Iron−Sulfur Cluster Biosynthesis. A Comparative Kinetic Analysis of Native and Cys-Substituted ISA-Mediated [2Fe-2S]$^{2+}$ Cluster Transfer to an Apoferredoxin Target†

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Received October 2, 2002; Revised Manuscript Received March 17, 2003

ABSTRACT: ISA type proteins mediate cluster transfer to apoprotein targets. Rate constants have been determined for cluster transfer from *Schizosaccharomyces pombe* ISA to apo Fd. Substitution of the cysteine residues of ISA produced derivative proteins (C72A, C136A, and C138A) that were found to be at least as active in cluster transfer reactions as the native form at 25 °C ($k_2 \sim 170$ M^{-1} min^{-1} for native, $k_2 \sim 169$ M^{-1} min^{-1} for C72A, $k_2 \sim 206$ M^{-1} min^{-1} for C136A, and $k_2 \sim 242$ M^{-1} min^{-1} for C138A), although the yield of cluster transfer was found to be lower as a consequence of the enhanced lability of clusters in the derivative proteins. Minor variations in rate constant for the ISA Cys derivatives do not reflect any change in the affinity of binding to the apo Fd since k_2 was found to be independent of the concentration of apo Fd over the range of 1−25 μM. The pH dependence of cluster transfer rates was found to be similar for native and C136A ISA, with an observed pK_a of 7.8 determined from the pH profiles for cluster transfer activity of each protein. The temperature dependence of the rate constant defining the cluster transfer reaction for the wild type versus this C136A ISA derivative is distinct ($\Delta H^* \sim 6.3$ kcal mol^{-1} and $\Delta S^* \sim -27.3$ cal K^{-1} mol^{-1} for native and $\Delta H^* \sim 2.7$ kcal mol^{-1} and $\Delta S^* \sim -38.9$ cal K^{-1} mol^{-1} for C136A ISA). Instability of the protein-bound cluster precluded a comparison with data from pH and temperature dependencies for the two other Cys derivatives. Experiments to determine the dependence of reaction rate constants on viscosity indicate cluster transfer is rate-limiting. A comparison of cross-species rate constants for cluster transfer to apo Fd targets from *Homo sapiens* and *S. pombe* demonstrated that the identity of the Fd is less critical for promoting cluster transfer from *Sp* ISA (at 25 °C, $k_2 \sim 170$ M^{-1} min^{-1} for *Sp* Fd and $k_2 \sim 169$ M^{-1} min^{-1} for *Hs* Fd). This contrasts with an earlier observation for ISU-mediated cluster assembly [Wu, S., et al. (2002) *Biochemistry 41*, 8876−8885], where the rates differed for *Hs* and *Sp* target Fd's, suggesting distinct binding sites for binding of holo ISA and ISU to apo Fd.

The biosynthesis of iron−sulfur clusters is a multistep process that requires multiple gene products to mediate assembly of these complex metal cofactors. Three bacterial operons that encode critical proteins for assembly have been identified. The nif operon specifically concerns maturation of the cluster cofactors of nitrogenase (*1*−*4*). In contrast, the isc operon encodes proteins that appear to mediate a general assembly pathway for the biosynthesis of a variety of cellular proteins that contain Fe−S clusters (*5*−*8*). More recently a third operon (termed suf) has been identified and appears to represent a minor general pathway for the assembly of Fe−S cofactors (*9*). Key proteins that have been implicated in bacterial Fe−S cluster assembly include sulfur donor proteins, chaperones, electron transport proteins, and transient carriers of intermediate Fe−S cluster building blocks (*7*, *10*, *11*). The latter include two families of proteins [IscU (or ISU) and IscA (or ISA)], each of which has been demonstrated to carry a [2Fe-2S] cluster (*12*−*14*) that can be transferred to an apo target. Proteins homologous to isc gene products have been identified in eukaryotes (*5*, *6*, *15*−*18*) and have been implicated in mitochondrial Fe−S cluster biosynthesis (*5*, *6*, *15*, *16*).

