¹ cm⁻¹), and 442 nm ($\epsilon = 15990$ M⁻¹ cm⁻¹) and a shoulder at ∼550 nm. The extinction coefficients are evaluated per mole of dimeric protein. The pattern of absorption features and relative intensities is very similar to those of human ISU, which also contains a [2Fe-2S]²⁺ cluster (*25*). The extinction coefficients suggest two clusters per dimeric protein when compared with the value of known [2Fe-2S]²⁺ proteins (*26*). This conclusion is consistent with the results of iron and protein quantification.

The Mössbauer spectrum of D37A ISU1 (Table 1 and Supporting Information) shows a single iron-containing species with an isomer shift, $\delta = 0.29 \pm 0.05$ mm/s, and quadrupole splitting, $\Delta E_Q = 0.59 \pm 0.05$ mm/s. A minor contribution from adventitiously bound ferric ion is also noted. The aforementioned parameters are consistent with a diferric [2Fe-2S]²⁺ cluster (*27*) and with earlier reports of both ISU proteins and a related Isa protein that we have recently characterized from *S. pombe* (*28*) (Supporting Information). For example, human ISU shows $\delta = 0.29$ mm/s and $\Delta E_Q = 0.59$ mm/s (*25*), while the *A. vinelandii* IscU has two nonequivalent iron signals with $\delta = 0.26$ and 0.32 mm/s and $\Delta E_Q = 0.66$ and 0.94 mm/s, respectively (*18*). The experimental values for holo D37A Isa1 are consistent with a tetrahedral Fe(III) and predominantly sulfur ligation; however, these data cannot exclude the presence of one or two non-cysteinyl (O/N) ligands. The possibility of a rubredoxin or a [3Fe-4S]⁺-type center is precluded by the EPR silence of the protein. Data obtained for the wild-type protein ($\delta = 0.31 \pm 0.05$ mm/s; $\Delta E_Q = 0.64 \pm 0.05$ mm/s) were similar to those obtained for the D37A derivative. This is the first time that Mössbauer data for wild-type and derivative forms of an ISU protein have been directly compared.

The [2Fe-2S] form is the principal product of reconstituted ISU-type proteins that have been characterized thus far. Dean, Johnson, and co-workers have established conditions for both [2Fe-2S] and [4Fe-4S] cluster assembly in *A. vinelandii* IscU (*13*); however, we have optimized conditions for only [2Fe-2S] formation in *S. pombe* ISU1. While no information is yet available on the functional activity of the [4Fe-4S] form of such proteins, the [2Fe-2S] form of *S. pombe* ISU1 is very active in the delivery of the [2Fe-2S] cluster to an apo Fd target, relative to similar reactions with other proteins (*29*). The abundance of [2Fe-2S] clusters in bacterial cells, relative to [4Fe-4S] forms, further supports the idea that the [2Fe-2S] building block is the critical functional form of the ISU family of proteins. Identification of single iron binding sites on *A. vinelandii* NifU (*18*) has been characterized as weak and of no functional relevance (*30*).

Cluster Coordination and Stability. The stability of the bound cluster appears to vary considerably with the source of the ISU and most likely reflects minor variations in the environment of the [2Fe-2S] center according to the primary sequence. Nevertheless, it has commonly been found that substitution of a highly conserved aspartate residue results in stabilization of the bound cluster (*18*, *25*). The ability of the D37A derivative to stabilize the ISU-bound $[2Fe-2S]^{2+}$ cluster is a curious phenomenon with only a few possible explanations. First, the substitution might effect a structural change that serves to decrease the solvent accessibility of the cluster, thereby increasing its stability. Second, the aspartate might engage in internal salt bridge or hydrogen bond formation that serves to destabilize the labile cluster. Third, aspartate 37 might serve as a ligand to the $[2Fe-2S]^{2+}$ in its labile form, with substitution resulting in recruitment of an alternative binding residue. Finally, aspartate 37 might mediate degradation of the ISU-bound cluster by protonation of inorganic sulfide or cysteine thiolate. Several observations that are relevant to a discussion of the factors influencing the stability of ISU-bound cluster are described next.

