

# Iron–Sulfur Cluster Biosynthesis. Kinetic Analysis of [2Fe-2S] Cluster Transfer from Holo ISU to Apo Fd: Role of Redox Chemistry and a Conserved Aspartate<sup>†</sup>

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**ABSTRACT:** ISU-type proteins mediate cluster transfer to apo protein targets. Rate constants have been determined for cluster transfer from ISU to apo Fd for both *Homo sapiens* and *Schizosaccharomyces pombe* proteins, and cross reactions have also been examined. Substitution of a key aspartate residue of ISU is found to decrease the rate of cluster transfer by at least an order of magnitude (for wild-type *Hs* ISU cluster transfer to *Hs* apo Fd,  $k_2 \sim 540 \text{ M}^{-1} \text{ min}^{-1}$ , relative  $56 \text{ M}^{-1} \text{ min}^{-1}$  for D37A ISU). This change in rate constant does not reflect any change in binding affinity of the ISU and Fd proteins. The pH dependencies of cluster transfer rates are similar for WT and D37A ISU, arguing against a role for Asp37 as a catalytic base, although evidence for general base catalysis mediating deprotonation of Cys from the apo target is supported by an observed  $\text{pK}_a$  of 6.9 determined from the pH profiles for both WT and D37A ISU. Such a  $\text{pK}_a$  value is at the lower limit for Cys and is common for solvent-accessible Cys thiols. The temperature dependence of the rate constant defining the cluster transfer reaction for wild type versus the aspartate derivative is distinct. Thermal activation parameters ( $\Delta H^*$  and  $\Delta S^*$ ) are consistent with a solvent-accessible ISU-bound cluster, with desolvation as a principle barrier to cluster transfer. Experiments to determine the dependence of reaction rate constants on viscosity indicate cluster transfer to be rate-limiting. Fully oxidized cluster appears to be the natural state for transfer to target proteins. Reduced Fd does not readily reduce ISU-bound  $[2\text{Fe-2S}]^{2+}$  and does not promote cluster transfer to an apo Fd target.

Proteins that deliver discrete metal ions to target proteins [including copper, nickel, and manganese (1–3)] constitute a growing family of metal transporters. However, many proteins and enzymes contain complex metal cofactors that must themselves be the product of a biosynthetic assembly apparatus. Recent advances in this area include the characterization of operons that define gene products responsible for the biosynthesis of nitrogenase cofactors (4–7), heme (8), and iron–sulfur clusters (9–12). For example, key proteins implicated in Fe–S cluster assembly have been identified in bacterial *isc* operons (11, 13, 14). These include sulfur donor proteins, chaperones, electron transport proteins, and transient carriers of intermediate Fe–S cluster building blocks. The latter include two families of proteins (IscU or ISU, and IscA or ISA), each of which has been demonstrated to carry a  $[2\text{Fe-2S}]$  cluster (15–17). While the ability of certain ISU-type proteins to carry  $[4\text{Fe-4S}]$  centers has been demonstrated (16), the generality of this observation and the ability of such centers to be transferred to a target remain to be established. Homologous proteins to *isc* gene products

have been identified in the *nif* operon for assembly of Fe–S centers of nitrogenase (11, 17), and also in eukaryotes (9, 10, 18–21). For the latter cell types, proteins involved in assembly of Fe–S centers are located in the mitochondrion.

Our goals in this field have focused on understanding the biosynthetic apparatus for assembly of the 2Fe and 4Fe iron–sulfur cluster centers in two eukaryotic organisms, specifically *Homo sapiens* (22) and the yeast *Schizosaccharomyces pombe* (23). Assembly of such clusters may occur exclusively in the mitochondrion (9, 10, 18, 19) with relevant proteins imported following ribosomal synthesis from nuclear transcribed mRNA. However, the possibility of cytosolic assembly pathways has also been noted (21). Herein our attention is focused on understanding the functional chemistry of a class of proteins belonging to the ISU family. Recent reports have focused on the characterization of the holo state of the ISU-type proteins (16, 22, 24, 25); however, the mechanisms of cluster transfer have barely been addressed. In this paper, we establish kinetic methodologies for assaying cluster transfer chemistry. Temperature, pH, and viscosity dependency measurements allow a basic kinetic and mechanistic framework to be established, and the role of redox chemistry in mediating cluster transfer is also addressed. A comparison of the reactivity of a substituted protein relative to the WT<sup>1</sup> form provides insight on the functional role of a highly conserved aspartate residue. Prior investigations of the IscU domain of *A. vinelandii* NifU had led to the finding that this substitution stabilized the bound

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[2Fe-2S] cluster (17, 22) and led us to consider the possible role of this residue in mediating cluster transfer chemistry.

## 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. Other inorganic salts were obtained from Aldrich (Milwaukee, WI). Ni-NTA resin was purchased from QIAGEN (Valencia, CA). CM-32 and DE-52 were from Whatman (Aston, PA). Homogenous-20 precast polyacrylamide gels, G-75, and Superose-12 resin were from Pharmacia (Uppsala, Sweden). NADPH and cytochrome *c* were purchased from Sigma (St. Louis, MO).

**Purification and Reconstitution of Proteins.** Cloning and expression of *Hs* ISU and *Sp* ISU1 proteins and derivatives have been described previously by us (22, 25). Each protein contains one [2Fe-2S]<sup>2+</sup> center, consistent with Fe quantitation and optical spectra obtained for these and related family members (22, 25). *Sp* and *Hs* ISU proteins were isolated and reconstituted as follows. 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 stirred on ice for 1 h. The cellular debris was removed by centrifugation at 15 000 rpm and 4 °C for 15 min, and the supernatant was loaded onto a Ni<sup>2+</sup>-chelation column (Qiagen) previously equilibrated with binding buffer and 6 M urea. After loading, 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, 3 M urea, and reconstituted by addition of 50 mM DTT, 1.0 mM Fe<sup>3+</sup>, 1.0 mM S<sup>2-</sup> under rigorously anaerobic conditions. After 30 min, the refolded holo protein 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 was eluted with buffer A + 150 mM NaCl. Fractions containing holo protein (as judged by UV-vis absorbance) were pooled and concentrated by ultrafiltration. CM32-purified D37A ISU (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<sub>278</sub>/A<sub>324</sub> ratio of <1.1 were pooled, concentrated, and stored at –80 °C. Protein purity at each stage of purification was monitored by SDS-PAGE.

Wild-type and D37A *Hs* ISU were prepared in an analogous fashion. Expression vectors for other proteins were obtained as follows, and proteins were prepared following literature procedures: an expression plasmid for *Hs* Fd (26) was kindly provided by J. Markley (cloning and expression of the yeast homologue has been achieved in this laboratory and details will be reported elsewhere); and an expression plasmid for a bovine NADPH Fd reductase (27) was kindly provided by G. Schulz.

**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 0.5 cm path-length cuvette was used for measurements, which were recorded anaerobically at room temperature. Throughout we define the concentrations of holo Fd and holo ISU proteins in terms of cluster concentration.

**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. (28). 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 incubating 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<sub>3</sub> solutions.

**Demonstration of Direct Cluster Transfer from Holo ISU to Apo Fd by Mössbauer Spectroscopy.** *Hs* D37A ISU was reconstituted with <sup>57</sup>Fe to form [2<sup>57</sup>Fe-2S] holo protein. An <sup>57</sup>Fe<sup>3+</sup> solution was prepared by dissolving <sup>57</sup>Fe metal 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<sup>3+</sup> with 50 mM Tris-HCl buffer (pH 7.5) before use. The cluster transfer reaction was carried out in six Eppendorf tubes, each containing the same solution composition, in Tris-HCl (50 mM), pH 7.5 at 4 °C. In each tube, 0.5 mL of apo Fd (500 µM) was incubated with DTT (2.3 mg) for 1 h, followed by addition of 1.0 mL of <sup>57</sup>Fe-labeled holo *Hs* ISU (50 µM). A 10-fold excess (10 mM, 200 µL) of regular Fe<sup>2+</sup> (FeSO<sub>4</sub>·7H<sub>2</sub>O) was added to each reaction solution, and the final concentrations of each species were: [apo Fd] = 147 µM, [DTT] = 8.8 mM, [<sup>57</sup>Fe in the Fe–S cluster] = 116 µM, and [Fe<sup>2+</sup> in the solution] = 1.17 mM. After 24 h, the mixture was loaded onto a DE52 column, which was washed with 3 volumes of buffer (50 mM Tris-HCl, 50 mM NaCl) before eluting the holo Fd with 50 mM Tris-HCl, 150 mM NaCl. The colored fraction was collected in a volume of 4.0 mL of elution buffer, and the concentration determined by UV-vis was 84.5 µM.