In an earlier paper, we presented results from a detailed kinetic analysis of ISU-mediated cluster transfer to an apo Fd[1] target (*19*). Following our recent report of the isolation and characterization of a yeast ISA protein, we sought to make a detailed comparison of the cluster transfer properties of ISA with ISU type proteins. As for ISU, we have shown that ISA serves as a platform for assembly of [2Fe-2S]$^{2+}$ building blocks that can then be transferred to apoprotein targets. In this paper, we address the functional similarities and variations of ISA versus ISU activity to determine reasons for the apparent redundancy in protein function. The availability of Cys derivatives of *Schizosaccharomyces pombe* (*Sp*) ISA (*20*) also allowed the roles of critical residues in the cluster binding pocket to be explored. In this

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

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[1] Abbreviations: CBB, Coomassie brilliant blue; Cyt *c*, cytochrome *c*; DTT, dithiothreitol; Fd, ferredoxin; NADPH, nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UV−vis, ultraviolet−visible.

10.1021/bi026939+ CCC: $25.00
Published on Web 04/25/2003

paper, we use our previously established kinetic methodologies for assaying cluster transfer chemistry to examine the temperature, pH, and viscosity dependencies of cluster transfer to *S. pombe* and *Homo sapiens* (*Hs*) apo Fd. This allows extension of our working model of the mechanism of cluster transfer to this family of proteins.

MATERIALS AND METHODS

General Chemicals. Solutions were purged with argon and handled under positive Ar(g) pressure using standard Schlenk line techniques. 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).

Protein Purification. Protocols for purification of native ISA and derivatives, and for the target ferredoxins, have been described elsewhere (*19*−*21*). An expression plasmid for *Hs* Fd was kindly provided by J. Markley, and an expression plasmid for a bovine NADPH Fd reductase was kindly provided by G. Schulz (*22*).

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

Iron Quantitation. Protein concentrations were quantitatively assessed from the measured extinction coefficients (*19*). For mutants, the cluster content was determined by quantitation of iron. While formation of the apoprotein was observed during chromatographic purification of mutants, the holo form was sufficiently stable in solution for the kinetic experiments discussed herein. Certain pH and VT dependence studies were not carried out on mutants where cluster stability was inconsistent with rates of cluster transfer to be monitored. 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 and precipitated material removed by centrifugation. The supernatant (100 μL) was diluted in 1.3 mL of 0.5 M Tris-HCl (pH 8.5), and 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 the absorbance was compared to a calibration curve made with 0.01−0.3 mM $FeCl_3$ solutions.

Determination of Reaction Rate Constants for [2Fe-2S] Cluster Transfer from Holo ISA to Apo Fd by Native PAGE. Native PAGE analyses of Fe−S cluster transfer from holo ISA to apo Fd were performed as follows. Apo Fd was incubated with 4.5 mM DTT for 30 min, followed by addition of holo ISA. The holo ISA concentration was evaluated from the cluster content. At intervals of 30 or 60 min, an aliquot of the reaction mixture at 4 °C 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 is run. A greater than 10-fold excess of holo ISA over apo Fd was used in cluster transfer reactions, with overall concentrations of apo Fd and Sp ISA of 16 and 167 μM in 18 μL, respectively. Following Fe−S cluster transfer from holo ISA to apo Fd, the concentration of holo Fd was observed to increase, as determined by both Coomassie and iron staining, following quantitative evaluation of band intensity by use of a Bio-Rad gel doc 1000 instrument. 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_{obs}) for Fe−S cluster transfer from holo ISA 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.

Determination of Reaction Rate Constants for [2Fe-2S] Cluster Transfer from Holo ISA to Apo Fd by a Cytochrome c Assay. Apo Fd (170 μM, 20 μL) was incubated with DTT (50 mM, 20 μL) for 30 min. Subsequently, 11.8 μL of this mixture was added to 50 μL of 300 μM holo *Sp* ISA or cysteine mutants. The holo ISA concentration was evaluated from the cluster content. At 10 or 20 min intervals, 6.2 μL 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 μM 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 6.2 μL of a holo ISA/apo Fd mixture and NADPH under anaerobic conditions. The final concentrations of Fd, *Sp* ISA, and NADPH were 0.1, 1.5, and 400 μM, 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 level of formation of reconstituted Fd according to constructed calibration curves that were produced as previously described (*19*). 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_{obs}) was determined. The second-order rate constant k_2 was determined from eq 1

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

As a control, the activity levels for apo Fd, apo ISA, and holo ISA were separately analyzed, and all showed no activity. Furthermore, to correct for the influence of DTT, which can reduce cytochrome *c* at a rate that is low compared to the rate of reduction of NADPH, Fd reductase, and Fd, 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 for Fe−S cluster transfer from holo *Sp*