An important feature of the isolated wild-type and D37A holo *S. pombe* ISU1 proteins is their limited solubility at ambient temperatures, with some limited solubility achievable at 4 °C. This contrasts with the behavior of the human homologue, which is significantly more soluble and therefore a more effective tool for detailed functional studies. For this reason, studies of the pH dependence of cluster stability and cross-linking to Fd were carried out on the homologous human ISU. However, under conditions where cluster stability could be compared for the *S. pombe* and human proteins, then the observed trends were found to be similar, suggesting conserved mechanisms for control of cluster stability and lability in this homologous family of proteins.

Recently, we have reported a detailed study of proton-mediated cluster degradation (*31*). The stabilizing influence of the D37A substitution is consistent with a role for the conserved Asp in proton-mediated cluster degradation (*31*). Given the potential for the conserved aspartate to act as an acid catalyst, we examined the pH dependence of cluster degradation for both human and *S. pombe* ISU in comparison with that of ferredoxin (Fd). For the wild-type forms the clusters are significantly less stable than the D37A derivatives and are immediately degraded below pH 6. As Figure 5 shows, this contrasts markedly with results for the D37A derivatives where we find relatively slow rates of degradation
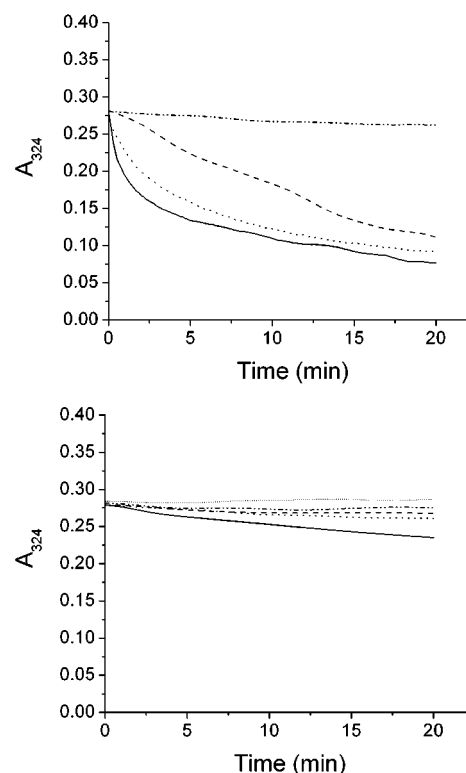

FIGURE 5: Influence of pH on cluster stability for D37A ISU1's determined through the time dependence of cluster absorbance with pH. Spectra were taken in 50 mM buffer (pH 8, Tris-HCl; pH 7, phosphate; pH 6, Bis-Tris; pH 5, acetate; pH 4, formate) and 50 mM NaCl. (Upper) Data for *S. pombe* ISU1 at 4 °C showing data from pH 7 to pH 4. (Lower) Human ISU at 4 °C showing data from pH 8 to pH 4. The cluster is more stable at higher pHs.

at pH 6 and below for both human and *S. pombe* ISU. This behavior is not observed for, and contrasts with, Fd (although the latter does precipitate with retention of cluster at pH 4). As previously discussed by us (*31*), this is consistent both with a role for protonation in mediating cluster degradation and with enhanced solvent accessibility resulting in facile protonation of sulfides. This is not normally observed for ferredoxin-like proteins in this pH range since solvent accessibility to the cluster demands structural perturbations of the protein that only occur at significantly lower pHs. Presumably, the ISU cluster is in fact close to the protein surface and solvent accessible inasmuch as it must be attacked by Cys residues on apoferredoxin and other target apo Fe-S proteins. We have previously demonstrated that solvent accessibility does in fact provide a critical criterion for regulating the stability of oxidized Fe-S clusters toward solvolysis (*31−33*) and is consistent with the considerable stabilizing influence of the D37A substitution. The variation in degradation rates for human and *S. pombe* ISU (Figure 5) is also consistent with minor changes in solvent accessibility for each protein. Finally, we note that the pH dependence of cluster stability that we observe is inconsistent with either internal salt bridge or hydrogen bond formation for Asp 37 or with direct ligation. In both cases the pK_a for the carboxylate would be significantly lowered relative to a free carboxylate, and this is not observed in the data shown in Figure 5. The pH data are more consistent with possibilities 1 and 4, described earlier.