The concentration of <sup>57</sup>Fe-labeled [2Fe-2S] was also measured by Mössbauer spectroscopy, and the concentrations measured by each method were similar to within 3%. Mössbauer characterization of the holo Fd resulting from

<sup>1</sup> Abbreviations: CBB, Coomassie brilliant blue; cyt *c*, cytochrome *c*; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Fd, ferredoxin; FPLC, fast protein liquid chromatography; IPTG, isopropyl thiogalactoside; LB medium, Luria-Bertani medium; NADPH, nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UV-vis, ultraviolet-visible; WT, wild type.

cluster transfer from ISU was carried out on 500  $\mu\text{L}$  of concentrated protein solution and frozen at  $-80^\circ\text{C}$ . 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  $^{57}\text{Co}$  source in a rhodium foil purchased from Isotope Products Laboratory. All isomer shifts are quoted relative to iron metal at room temperature.

Concentrations of components in Mössbauer spectra were estimated by comparison as follows. A standard spectrum was recorded at 4.2 K of a sample such as iron(II) chloride that consists of a single quadrupole doublet. The intensity and line width were determined and correlated to the amount of  $^{57}\text{Fe}$  present in the sample. For other samples, the intensity and line widths of each quadrupole doublet species were determined and compared to the standard to estimate the  $^{57}\text{Fe}$  concentration. This process does assume that the iron species being compared have approximately the same recoilless fraction, and thus far there has been no reported case of an iron species in a protein with a significantly different recoilless fraction at 4.2 K.

**Determination of Reaction Rate Constants for [2Fe-2S] Cluster Transfer from Holo ISU to Apo Fd by Native PAGE.** Native PAGE analyses of Fe-S cluster transfer from holo ISU to apo Fd were performed as follows. Apo Fd was incubated with 4.5 mM DTT for 30 min, followed by addition of holo ISU. At intervals of 10 min (wild-type ISU) or 60 min (D37A ISU), an aliquot of the reaction mixture was withdrawn and frozen for later assay by native-PAGE. Reaction aliquots were thawed and immediately loaded on the gel. The short time between thawing, loading, and running the gel and dilution with gel loading buffer eliminates the possibility of significant cluster transfer chemistry occurring before the gel was run. A greater than 10-fold excess of holo ISU to apo Fd was used in cluster transfer reactions, with overall concentrations of apo Fd and Hs ISU of 16 and 167  $\mu\text{M}$  (327  $\mu\text{M}$  for *Sp* ISU1) in 18  $\mu\text{L}$ , respectively. Following Fe-S cluster transfer from holo ISU to apo Fd, the concentration of holo Fd was observed to increase, as determined by both Coomassie and iron staining (29), following quantitative evaluation of band intensity by use of a BIO-RAD gel doc 1000. Figure 1 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 ( $k_{\text{obs}}$ ) for Fe-S cluster transfer from holo ISU to apo Fd were obtained, and  $k_2$  was 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.

**Calibration of Hs Fd and Sp Fd Concentrations for Use in a Cytochrome *c* Assay.** In the pre-steady-state, the velocity of reduction of cytochrome *c* via a NADPH/Fd-reductase/Fd reduction pathway is related to the concentration of holo Fd. Reaction mixtures contained 80  $\mu\text{M}$  cytochrome *c* and 200 nM Fd reductase in 1 mL of potassium phosphate buffer (10 mM, pH 7.5). Reaction was initiated by addition of holo Fd and NADPH (400  $\mu\text{M}$ ) under anaerobic conditions and monitored by measuring the increase in absorbance at 550 nm from reduced cytochrome *c*. By varying the concentration of holo Fd (5–100 nM), calibration curves for reaction

velocity (OD/30 s) versus concentration of Fd were constructed.

**Determination of Reaction Rate Constants for [2Fe-2S] Cluster Transfer from Holo ISU to Apo Fd by a Cytochrome *c* Assay.** Apo Fd (200  $\mu\text{M}$ , 20  $\mu\text{L}$ ) was incubated with DTT (50 mM, 20  $\mu\text{L}$ ) for 30 min. Subsequently, 7  $\mu\text{L}$  of this mixture was added to 35  $\mu\text{L}$  of 200  $\mu\text{M}$  holo Hs ISU or 400  $\mu\text{M}$  holo *Sp* ISU1. At 10 min intervals (for WT ISU) or 60 min intervals (for D37A ISU), 6  $\mu\text{L}$  of this mixture was withdrawn for evaluation of holo Fd formation by the cytochrome *c* assay. To eliminate the possibility of significant cluster transfer chemistry occurring in this transition phase, the sample was immediately diluted and assayed. The reaction mixture contained 80  $\mu\text{M}$  cytochrome *c* and 200 nM Fd reductase in 1 mL of potassium phosphate buffer (10 mM, pH 7.5). The assay was initiated by addition of 6  $\mu\text{L}$  of holo ISU-apo Fd mixture and NADPH (400  $\mu\text{M}$ ) under anaerobic conditions. The final concentrations of Fd and Hs ISU were 0.1 and 1  $\mu\text{M}$  (or 2  $\mu\text{M}$  for *Sp* ISU1), respectively. The reduction of cyt *c* was monitored by measuring the increase in absorbance at 550 nm. The pre-steady-state velocity (OD/30 s) was measured and used to determine the formation of reconstituted Fd according to the previously constructed calibration curves. The resulting plot of the yield of reconstituted Fd versus time was fitted to a first-order decay curve, and the observed rate constant ( $k_{\text{obs}}$ ) was determined. The second-order rate constant  $k_2$  was determined from eq 1 (30):

$$k_{\text{obs}} = k_2[\text{ISU-bound cluster}] \quad (1)$$

Reactions with Hs proteins were examined at  $25^\circ\text{C}$ , while reactions of *Sp* proteins were monitored at  $4^\circ\text{C}$  as a result of the poor solubility of *Sp* ISU1 at the higher temperature. Kinetic studies of cross reactions of Fe-S cluster transfer between proteins from each species (*Hs-Sp*) were also examined. As a control, the activity levels for apo Fd, apo ISU, and holo ISU were separately analyzed, and all showed no activity. Furthermore, to correct for the influence of DTT, which can reduce cytochrome *c* at a low rate compared to NADPH/Fd-reductase/Fd reduction, the reaction mixture without NADPH and Fd reductase was used to determine the velocity of reduction of cytochrome *c* by DTT.