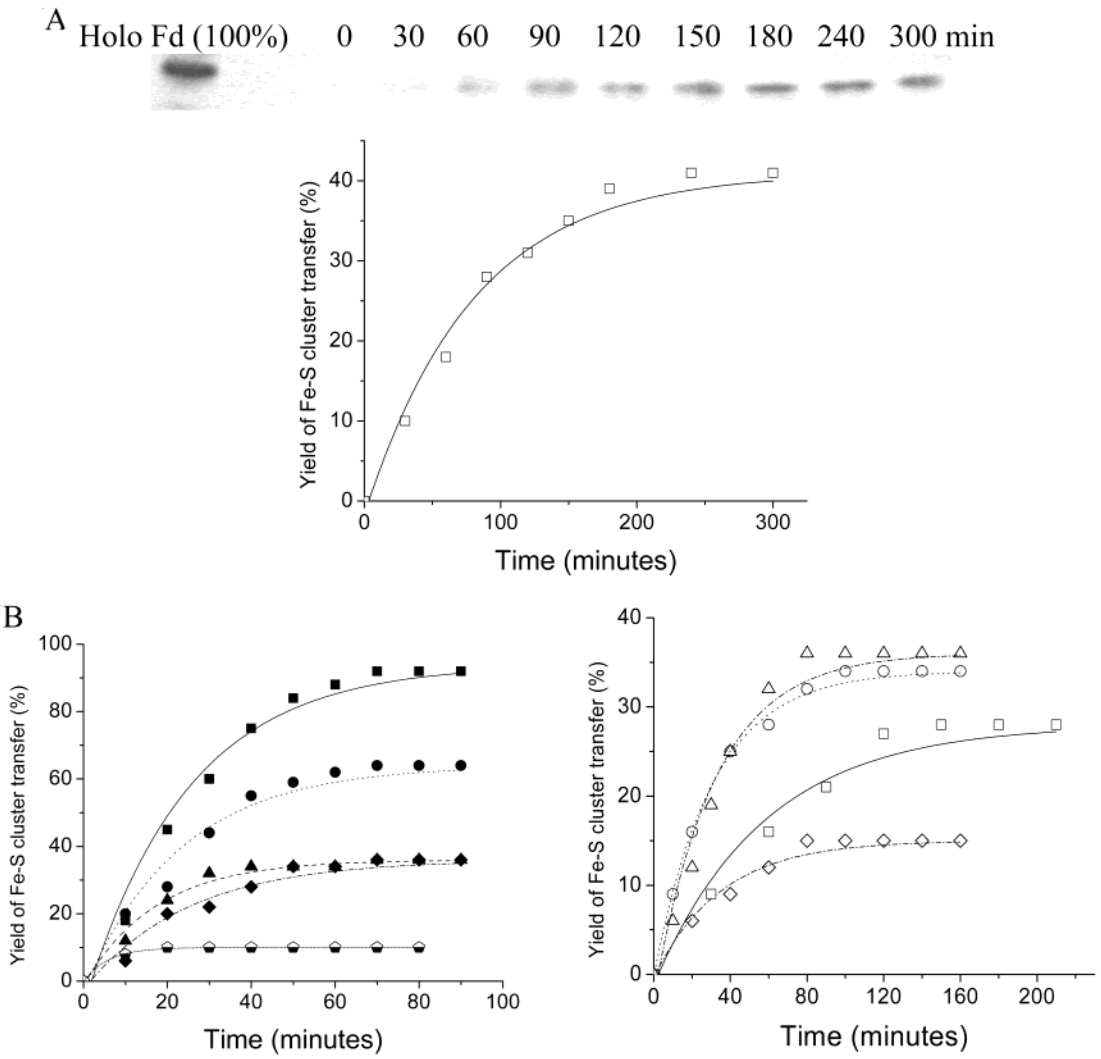

FIGURE 1: (A) Native PAGE. The top panel shows $[2Fe\text{-}2S]^{2+}$ cluster transfer from *Sp* holo ISA to *Hs* apo Fd at 4 °C, showing the formation of holo Fd, which was followed by native PAGE and CBB staining. Overall concentrations of apo Fd and ISA were 16 and 167 μM, respectively. The bottom panel shows a plot of the yield of Fe−S cluster transfer (the ratio of intensity) as a function of reaction time fitted to a rate equation for a first-order decay process. The apparent rate constant (k_{obs}) for cluster transfer is 0.012 (4 °C). (B) Cytochrome *c* assay. $[2Fe\text{-}2S]^{2+}$ cluster transfer from *Sp* holo ISA or cysteine mutants to apo *Sp* Fd at 25 °C (left) and 4 °C (right). Overall concentrations of apo Fd and ISA were 16 and 243 μM, respectively: wild type ISA (squares), C72A ISA (diamonds), C136A ISA (circles), and C138A ISA (triangles). A control experiment (pentagon) illustrates the low reconstitution yield of holo Fd in the presence of $FeCl_3$ (486 μM) and Na_2S (486 μM), but with no added holo ISA.