The thermal stability of ISU's also differs markedly from Fd. In particular, the isolated human ISU exhibits the onset

of cluster degradation at temperatures above 40 °C at neutral pH, while Fd is stable up to ~60 °C, at which time cluster loss is triggered, presumably by loss of key structural components. These observations are consistent with a state of conformational flexibility for the ISU proteins and are an additional factor underlying the observed lability of the bound [2Fe-2S] center, a prerequisite for its proposed role as a cluster transfer agent. The temperature dependence of cluster stability does not exclude possibilities 2 and 3 as roles for the conserved aspartate residue (described earlier). However, substitution of a ligating with a nonligating residue does not normally promote cluster stability. Moreover, neither recruitment of an alternative ligand residue from another part of the protein chain nor disruption of an internal salt bridge or hydrogen bond would typically result in a stabilization of protein structure or cluster binding, although neither possibility can be excluded in this case. As noted earlier, however, the pH dependence data are inconsistent with either of these possibilities.

In general, the human ISU displays greater stability over the *S. pombe* homologue, as reflected by the dependence of stability on pH (Figure 5) and temperature; nevertheless, both are labile relative to the [2Fe-2S] cluster in ferredoxin. Also, the clusters in wild-type ISU1 are significantly less stable than those in the D37A derivative forms in terms of pH and temperature dependence. Inasmuch as the cluster is most likely an intermediate that is transferred to other target proteins, the lability of the [2Fe-2S] center is most likely of functional significance. The cluster binding site presumably lies close to the surface and is accessible to nucleophilic attack by Cys residues on the target protein. Accordingly, we observe a set of design features of this class of protein that favor transient capture and release of an iron–sulfur cluster, including the possibility of proton-mediated cluster release coupled to solvent accessibility and conformational flexibility. These features stand in marked contrast to typical structural constraints for other redox and catalytic iron–sulfur proteins (*32*, *33*), and atypical Cys sequence alignments, coordination chemistries, and protein pockets defining cluster environments would be expected.

Chemical Cross-Linking with Ferredoxin. A. vinelandii NifU is a modular protein that consists of three domains. The N-terminal domain shows some similarity to the IscU type proteins and carries a labile cluster, while the central domain carries a [2Fe-2S] ferredoxin cluster center and has a putative role as an electron-transfer mediator between external reductase and the labile cluster center. A ferredoxin protein has also been characterized in the *isc* bacterial operon, and homologous mitochondrial ferredoxins have been identified in yeast and human cell lines. It has been hypothesized that these ferredoxins might complex with their IscU partners, mimicking the intramolecular complex already defined in NifU, and that redox chemistry might play an important role in mediating cluster assembly reactions. Such a hypothesis has not previously been tested, and to this end we sought to characterize complex formation between ISU1 and yeast/human Fd proteins using standard conditions.

Both the yeast and highly homologous human Fd's were found to form cross-linked complexes with *S. pombe* ISU1. The human homologue did in fact provide better defined bands on SDS–PAGE gels (Figure 6), with *S. pombe* Fd tending to be streaky. Cross-linking resulted in the appear-
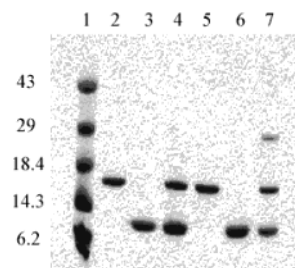

FIGURE 6: ISU1 EDC cross-linking with human ferredoxin. A 40 μM sample of each protein in 25 mM Hepes, pH 7.4, and 50 mM NaCl was incubated at room temperature for 2 h with or without 5 mM EDC. Lanes: 1, low molecular weight marker; 2, ISU1; 3, ferredoxin; 4, ISU1 + ferredoxin; 5, ISU1 + EDC; 6, ferredoxin + EDC; 7, ISU1 + ferredoxin + EDC. Masses of MW markers (in kDa) are indicated on the left.