**Dependence of  $k_2$  on Temperature.** The temperature dependence of  $k_2$  on the process of Fe-S cluster transfer from holo Hs ISU (or D37A) to apo Hs Fd was examined at several temperatures (25, 20, 15, 10, and  $4^\circ\text{C}$ ) by cytochrome *c* assay. The process was the same as the procedures described in the previous section. The plot ( $\ln k_2$  versus  $1/T$ ) was fitted to a standard Eyring equation (eq 2) [where all parameters have their usual meanings (31)], and the activation enthalpy ( $\Delta H^*$ ) and entropy ( $\Delta S^*$ ) for cluster transfer were determined:

$$k_2 = (kT/h) \exp(-\Delta G^*/RT) = (kT/h) [\exp(-\Delta H^*/RT) \exp(-\Delta S^*/R)] \quad (2)$$

**Dependence of  $k_2$  on pH for Cluster Transfer from WT and D37A ISU.** The pH dependence of  $k_2$  of the WT and D37A Hs ISU was determined by performing the cytochrome *c* assay described above. The reaction temperature was  $25^\circ\text{C}$ , and the buffers (50 mM) were MES (pH 5.5–7.0) and

Tris-HCl (pH 7.0–9.0). The variation of  $k_2$  with pH was fit to eq 3 to yield the ionization constant ( $pK_a$ ) and the limiting rate constants at low and high pH,  $k_2(\text{HA})$  and  $k_2(\text{A})$ , respectively:

$$k_2 = \{k_2(\text{HA})10^{-\text{pH}} + k_2(\text{A})10^{-\text{p}K_a}\}/(10^{-\text{pH}} + 10^{-\text{p}K_a}) \quad (3)$$

**Dependence of  $k_2$  on Viscosity.** Kinetic studies of Fe–S cluster transfer from holo *Hs* ISU to apo *Hs* Fd in the presence of varying amounts of viscogen [0, 25%, and 40% (w/v) sucrose] were examined. The reaction temperature was 25 °C, and the buffer was 50 mM Tris-HCl (pH 7.5).

**NADPH/Fd Reductase-Mediated Fe–S Cluster Transfer.** To examine the role of Fd-mediated ISU reduction in promoting cluster transfer, catalytic amounts of Fd reductase and NADPH were added to the reaction mixture for Fe–S cluster transfer from holo ISU to apo Fd. The reaction mixture contained holo *Hs* ISU (167  $\mu\text{M}$ ), apo *Hs* Fd (16.7  $\mu\text{M}$ ), Fd reductase (48 nM), and NADPH (120  $\mu\text{M}$ ). Additionally, the reaction was carried out with a molar equivalent of added Fd in addition to apo Fd. This reaction mixture contained holo *Hs* ISU (167  $\mu\text{M}$ ), apo *Hs* Fd (8.35  $\mu\text{M}$ ), holo *Hs* Fd (8.35  $\mu\text{M}$ ), Fd reductase (0.48  $\mu\text{M}$ ), and NADPH (120  $\mu\text{M}$ ). Both reactions were assayed in a similar fashion. After 10 min, equivalent volumes (6  $\mu\text{L}$ ) were taken from each solution for evaluation by use of the cytochrome *c* assay (1 mL of assay solution) at 25 °C. A control experiment was conducted without NADPH and Fd reductase.

## RESULTS

**Demonstration of Direct  $[2\text{Fe-2S}]^{2+}$  Cluster Transfer by Mössbauer Spectroscopy.** Direct  $[2\text{Fe-2S}]^{2+}$  cluster transfer from ISU to apo Fd was demonstrated by use of Mössbauer spectroscopy. As a result of the enhanced stability of D37A ISU, relative to WT, the former was used in these experiments. The  $[2\text{Fe-2S}]$  cluster of holo *Hs* ISU (D37A) was labeled with  $^{57}\text{Fe}$  under standard reconstitution conditions. The labeled *Hs* D37A ISU was mixed with 1 equiv of apo Fd in the presence of a 10-fold excess of regular ferrous ion, which was added to the reaction mixture for cluster transfer. Quantitation of the  $^{57}\text{Fe}$  content in the reconstituted Fd by analysis of the Mössbauer peak intensity, and independent determination of the concentration of holo Fd from the electronic absorbance of the cluster, revealed that only  $^{57}\text{Fe}$  was loaded into the apo Fd. Cluster transfer yield was estimated as 57% based on the conversion of holo ISU to apo Fd. The efficiency of transfer, based on the amount of  $^{57}\text{Fe}$  transferred directly from ISU to apo Fd in the presence of background iron, was greater than 97%.

**Cluster Transfer Yields.** WT *Hs* ISU and D37A *Hs* ISU were used to compare the efficiency of Fe–S cluster transfer to apo *Hs* Fd. The yield of holo Fd following addition of WT holo *Hs* ISU was significantly greater than that obtained by addition of D37A *Hs* ISU. Product holo Fd was also readily isolated following chromatographic separation, and the yield of cluster transferred was determined to be 69% for D37A *Hs* ISU and greater than 90% for *Hs* ISU based on quantitation of holo Fd by absorption spectroscopy. Control experiments carried out in the absence of ISU, but with addition of an equivalent concentration of iron and

sulfide, yielded negligible yields of holo Fd under analogous conditions to those used for ISU-mediated reconstitution.

**Native PAGE Assay.** Since *Hs* ISU and *Sp* ISU1 are basic proteins ( $pI \sim 8\text{--}9$ ) and Fd is an acidic protein ( $pI \sim 4\text{--}5$ ), ISU and Fd are readily separable by native polyacrylamide gel electrophoresis. Holo and apo ISU proteins remain in the loading lane, and apo and holo Fd are well resolved following Coomassie staining. Holo Fd and holo ISU can also be distinguished from apo protein by use of established iron staining techniques (Figure 1). Under conditions where cluster transfer from ISU to apo ferredoxin is slow ( $t_{1/2} > 1$  h), nondenaturing gel electrophoresis can be applied to kinetic studies of the reconstitution of apo Fd from holo ISU (Figure 1) by monitoring the formation of holo Fd from quantitation of band intensity. An excess of at least 10-fold in  $[2\text{Fe-2S}]$  cluster of ISU was used relative to apo Fd to ensure pseudo-first-order kinetics. Consequently, second-order rate constants ( $k_2$ ) were determined from  $k_{\text{obs}}$  by use of eq 1. Experiments to determine the reaction rate constants for cluster transfer show that the rate of  $[2\text{Fe-2S}]^{2+}$  cluster transfer from wild-type holo ISU 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, and so other assay methods (described next) were used. 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. Native PAGE proved useful for kinetic studies of the relatively slow rate of Fe–S cluster transfer from D37A ISU to apo Fd, and was especially valuable for *S. pombe* reactions, which tended to be significantly slower than the human equivalent. Under the reaction conditions used (see legend to Figure 1), a reaction rate constant  $k_2 \sim 18 \text{ M}^{-1} \text{ min}^{-1}$  was determined for *Sp* ISU1 (D37A) cluster transfer. Other data are summarized in Table 1. It is important to note that when the concentration of apo Fd was significantly increased or decreased (while maintaining pseudo-first-order conditions), there was essentially no change in  $k_2$ , consistent with a saturation of available apo Fd. That is, variations in  $k_2$  values reported in Table 1 reflect variations in reactivity within the ISU–apo Fd complex rather than variations in the binding affinity of ISU for the target protein.

**Cytochrome *c* Assay.** Preliminary kinetic studies showed that the rate of Fe–S cluster transfer from WT holo *Hs* ISU to apo *Hs* Fd was too fast to be monitored by nondenaturing PAGE. The concentration of protein required for either iron or Coomassie staining methods to provide reliable quantitation of bands for determination of rate constants (such as in Figure 1) resulted in too rapid a reaction, and so other possibilities were explored. A cytochrome *c* assay is commonly used to measure the activity of Fd and Fd reductase. In turn, Fd functions as an electron carrier for reduction of cytochrome *c* via a NADPH/Fd reductase electron donor. The velocity of reduction of cytochrome *c* is related to the concentration of Fd if the concentrations of NADPH, Fd reductase, and cytochrome *c* are held constant over a series of comparative reactions. To provide an additional assay mechanism to Fe–S cluster transfer from holo ISU to apo Fd at lower concentrations of reactant proteins, we have modified the cytochrome *c* assay to allow quantitation of the concentration of reconstituted Fd. The reaction mixture contained 80  $\mu\text{M}$  cytochrome *c* and 200 nM Fd reductase in