ISA (or C136A) to apo *Sp* Fd was examined at several temperatures (25, 20, 15, 10, and 4 °C) by the cytochrome *c* assay. Experimental conditions similar to those defined earlier were used. The resulting plot of ln k_2 versus $1/T$ was fitted to a standard Eyring equation (eq 2) [where all parameters have their usual meanings and the activation enthalpy (ΔH^*) and entropy (ΔS^*) for cluster transfer have been 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 Native and C136A ISA. The pH dependence of k_2 of the native and C136A *Sp* ISA was determined by performing the cytochrome *c* assay described above. The reaction temperature was 25 °C, and the buffers (50 mM) were MES (pH 5.5−6.5) 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(HA) and k_2(A), respectively.

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

Dependence of k_2 on Viscosity for Cluster Transfer from Native and C136A Sp ISA. Kinetic studies of Fe−S cluster transfer from holo *Sp* ISA to apo *Sp* Fd in the presence of varying amounts of viscogen [0, 20, and 40% (w/v) sucrose] were carried out. The reaction temperature was 25 °C, and the buffer was 50 mM Tris-HCl (pH 7.5).

NADPH- and Fd Reductase-Mediated Fe−S Cluster Transfer. To examine the role of Fd-mediated ISA 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 ISA to apo Fd. The reaction mixture contained holo ISA (243 μM), apo Fd (16 μM), Fd reductase (48 nM), and NADPH (120 μM).

Additionally, the reaction was carried out with a molar equivalent of added Fd in addition to apo Fd. This reaction mixture contained holo ISA (243 μM), apo Fd (8 μM), holo Fd (8 μM), Fd reductase (48 nM), and NADPH (120 μM). Both reaction mixtures were assayed in a similar fashion. After 10 min, equivalent volumes (6.2 μL) 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

Cluster Transfer Pathway and Reaction Yields. By use of Mossbauer spectroscopy, we have previously demonstrated that the ISU-bound $[2Fe\text{-}2S]^{2+}$ cluster is transferred intact to an apo Fd target. This conclusion was also supported by the fact that no significant quantity of holo Fd was formed under reconstitution conditions where free iron and sulfide ions were provided in the absence of ISU. Similarly, ISA-mediated $[2Fe\text{-}2S]^{2+}$ cluster delivery to apo Fd is almost quantitative under the correct reaction conditions (Figure 1B, left). We again observed minimal (<7%, Figure 1) cluster formation in apo Fd when providing only free iron and sulfide at the same concentrations used for the ISA-promoted reaction. Accordingly, cluster transfer can again be assumed to arise via direct transfer of the intact cofactor from ISA to apo Fd.

The efficiency of Fe−S cluster transfer from native *Sp* ISA to apo Fd was compared to that of the cysteine mutants of ISA. The yield of holo Fd following addition of native holo *Sp* ISA was significantly greater than that obtained by addition of Cys to Ala mutants (C72A, C136A, and C138A). Product holo Fd was also readily isolated following chromatographic separation, and the yield of the cluster transferred at 25 °C was determined to be 35% for C72A *Sp* ISA, 65% for C136A *Sp* ISA, 35% for C138A *Sp* ISA, and >90% for native *Sp* ISA based on quantitation of holo Fd by absorption spectroscopy. As stated earlier, control experiments carried out in the absence of ISA, but with addition of an equivalent concentration of iron and sulfide, produced negligible yields of holo Fd under conditions analogous to those used for ISA-mediated reconstitution.