ance of a new high molecular weight species that ran slightly faster than the 29 kDa marker (Figure 6), in agreement with the predicted MW of ~27 kDa for the ISU1·Fd cross-linked species. Incubation of either ISU1 or human Fd alone did not give rise to any cross-linked products, suggesting that the observed product corresponds to an ISU1·Fd species. Since EDC is a specific, zero-length cross-linker that forms amide bonds between Lys and Asp/Glu side chains, the appearance of a cross-linked product is good initial evidence for a well-defined interaction between these two Fe-S proteins that is substantially brought about by a complementarity of acidic and basic residues. Formation of the cross-linked product is unlikely to arise from nonspecific interactions since cross-linking is observed also at significantly lower concentrations, while ISU1 and Fd show no self-cross-linking from nonspecific self-binding at these concentrations. It is of further interest and likely significant that we find holo ISU1 also able to form a complex with apo Fd, while apo ISU1 complexes with neither holo nor apo Fd. While the absence of cross-linking does not prove that these proteins do not interact, these data are consistent with an Fe-S transfer pathway from holo ISU1 to apoferredoxin. We have in fact demonstrated direct cluster transfer between these two proteins (described next). These cross-linking data also provide support for holo ISU1 and holo ferredoxin as potential redox partners. In this regard, the stable [2Fe-2S]$^{2+}$ in the central domain of the *A. vinelandii* NifU has been proposed to provide reducing equivalents to labilize the transient [2Fe-2S] cluster during transfer of Fe-S equivalents from NifU to its target apoprotein. A similar suggestion can be made for the yeast ISU1·Fd complex, with reduced Fd serving to reduce the [2Fe-2S]$^{2+}$ cluster on ISU1, thereby facilitating degradation of the cluster and release of iron and sulfide equivalents. However, under conditions where reduced Fd is generated during cluster transfer reactions (by addition of NADPH/Fd reductase), we find no change in the observed rate of cluster transfer. Perhaps the physiological relevance of the holo ISU1·holo Fd complex serves rather to remove electrons from the nascent ISU1 cluster, thereby stabilizing the reductively labile reduced state of the ISU-bound [2Fe-2S] center. Finally, it is noteworthy that the binding of ferredoxin by ISU1- and Isa-type proteins is consistent with the sequence of basic residues found in the latter two (*25*), which possibly interact with acidic residues on Fd that have been previously implicated in complex formation with other cellular proteins (*34*). It is
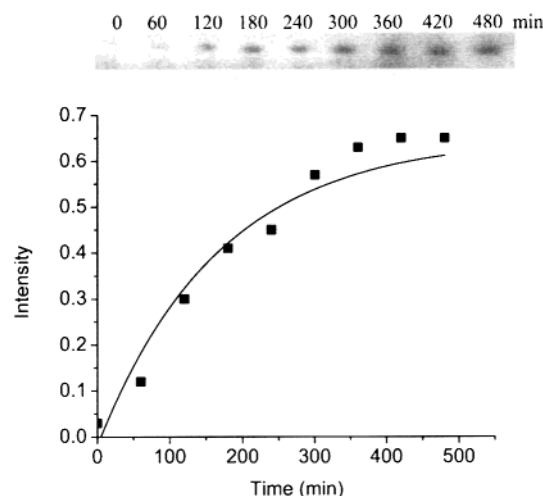
FIGURE 7: (Top) The $[2Fe-2S]^{2+}$ cluster transfer from *S. pombe* holo D37A ISU1 to *S. pombe* apo Fd, showing the formation of holo Fd, is followed by native PAGE and iron staining. Overall concentrations of apo Fd and ISU1 were 16 and 327 μM, respectively. (Bottom) A plot of the time dependence of the intensity of the band, corresponding to holo Fd, from a scanned iron-stained gel was fitted to a rate equation for a first-order decay process. The apparent rate constant (k_{obs}) for cluster transfer is 0.006 min^{-1}.

Table 2: Observed (k_{obs}) and Determined Second-Order Rate Constants (k_2) for Fe-S Cluster Transfer

ISU/Fd complex	k_{obs} (min^{-1}) (temp, °C)	k_2 (M^{-1} min^{-1}) (temp, °C)
S. pombe WT ISU1	0.03 (4)	80 $\pm$ 5 (4)
S. pombe D37A ISU1	0.006 (4)	18 $\pm$ 2 (4)
Synechocystis	0.07 (30)[a]	—[b]
E. coli IscA/apo Fd	0.008 (25)[a]	—[b]

[a] Estimated from published data (*16, 29*), but lack of information on concentrations precludes a detailed comparison. [b] Not determined.

important to note, however, that these data do not exclude the possibility of another physiological target for ISU, since many other iron−sulfur proteins are presumably targets for cluster insertion reactions.