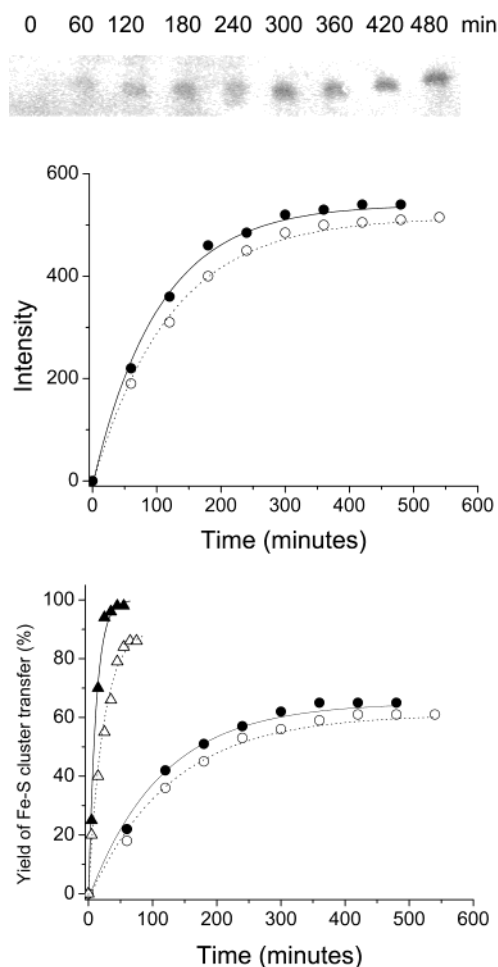


FIGURE 1: (Top)  $[2\text{Fe-2S}]^{2+}$  cluster transfer from *Hs* holo D37A ISU to *Hs* apo Fd at 25 °C, showing the formation of holo Fd, is followed by native-PAGE and CBB staining. Overall concentrations of apo Fd and ISU were 16 and 167  $\mu\text{M}$ , respectively. (Middle) Plot of the intensity of holo Fd as a function of reaction time was fitted to a rate equation for a first-order decay process (25 °C, solid circles and solid line; 4 °C, empty circles and dotted line). The apparent rate constants ( $k_{\text{obs}}$ ) for cluster transfer are 0.012  $\text{min}^{-1}$  (25 °C) and 0.010  $\text{min}^{-1}$  (4 °C). (Bottom)  $[2\text{Fe-2S}]^{2+}$  cluster transfer from *Hs* holo D37A ISU to *Hs* apo Fd was monitored by the cytochrome *c* assay. Overall concentrations of apo Fd and ISU were 16 and 167  $\mu\text{M}$ , respectively. A plot of the yield (%) of holo Fd as a function of reaction time was fitted to a rate equation for a first-order decay process (25 °C, solid circles and solid line; 4 °C, empty circles and dotted line). The apparent rate constants ( $k_{\text{obs}}$ ) for cluster transfer are 0.009  $\text{min}^{-1}$  (25 °C) and 0.008  $\text{min}^{-1}$  (4 °C). Fe-S cluster transfer from WT holo *Hs* ISU to apo *Hs* Fd was monitored by the cytochrome *c* reduction assay at 25 °C (solid triangles, solid line) and 4 °C (empty triangles, dotted line). Overall concentrations of apo Fd and ISU were 16 and 167  $\mu\text{M}$ , respectively. A plot of the yield (%) of holo Fd as a function of reaction time was fitted to a rate equation for a first-order decay process. The apparent rate constants ( $k_{\text{obs}}$ ) for cluster transfer are 0.09  $\text{min}^{-1}$  (25 °C) and 0.04  $\text{min}^{-1}$  (4 °C).

1 mL of potassium phosphate (pH 7.5, 10 mM). The reaction was initiated by addition of holo Fd and NADPH (400  $\mu\text{M}$ ) under anaerobic conditions and monitored by measuring the increase in absorbance at 550 nm from reduced cyt *c*. By varying the concentration of holo Fd, a calibration curve (velocity versus concentration of Fd) was constructed. The velocity is determined by measuring the difference of absorbance within the first 30 s and is reported in units of OD/30 s. While bovine Fd reductase (27) has been used in

Table 1: Observed ( $k_{\text{obs}}$ ) and Determined Second-Order Rate Constants ( $k_2$ ) for Fe-S Cluster Transfer<sup>a</sup>

ISU/Fd complex <sup>b</sup>	$k_{\text{obs}}$ ( $\text{min}^{-1}$ )	$k_2$ ( $\text{M}^{-1} \text{min}^{-1}$ ) (temp, °C)	assay method
<i>Hs</i> ISU/ <i>Hs</i> Fd	0.09 (25 °C)	540 ± 23 (25)	cyt <i>c</i>
	0.04 (4 °C)	238 ± 18 (4)	cyt <i>c</i>
<i>Sp</i> ISU1/ <i>Hs</i> Fd	0.08 (4 °C)	237 ± 19 (4)	cyt <i>c</i>
<i>Hs</i> ISU/ <i>Sp</i> Fd	0.05 (25 °C)	294 ± 21 (25)	cyt <i>c</i>
	0.016 (4 °C)	94 ± 6 (4)	cyt <i>c</i>
<i>Sp</i> ISU1/ <i>Sp</i> Fd	0.03 (4 °C)	80 ± 5 (4)	cyt <i>c</i>
<i>Hs</i> ISU (D37A)/ <i>Hs</i> Fd	0.009 (25 °C)	56 ± 5 (25)	cyt <i>c</i>
	0.008 (4 °C)	45 ± 4 (4)	cyt <i>c</i>
<i>Sp</i> ISU1 (D37A)/ <i>Sp</i> Fd	0.005 (4 °C)	15 ± 2 (4)	cyt <i>c</i>
<i>Hs</i> ISU (D37A)/ <i>Hs</i> Fd	0.012 (25 °C)	74 ± 9 (25)	native PAGE
	0.010 (4 °C)	62 ± 6 (4)	native PAGE
<i>Sp</i> ISU1 (D37A)/ <i>Sp</i> Fd	0.006 (4 °C)	18 ± 2 (4)	native PAGE

<sup>a</sup> The concentrations of ISU-bound  $[2\text{Fe-2S}]$  cluster used are: [*Hs* ISU] = [*Hs* ISU (D37A)] = 167  $\mu\text{M}$  and [*Sp* ISU1] = [*Sp* ISU1 (D37A)] = 167  $\mu\text{M}$ . The concentrations of apo Fd were 16  $\mu\text{M}$ . <sup>b</sup> Two other  $k_{\text{obs}}$  values can be estimated from literature data, although lack of information on concentrations precludes a detailed comparison and calculation of  $k_2$ . These include  $k_{\text{obs}} \sim 0.07$  at 30 °C for a *Synechocystis* protein (32) and  $k_{\text{obs}} \sim 0.008$  at 25 °C for *E. coli* IscA-mediated reconstitution of apo Fd (15).

these assays, both *Hs* and *Sp* Fd carry out facile electron exchange. Any variations in redox chemistry stemming from variations in binding affinity for each Fd to the reductase are taken into account through use of the calibration plots. When the concentrations of Fd are higher than 20 nM, the calibration curves are linear for *Hs* Fd and *Sp* Fd (presumably reflecting saturation of the Fd reductase binding sites), with *Hs* Fd showing a higher velocity of reduction of cytochrome *c*. It is also important to note that under conditions where rates of reaction could be measured by both the native PAGE and cyt *c* assay methods, there was good agreement between the methods (Table 1).

Finally, it is again important to note that when the concentration of apo Fd was significantly increased or decreased (while maintaining pseudo-first-order conditions),  $k_2$  was again essentially unchanged. That is, variations in  $k_2$  values reported in Table 1 reflect variations in reactivity within the ISU-apo Fd complex rather than variations in binding affinity of ISU for the target protein.

**Measurement of  $k_{\text{obs}}$  for  $[2\text{Fe-2S}]$  Cluster Transfer from Holo ISU to Apo Fd.** For kinetic studies of Fe-S cluster transfer from holo ISU to apo Fd, apo Fd was incubated with DTT for 30 min, followed by addition of holo ISU. At intervals of 10 min (WT ISU) or 60 min (D37A ISU), an aliquot of the reaction mixture was withdrawn for assay. A 10-fold excess of holo ISU to apo Fd was used in cluster transfer reactions, with overall concentrations of Fd and ISU of 1000 and 100 nM, respectively. Following Fe-S cluster transfer from holo ISU to apo Fd, the concentration of holo Fd increases, and the velocity of reduction of cytochrome *c* determined from the kinetic assay (Figure 1) was also found to increase in a manner consistent with a higher yield of holo Fd. Since DTT can reduce cytochrome *c*, the overall velocity of reduction of cytochrome *c* must be corrected for this background rate. Accordingly, the velocity from a control assay carried out in the absence of apo Fd was subtracted from the measurement obtained under regular assay conditions. The concentration of DTT in the assay was  $\sim 4$  mM, and the velocity of reduction of cytochrome *c* by DTT was

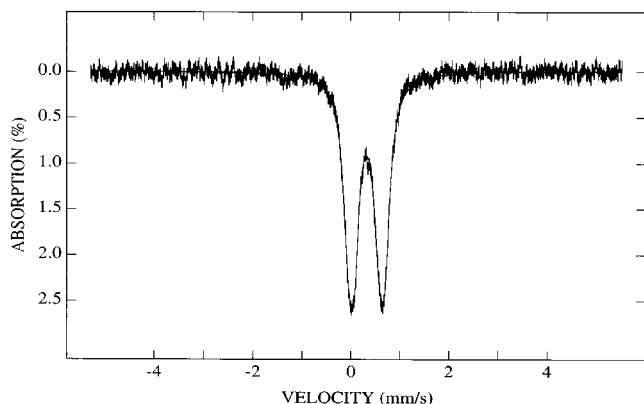


FIGURE 2: Mössbauer spectrum of holo Fd following [2<sup>57</sup>Fe-2S] cluster transfer from ISU is shown. Cluster transfer was carried out in the presence of an excess of unlabeled ferrous ion. The spectrum was recorded at 4.2 K in a 450 G parallel applied field. The two irons are distinguishable, and the fitted parameters are  $\Delta E_Q(1) = 0.51$  mm/s,  $\delta(1) = 0.32$  mm/s,  $\Delta E_Q(2) = 0.75$  mm/s,  $\delta(2) = 0.32$  mm/s.

determined to be 0.03 (OD/30 s), which is low relative to the value of interest and changed smoothly with time. The actual velocity, corrected for background, was converted to the concentration of holo Fd by use of a calibration curve. The yield of holo Fd was plotted as a function of time and fitted to a first-order decay. The rate constant ( $k_2$ ) of Fe–S cluster transfer from holo ISU to apo Fd was obtained from eq 1 following determination of  $k_{\text{obs}}$ . Control experiments showed no measurable reduction of cytochrome *c* in the absence of either holo Fd or DTT.

In Table 1, the observed and derived second-order rate constants of the complex formed between WT ISU and Fd are shown to be larger than those formed with D37A ISU. For example, the second-order rate constant ( $k_2$ ) for the complex of WT *Hs* ISU and *Hs* Fd is 10-fold larger than that of the complex formed between D37A *Hs* ISU and *Hs* Fd. This provides direct evidence that substitution of Asp37 influences the pathway for Fe–S cluster transfer from holo ISU to apo Fd and presumably serves a role in mediating cluster transfer. This result is also independent of the lower yield obtained for cluster transfer mediated by D37A ISU (discussed later). As a result of the poor solubility of *Sp* ISU1 at ambient temperatures, the rate constants for cluster transfer from *Sp* ISU1 to target Fd's were determined at 4 °C. When the temperature is decreased, the value ( $k_2$ ) is also found to decrease.

Cluster transfer rates for *Homo sapien* proteins were inevitably greater than the rates determined for *Schizosaccharomyces pombe* pairs (Table 1). Cross-species Fe–S cluster transfer was also examined, with *Hs* ISU displaying larger rate constants for Fe–S cluster transfer to *Hs* Fd relative to *Sp* Fd (238 versus 94 M<sup>−1</sup> min<sup>−1</sup>, respectively). Similarly, *Sp* ISU1 displayed larger rate constants for Fe–S cluster transfer to *Hs* Fd relative to *Sp* Fd. Remarkably, a comparison of either Fd as the common acceptor with *Hs* ISU or *Sp* ISU1 as the donor revealed similar rate constants. For example,  $k_2$  is 238 M<sup>−1</sup> min<sup>−1</sup> for *Hs* ISU transfer to *Hs* Fd at 4 °C, which compares well with 237 M<sup>−1</sup> min<sup>−1</sup> for *Sp* ISU1 transfer to *Hs* Fd at 4 °C. That is, the identity of the apo Fd appears to be the critical factor, with *Hs* apo Fd as the more efficient acceptor of the [2Fe-2S] cluster core.

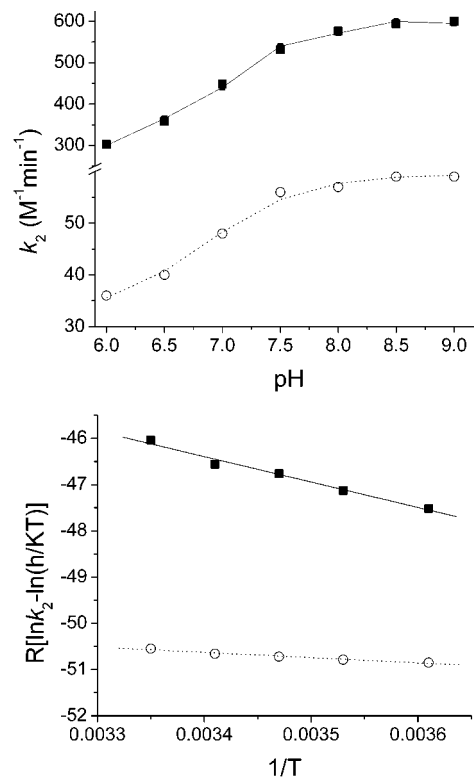


FIGURE 3: (Top) pH dependence of Fe–S cluster transfer from wild-type holo *Hs* ISU (solid line) or D37A holo *Hs* ISU (dotted line) to apo *Hs* Fd at room temperature. (Bottom) Plots ( $\ln k_2$  versus  $1/T$ ) of Fe–S cluster transfer from WT holo *Hs* ISU (squares) or holo D37A *Hs* ISU (circles) to apo *Hs* Fd. Enthalpic and entropic components ( $\Delta H^*$  and  $\Delta S^*$ ) were obtained from the slope and intercept.

**Influence of pH on [2Fe-2S]<sup>2+</sup> Cluster Transfer.** Fe–S cluster transfer from holo *Hs* ISU to apo *Hs* Fd was examined at various pH values. For WT ISU,  $k_2$  showed a pH dependence (Figure 3, top panel) that could be fit to standard eq 3 to yield the ionization constant for a titratable residue with a  $pK_a$  of 6.9. The maximum value of  $k_2$  was obtained at pH 8.5 and was observed to decrease at lower pH values. At pH 5.5, the yield of Fe–S cluster was 8% at the first 5 min, and then the yield decreased at the following times according to cytochrome *c* assay. The initial velocity for Fe–S cluster transfer determined within the first 5 min of reaction was low compared to the velocity determined at higher pH's. Subsequently, the Fe–S cluster transfer reaction ceased, and clusters in both Fd and ISU were observed to degrade. For D37A ISU,  $k_2$  displayed a similar pH dependence (Figure 3, top panel). It is important to note that these pH variations of cluster transfer yield do not reflect a pH dependence of the assay methods per se. The latter are carried out under standard pH 7.5 conditions. Only the cluster transfer chemistry is subject to variations in pH.

**Influence of Temperature on [2Fe-2S]<sup>2+</sup> Cluster Transfer.** Fe–S cluster transfer from WT ISU to apo Fd showed a more pronounced temperature dependence relative to the D37A ISU (Table 2A). Data were readily fit to standard Eyring eq 2 as shown in Figure 3 (bottom panel). The magnitude of  $\Delta H^*$  (WT ISU) is 5.5 kcal/mol, relative to 1.1 kcal/mol for D37A ISU (Table 2B).