[2Fe-2S]$^{2+}$ Cluster Transfer from ISU1 to Fd. Reconstitution of apo Fd from holo ISU1 was easily followed by native polyacrylamide gel electrophoresis. Since ISU-type proteins are basic (p$I \sim 8-9$) and Fd's are acidic (p$I \sim 4-5$), they are readily separable by native polyacrylamide gel electrophoresis. Holo and apo ISU1 proteins remain in the loading lane, while apo and holo Fd are well resolved following Coomassie staining. Holo Fd (Figure 7) and holo ISU1 can also be distinguished from the apoprotein by use of an established iron staining technique (*29*). Product holo Fd was readily isolated following chromatographic separation. The yield of holoprotein following addition of wild-type holo ISU1 to apo Fd (better than 90%) was significantly greater than that obtained by addition of D37A ISU1 ($\sim$70%) and may be ascribed to the more rapid reaction of the former that precluded significant disulfide bond formation on apo Fd. Control experiments carried out in the absence of ISU1, but with addition of an equivalent concentration of iron and sulfide, yielded negligible yields of holo Fd under conditions analogous to those used for ISU1-mediated reconstitution.

Experiments to determine the reaction rate constants for cluster transfer show that the rate of $[2Fe-2S]^{2+}$ cluster transfer from wild-type holo ISU1 is greater than that of the D37A derivative, although the former is too rapid to accurately determine under the concentration conditions required for gel analysis. As a result of the poor solubility of *S. pombe* ISU1 at ambient temperatures, the rate constants for cluster transfer to a target Fd were determined at 4 °C. Under the reaction conditions used (see legend to Figure 7), a reaction rate constant $k_{obs} \sim 18$ M^{-1} min^{-1} was determined for the D37A ISU1 cluster transfer, and a lower limit of 80 M^{-1} min^{-1} was assigned for WT (Table 2). The significant difference in the measured rate constants (k_2) of wild-type ISU1 and D37A ISU1 provides evidence that the residue,

aspartate 37, plays an important role in the process of Fe-S cluster transfer. The variation in the pH dependence of cluster stability for human and yeast ISU proteins (Figure 5) suggests that protonation of the cluster is most likely not a role for Asp37 in mediating cluster transfer. Protonation, in fact, tends to cause Fe-S clusters to degrade, which would not be favored for cluster transfer in this instance. We have previously reported on the protein-mediated cluster degradation pathways for Fe-S centers (*31*). However, it is possible that the carboxylate of Asp37 may function as a base catalyst for proton removal from the attacking Cys nucleophiles from the apo Fd target.

ISU domains are among the most highly conserved in nature, spanning all three kingdoms of life. They appear to provide a platform for assembly of $[2Fe-2S]^{2+}$ clusters prior to transfer to target apoproteins, and so a detailed understanding of cluster transfer from one organism is likely to be of general value. Previously, Nishio and Nakai have reported [2Fe-2S] cluster transfer from *Synechocystis* NifU to an apo Fd target (*29*). This is distinct from the reaction chemistry reported herein, insofar as the NifU fragment studied by these workers corresponded to the C-terminal domain of NifU, which is not a member of the ISU family of proteins and is nonhomologous to such. In fact, yeast and human cells carry a discrete protein that is homologous to this domain. Nevertheless, it is of interest that apo Fd can be reconstituted by a distinct family of proteins. In fact, yet another class of proteins has also been demonstrated to reconstitute apo Fd. Takahashi, Fontecave, et al. have demonstrated that *E. coli* IscA can reconstitute apo Fd (*16*). Similar to ISU-type proteins, the *Synechocystis* NifU C-terminal domain and *E. coli* IscA proteins also carry [2Fe-2S] cluster centers. While evidence in support of direct cluster transfer has not, as yet, been presented for these latter two proteins, nevertheless they do support the idea of [2Fe-2S] building blocks as the fundamental unit in all such reactions and as the critical functional state of the ISU family of proteins. A comparison of available cluster transfer data is summarized in Table 2 and clearly shows that the rates of cluster transfer that we determine for *S. pombe* ISU1 to a target Fd are significant, relative to other published data. However, these data also underscore the importance of defining reaction conditions to allow a faithful comparison of kinetic data from various laboratories working in this area. As the focus of study increasingly moves from definition and characterization of cofactor content to the understanding of cluster transfer mechanism, the accurate reporting of kinetic data will provide for informative comparisons across species and protein platforms. It will also provide a foundation for examining the influence of chaperone proteins on cluster transfer rates (*15, 35*).

Wu et al.

SUPPORTING INFORMATION AVAILABLE

One figure showing Mössbauer spectra of holo wild-type ISU1, D37A ISU1, and Isa. This material is available free of charge via the Internet at http://pubs.acs.org.

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