**Influence of Viscosity on [2Fe-2S]<sup>2+</sup> Cluster Transfer.** Fe–S cluster transfer from holo *Hs* ISU to apo *Hs* Fd was

Table 2: Dependence of Cluster Transfer on Temperature

(A) Temperature Dependence of $k_2$ for Cluster Transfer from <i>Hs</i> ISU to Fd <sup>a</sup>			
WT ISU		D37A ISU	
temp (°C)	$k_2$ (M <sup>-1</sup> min <sup>-1</sup> )	temp (°C)	$k_2$ (M <sup>-1</sup> min <sup>-1</sup> )
25	540	25	56
20	407	20	52
15	362	15	49
10	296	10	47
4	238	4	45

(B) Activation Parameters for Fe–S Cluster Transfer from <i>Hs</i> ISU			
ISU/Fd complex	$\Delta H^*$	$\Delta S^*$	$\Delta G^*$
	(kcal mol <sup>-1</sup> )	(K <sup>-1</sup> mol <sup>-1</sup> )	(kcal mol <sup>-1</sup> ) <sup>b</sup>
<i>Hs</i> ISU/ <i>Hs</i> Fd	5.5 ± 1.0	−27.7 ± 3.1	13.7 ± 1.8
<i>Hs</i> ISU (D37A)/ <i>Hs</i> Fd	1.1 ± 0.2	−46.8 ± 5.2	15.0 ± 1.4

<sup>a</sup> Errors in kinetic rate constants are not explicitly stated since the error in the fitted Eyring plots is explicitly determined for the fitted activation parameters noted in (B). <sup>b</sup> Calculated for 298 K.

examined as a function of solution viscosity. Both 25% and 40% (w/v) sucrose were applied to study the influence of viscogen and yielded  $k_2$  values of 515 and 510 M<sup>-1</sup> min<sup>-1</sup>, respectively. These values do not show a significant difference from that with 0% sucrose (540 M<sup>-1</sup> min<sup>-1</sup>). Such viscosity studies show that diffusion is not the rate-limiting step, which is most likely the process of Fe–S cluster transfer between ISU and Fd. ISU and Fd have been previously shown to form a complex through chemical cross-linking experiments (25), and this complex is most likely the key intermediate in the process of Fe–S cluster transfer.

**NADPH/Fd Reductase-Mediated Fe–S Cluster Transfer.** To determine the role of redox chemistry on cluster transfer rates, the reaction was run under solution conditions that included catalytic amounts of NADPH, Fd reductase, and holo Fd. No enhancement of cluster transfer rate constant was observed. In fact, the value of  $k_2$  of NADPH/Fd reductase-mediated Fe–S cluster transfer from holo *Hs* ISU to apo *Hs* Fd was determined as 420 M<sup>-1</sup> min<sup>-1</sup> and found to be slightly lower than that of the reaction without NADPH/Fd reductase (540 M<sup>-1</sup> min<sup>-1</sup>). In detail, after the first 5 min of reaction, the yield from each set of conditions (with and without addition of NADPH/Fd reductase) was ~25%. However, after 15 min the yield of the NADPH/Fd reductase-mediated Fe–S cluster transfer was only 53%, and significantly lower than the yield (70%) of the reaction without NADPH/Fd reductase. The cluster transfer reaction appeared to slow after 5 min, most likely as a result of Fd reduction by NADPH/Fd reductase. There is most likely an equilibrium electron-transfer reaction between Fd and the cluster in ISU. Since the Fe–S clusters in ISU are reductively labile, this would result in degradation of the ISU-bound Fe–S clusters, making them unavailable for cluster transfer to apo Fd. This effect should become more significant as the reaction progresses and the concentration of holo Fd increases. This is indeed consistent with experimental observation. In the first 5 min, the concentration of holo Fd was low, and reduction of ISU by reduced Fd was slow. After 5 min, the increasing concentration of holo Fd facilitated catalytic reduction and degradation of holo ISU and slowed the second-order cluster reaction to apo Fd. This resulted in a

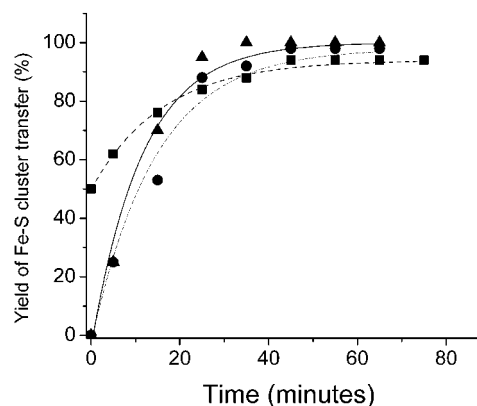


FIGURE 4: NADPH/Fd reductase-mediated Fe–S cluster transfer with no initial holo Fd (circles). The reaction mixture contained holo *Hs* ISU (167  $\mu$ M), apo *Hs* Fd (8.35  $\mu$ M), holo *Hs* Fd (8.35  $\mu$ M), Fd reductase (0.48  $\mu$ M), and NADPH (120  $\mu$ M). NADPH/Fd reductase-mediated Fe–S cluster transfer with 1 equiv of holo Fd added (squares). The reaction mixture contained holo *Hs* ISU (167  $\mu$ M), apo *Hs* Fd (16.7  $\mu$ M), Fd reductase (48 nM), and NADPH (120  $\mu$ M). A control reaction (triangles) where the reaction mixture contained holo *Hs* ISU (167  $\mu$ M), apo *Hs* Fd (16.7  $\mu$ M), and neither Fd reductase nor NADPH, represented Fe–S cluster transfer from holo *Hs* ISU to *Hs* Fd without mediation by NADPH/Fd reductase. Reaction progress was assessed by use of the cytochrome *c* assay at 25 °C. A plot of the yield (%) of holo Fd as a function of reaction time was fitted to a rate equation for a first-order decay process. Note that for the reaction including a molar equivalent of holo Fd the yield is recorded as the total holo Fd present in solution for fairness of comparison, and so the yield is 50% at time zero. The apparent rate constants  $k_{\text{obs}}$  for cluster transfer are 0.06 min<sup>-1</sup> ( $k_2 \sim 360$  M<sup>-1</sup> min<sup>-1</sup>), 0.07 min<sup>-1</sup> ( $k_2 \sim 420$  M<sup>-1</sup> min<sup>-1</sup>), and 0.09 min<sup>-1</sup> ( $k_2 \sim 540$  M<sup>-1</sup> min<sup>-1</sup>), respectively.

lower value of  $k_2$  for the NADPH/Fd reductase-mediated Fe–S cluster transfer.

While there is most likely an equilibrium electron-transfer reaction between Fd and the cluster in ISU, this appears to lie heavily on the side of Fd. In fact, NADPH/Fd reductase/Fd-mediated reduction of ISU showed no perceptible reduction of ISU over time frames that were considerably longer than those of a typical cluster transfer reaction, and only low levels of ISU cluster degradation. That is, reduction of ISU by Fd appears to be thermodynamically unfavorable. To provide further support for this conclusion, the cluster transfer reaction was also examined in the presence of NADPH and Fd reductase, and the addition of a molar equivalent of holo Fd. The resulting kinetic profile (Figure 4) is as expected. The pseudo-first-order  $k_{\text{obs}}$  of 0.06 min<sup>-1</sup> ( $k_2 = 360$  M<sup>-1</sup> min<sup>-1</sup>) and overall cluster transfer yields were again found to be lower than observed in the absence of reduced Fd.

## DISCUSSION

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]<sup>2+</sup> clusters prior to transfer to target apo proteins, and so a detailed understanding of cluster transfer from one organism is likely to be of general value. To this end, we have carried out a rigorous kinetic analysis of cluster transfer chemistry from representative eukaryotic ISU-type proteins to an apo Fd target. This has resulted in substantive mechanistic insight on the function of this class of protein that is likely to be of value in understanding other eukaryotic and bacterial ISU

proteins and may also be relevant to understanding ISA-type proteins.

**Transfer of an Intact [2Fe-2S] Core.** While we have shown that the Fe-S cluster transfer reaction from holo ISU to apo Fd can be monitored either by nondenaturing gel electrophoresis or by an optical cytochrome *c* assay, depending on the magnitudes of reaction velocities, neither of these assays directly demonstrates transfer of an intact cluster. That is, the possibility of cluster degradation with subsequent reassembly to form the intact cluster in a target protein has not yet been eliminated. We have established "direct" Fe-S cluster transfer from holo ISU to apo Fd by use of Mössbauer experiments to monitor the fidelity of Fe-S cluster transfer. Under conditions where a 10-fold excess of unlabeled Fe<sup>2+</sup> was added to the reaction mixture for cluster transfer, subsequent characterization of the reconstituted Fd revealed that only <sup>57</sup>Fe was loaded into apo Fd. This conclusion is based on quantitation of <sup>57</sup>Fe content in the reconstituted Fd by analysis of the Mössbauer peak intensity (Figure 2), and by independent determination of the concentration of holo Fd from the electronic absorbance of the cluster. Accordingly, cluster transfer from ISU to apo Fd involves the intact [2Fe-2S] core. The issue of the oxidation state of the cluster is addressed later.

**Cluster Transfer Yield and Kinetic Parameters for ISU-Mediated Reconstitution of Fd.** ISU-type proteins are basic (*pI* ~ 8–9), and Fd's are acidic (*pI* ~ 4–5), and so they are readily separable by native polyacrylamide gel electrophoresis and reconstitution of apo Fd from holo ISU can be followed by native PAGE. Holo and apo ISU proteins remain in the loading lane, while apo and holo Fd are well resolved following Coomassie staining (Figure 1). Holo Fd and holo ISU can also be distinguished from apo protein by use of established iron staining techniques (32). In larger scale reactions, the product holo Fd is readily isolatable by chromatographic separation. As previously noted, the yield of holo protein following addition of wild-type holo ISU to apo Fd (better than 90%) is significantly greater than that obtained by addition of D37A ISU (~70%) (25). This most likely reflects the more rapid reaction of the former that precludes significant disulfide bond formation in apo Fd over the shorter time course of the reaction. Control experiments carried out in the absence of ISU, but with addition of an equivalent concentration of iron and sulfide, yielded negligible yields of holo Fd under analogous conditions to those used for ISU-mediated reconstitution.

In preliminary experiments, we have shown that the rate of [2Fe-2S]<sup>2+</sup> cluster transfer from wild-type holo ISU is greater than that of the D37A derivative (25). 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. A reaction rate constant  $k_2 \sim 18 \text{ M}^{-1} \text{ min}^{-1}$  was determined for D37A ISU1 cluster transfer, and a lower limit of  $80 \text{ M}^{-1} \text{ min}^{-1}$  was assigned for WT (Table 1). The significant difference in the measured rate constants ( $k_2$ ) of wild-type and D37A ISU provides evidence that the residue aspartate 37 plays an important role in the process of Fe-S cluster transfer.

The absence of any significant influence of increasing viscosity on cluster transfer rates from holo *Hs* ISU to apo Fd provides evidence that diffusion of proteins to form an activated complex is not the rate-determining step. Conse-

quently, the rate-determining step must be found in the cluster transfer reaction. In turn, this indicates that  $k_2$  provides a relative measure of cluster transfer chemistry and provides a vehicle for monitoring the influence of pH, temperature, and viscosity on cluster transfer.

Cluster transfer from *Hs* ISU to apo Fd was found to be 3-fold faster than the corresponding reaction for *Sp* partners. Such differences in rates did not reflect any reaction of the Fd reductase with either Fd or cyt *c* since these were already accounted for by use of calibration plots. Significantly, the cross reaction of *Hs* ISU with *Sp* apo Fd yielded a rate constant ( $k_2$ ) that was similar to that of *Hs* apo Fd obtained under similar conditions, while the cross reaction of *Sp* ISU1 with *Hs* apo Fd yielded a rate constant ( $k_2$ ) that was similar to that of *Sp* apo Fd. These data demonstrate that the identity of the apo Fd is the most critical parameter in determining the rates of cluster transfer from ISU. This is consistent with the notion of a fairly accessible ISU-bound cluster, and critical structural parameters at the interface deriving principally from the Fd.

**Role for D37 in Mediating Cluster Transfer from ISU to Apo Fd.** Kinetic rate constants for cluster transfer from WT ISU are significantly different from those determined for D37A ISU (Table 1), providing evidence that aspartate 37 may play an important role in the chemistry of Fe-S cluster transfer. Such roles may include acid or base catalysis and structural influences through salt bridge formation or through regulation of solvent accessibility to the cluster core according to the polarity of the side chain. To both address the role of this residue and examine other pH-sensitive effects, a pH profile was determined.

The variation in the pH dependence of cluster stability for human and yeast ISU proteins (Figure 3, top panel) 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 (33). 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. Support for such an idea is provided by the pH dependence data shown in Figure 3 (top panel). Both WT and D37A ISU proteins display a similar rate profile, albeit with intrinsically lower  $k_2$ 's for the derivative protein. However, the apparent  $pK_a$  of 6.9 is consistent with deprotonation of solvent-accessible Cys, which tend to have smaller  $pK_a$ 's than a protein buried thiol (34), and would provide a more effective nucleophilic center to facilitate cluster transfer to apo Fd. The similarity in pH profiles for WT relative to D37A ISU also argues against a role for Asp37 in directly promoting cluster transfer, either by protonation of the ISU-bound cluster (or coordinating Cys residues) or by deprotonation of Cys nucleophiles on apo Fd. The experimental pH profile is also consistent with the involvement of a His, possibly through salt-bridge formation as a critical element of Fd recognition (either intra or inter), although this would not involve D37 since the profiles for WT and D37A ISU's are similar. The profiles do, however, support solvent accessibility and general base catalysis as factors contributing to cluster transfer reactivity.

*Activation Parameters for Cluster Transfer Provide Evidence for a Surface Solvent-Accessible Site on Holo ISU.* The temperature dependence (Figure 3, bottom panel) of cluster transfer reactions shows both enthalpic (+ve  $\Delta H^*$ ) and entropic (−ve  $\Delta S^*$ ) barriers (Table 2B). Enthalpic barriers dominate for WT ISU, and entropic barriers dominate the reactivity of the D37A derivative. Inasmuch as enthalpic terms typically reflect bond energies, the decrease in  $\Delta H^*$  measured for the derivative presumably reflects either weaker cluster binding in the ground state of holo D37A ISU, or loss of protein solvation energy. The latter reflects the need to displace solvent in the interfacial region during complex formation with apo Fd to achieve an activated transition state prior to cluster transfer. Since the cluster in the D37A derivative is known to be stabilized relative to WT protein, the first possibility, where  $\Delta H^*$  reflects weaker coordination, appears unlikely. The latter role, involving solvent, is, however, a very attractive hypothesis. Presumably the ISU-bound cluster is surface-localized to allow transfer to apo targets. As such, the cluster binding pocket would be expected to be solvent-accessible. We have previously demonstrated that solvent access to oxidized cluster centers promotes hydrolytic instability. Substitution of the polar D37 to a nonpolar Ala residue would diminish solvent access, stabilize the cluster, and result in loss of protein solvation energy. The latter would decrease  $\Delta H^*$ , but cause  $\Delta S^*$  to become more negative as a result of the loss of the entropy increase expected from displacement of bound water molecules. These ideas are consistent with our experimental observations (Table 2B).

*Role of Redox Chemistry and Oxidation State in Cluster Transfer.* *A. vinelandii* NifU is a modular protein that consists of three domains (7, 11). 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 (11), and homologous mitochondrial ferredoxins have been identified in yeast and human cell lines (19, 35). 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 (7). Such a hypothesis has not previously been tested.

Previously we have demonstrated complex formation between *Hs* ISU or *Sp* ISU1 and Fd proteins through cross-linking reactions. It was further observed that holo ISU forms a complex both with apo and with holo Fd, while apo ISU complexes with neither holo nor apo Fd. The absence of cross-linking does not prove that these proteins do not interact; however, these data are consistent with an Fe–S transfer pathway from holo ISU to apo ferredoxin, and also provide support for holo ISU and holo ferredoxin as potential redox partners. In this regard, the stable [2Fe-2S]<sup>2+</sup> 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 apo protein. A similar suggestion can be made for ISU complex formation with holo Fd, where reduced Fd serves to reduce the [2Fe-2S]<sup>2+</sup> cluster on ISU, thereby

facilitating degradation of the cluster and release of Fe and S<sup>2−</sup> 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-ISU–holo-Fd complex serves rather to remove electrons from the nascent ISU cluster, thereby stabilizing the reductively labile reduced state of the ISU-bound [2Fe-2S] center. In keeping with this idea is the apparent inability of reduced [2Fe-2S]<sup>+</sup> Fd to reduce ISU. The ISU cluster is relatively stable (and apparently not significantly reduced) in the presence of reduced Fd, but is immediately degraded upon dithionite reduction.

Finally it is noteworthy that the binding of ferredoxin by ISU- and ISA-type proteins is consistent with the sequence of basic residues found in the latter two (7), which possibly interact with acidic residues on Fd that have been previously implicated in complex formation with other cellular proteins (36). Importantly, however, these data do not necessarily suggest that Fd is the only physiological target for ISU. Undoubtedly ISU also targets other Fe–S proteins, and so the cross-linking that we observe here should not be interpreted as a specific interaction of ISU with Fd.

*Comparison with Published Data on ISU-Type and Other Protein Classes.* A comparison of available cluster transfer data is summarized in the legend to Table 1 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 the 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 (14, 37). It is noteworthy that the reaction for delivery of nickel to urease by the UreD–UreE–UreG–urease complex has a reported half-life of 6 h (38).

Previously, Nishio and Nakai have reported [2Fe-2S] cluster transfer from *Synechocystis* NifU to an apo Fd target (32). 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. Takahashi and Fontecave et al. have also demonstrated that *E. coli* IscA can reconstitute apo Fd (15). Similar to ISU-type proteins, the *Synechocystis* NifU C-terminal domain and *E. coli* IscA proteins also carry [2Fe-2S] cluster centers. While evidence for [4Fe-4S]<sup>2+</sup> cluster assembly has been reported for the ISU-type domain of *A. vinelandii* IscU (16), transfer of this cluster form to an apo target has not been demonstrated.

## REFERENCES

- Harrison, M. D., Jones, C. E., and Dameron, C. T. (1999) *J. Biol. Inorg. Chem.* 4, 145–153.

2. Hausinger, R. P., Eichorn, G. L., and Marzilli, L. G., Eds. (1996) in *Mechanisms of Metallocenter Assembly*, Wiley, New York.
3. Soriano, A., Colpas, G., and Hausinger, R. P. (2000) *Biochemistry* 39, 12435–12440.
4. Rangaraj, P., Shah, V. K., and Ludden, P. W. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 11250–11255.
5. Goodwin, P. J., Agar, J. N., Roll, J. T., Roberts, G. P., Johnson, M. K., and Dean, D. R. (1998) *Biochemistry* 37, 10420–10428.
6. Aguilar, O. M., Taormino, J., Thony, B., Ramseier, T., Hennecke, H., and Szalay, A. A. (1990) *Mol. Gen. Genet.* 224, 413–420.
7. Fu, W., Jack, R. F., Morgan, T. V., Dean, D. R., and Johnson, M. K. (1994) *Biochemistry* 33, 13455–13463.
8. Dailey, H. A., Dailey, T. A., Wu, C. K., Medlock, A. E., Wang, K. F., Rose, J. P., and Wang, B. C. (2000) *Cell Mol. Life Sci.* 57, 1909–1926.
9. Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C., and Kispal, G. (1999) *Biol. Chem. Hoppe-Seyler* 380, 1157–1166.
10. Lill, R., and Kispal, G. (2000) *Trends Biochem. Sci.* 25, 352–356.
11. Zheng, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) *J. Biol. Chem.* 273, 13264–13272.
12. Zheng, L., and Dean, D. R. (1994) *J. Biol. Chem.* 269, 18723–18726.
13. Takahashi, Y., and Nakamura, M. (1999) *J. Biochem. (Tokyo)* 126, 917–926.
14. Hoff, K. G., Silberg, J. J., and Vickery, L. E. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 7790–7795.
15. Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y., and Fontecave, M. (2001) *J. Biol. Chem.* 276, 22604–22607.
16. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) *Biochemistry* 39, 7856–7862.
17. Yuvaniyama, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 599–604.
18. Muhlenhoff, U., and Lill, R. (2000) *Biochim. Biophys. Acta* 1459, 370–382.
19. Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 1050–1055.
20. Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999) *EMBO J.* 18, 3981–3989.
21. Tong, W. H., and Rouault, T. (2000) *EMBO J.* 19, 5692–5700.
22. Foster, M. W., Mansy, S. S., Hwang, J., Penner-Hahn, J. E., Surerus, K. K., and Cowan, J. A. (2000) *J. Am. Chem. Soc.* 122, 6805–6806.
23. Wu, G., Mansy, S. S., Foster, M. W., Hille, R., Hemann, C. F., Surerus, K. K., and Cowan, J. A. (2002) *J. Biol. Inorg. Chem.* 7, 526–532.
24. Agar, J. N., Zheng, L., Cash, V. L., Dean, D. R., and Johnson, M. K. (2000) *J. Am. Chem. Soc.* 122, 2136–2137.
25. Wu, G., Mansy, S. S., Wu, S., Surerus, K. K., Foster, M. W., and Cowan, J. A. (2002) *Biochemistry* 41, 5024–5032.
26. Xia, B., Cheng, H., Bandarian, V., Reed, G. H., and Markley, J. L. (1996) *Biochemistry* 35, 9488–9495.
27. Ziegler, G. A., Vonrhein, C., Hanukoglu, I., and Schulz, G. E. (1999) *J. Mol. Biol.* 289, 981–990.
28. Moulis, J. M., and Meyer, J. (1982) *Biochemistry* 21, 4762–4771.
29. Lysionek, A. E., Zubillaga, M. B., Calmanovici, G. P., Salgueiro, M. J., Sarabia, M. I., Barrado, A. D., Caro, R. A., Weill, R., and Boccio, J. R. (1998) *Biol. Trace Elem. Res.* 65, 87–94.
30. Lui, S. M., Soriano, A., and Cowan, J. A. (1993) *J. Am. Chem. Soc.* 115, 10483–10486.
31. Lui, S. M., Liang, W., Soriano, A., and Cowan, J. A. (1994) *J. Am. Chem. Soc.* 116, 4531–4536.
32. Nishio, K., and Nakai, M. (2000) *J. Biol. Chem.* 275, 22615–22618.
33. Foster, M. W., Bian, S., Surerus, K. K., and Cowan, J. A. (2001) *J. Biol. Inorg. Chem.* 6, 266–274.
34. Dyson, H. J., Jeng, M.-F., Tennant, L. L., Slaby, I., Lindell, M., Cui, D.-S., Kuprin, S., and Holmgren, A. (1997) *Biochemistry* 36, 2622–2636.
35. Xia, B., Cheng, H., Skjeldahl, L., Coghlan, V. M., Vickery, L. E., and Markley, J. L. (1995) *Biochemistry* 34, 180–187.
36. Brandt, M. E., and Vickery, L. E. (1993) *J. Biol. Chem.* 268, 17126–17130.
37. Silberg, J. J., Hoff, K. G., Tapley, T. L., and Vickery, L. E. (2001) *J. Biol. Chem.* 276, 1696–1700.
38. Soriano, A., Colpas, G. J., and Hausinger, R. P. (2000) *Biochemistry* 39, 12435–12440.

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