Native PAGE Assay. Since native *Sp* ISA and the Cys derivatives are basic proteins (pI ∼8−9), and Fd is acidic (pI ∼4−5), ISA and Fd can be readily separated by native polyacrylamide gel electrophoresis. Holo and apo ISA proteins remain in the loading lane, while apo and holo Fd are well-resolved following Coomassie staining. Holo Fd and holo ISA can also be distinguished from the apoprotein by use of established iron staining techniques. Under conditions where cluster transfer from ISA to apoferredoxin at 4 °C is slow ($t_{1/2}$ > 1 h), nondenaturing gel electrophoresis can be applied to kinetic studies of the reconstitution of apo Fd from holo ISA (Figure 1) by monitoring the formation of holo Fd from quantitation of band intensity. An excess of at least 10-fold of the [2Fe-2S] cluster of ISA was used relative to apo Fd to ensure pseudo-first-order kinetics. Consequently, second-order rate constants (k_2) were determined from k_{obs} by use of eq 1. Native PAGE proved to be useful for kinetic studies of the relatively slow rate of Fe−S cluster transfer from holo ISA to apo Fd at 4 °C. Under the reaction conditions that were used (see the legend ofo Figure 1), a reaction rate constant k_2 of ∼72 M^{-1} min^{-1} was determined for *Sp* ISA 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 ISA−apo Fd complex rather than variations in the affinity of ISA for the target protein.

Table 1: Observed (k_{obs}) and Determined Second-Order Rate Constants (k_2) for Fe−S Cluster Transfer from Native and Derivative *S. pombe* ISA to Target Fd's

ISA−apo Fd complex[a]	k_{obs} (min^{-1})	k_2 (M^{-1} min^{-1})	assay method[b,c]
ISA−Fd	0.04 (25 °C)	170 ± 8 (25 °C)	cytochrome *c*
	0.017 (4 °C)	72 ± 6 (4 °C)	cytochrome *c*
ISA−*Hs* Fd	0.04 (25 °C)	169 ± 8 (25 °C)	cytochrome *c*
	0.016 (4 °C)	67 ± 6 (4 °C)	cytochrome *c*
ISA−Fd	0.012 (4 °C)	71 ± 9 (4 °C)	native gel
ISA−*Hs* Fd	0.012 (4 °C)	72 ± 8 (4 °C)	native gel
C72A ISA−Fd	0.04 (25 °C)	169 ± 8 (25 °C)	cytochrome *c*
	0.029 (4 °C)	120 ± 7 (4 °C)	cytochrome *c*
C136A ISA−Fd	0.05 (25 °C)	206 ± 12 (25 °C)	cytochrome *c*
	0.03 (4 °C)	135 ± 7 (4 °C)	cytochrome *c*
C138A ISA−Fd	0.06 (25 °C)	242 ± 12 (25 °C)	cytochrome *c*
	0.03 (4 °C)	131 ± 8 (4 °C)	cytochrome *c*

[a] Unless otherwise indicated, all proteins are from *S. pombe*. [b] In the cytochrome *c* assay, the concentrations of ISA-bound [2Fe-2S] cluster were as follows: [ISA] = [C72A ISA] = [C136A ISA] = [C138A ISA] = 243 μM. The concentrations of apo Fd were 16 μM. [c] In the native gel assay, the concentrations of ISA-bound [2Fe-2S] cluster were 167 μM. The concentrations of apo Fd were 16 μM.

Cytochrome c Assay. Preliminary kinetic studies showed that the rate of Fe−S cluster transfer from holo *Sp* ISA to apo Fd at a higher temperature (>4 °C) was too fast to be monitored by nondenaturing PAGE. The concentration of protein required for either an iron or Coomassie staining method to provide reliable quantitation of bands for determination of rate constants (such as in Figure 1) resulted in an excessively rapid reaction, and so other possibilities were explored. A cytochrome *c* assay is commonly used to measure the activity of Fd and Fd reductase. Since the velocity of reduction of cytochrome *c* is related to the concentration of Fd (assuming the concentrations of NADPH, Fd reductase, and cytochrome *c* are held constant), this provides a method for evaluating the rate of formation of holo Fd during cluster assembly reactions. The rationale for this assay has previously been described in detail (*19*). 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). Furthermore, 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 ISA−apo Fd complex rather than variations in the affinity of ISA for the target protein.

Measurement of k_{obs} for [2Fe-2S] Cluster Transfer from Holo ISA to Apo Fd. For kinetic studies of Fe−S cluster transfer from holo ISA to apo Fd, apo Fd was incubated with DTT for 30 min, followed by addition of holo ISA. At

intervals of 10 or 20 min, an aliquot of the reaction mixture was withdrawn for assay. A 10-fold excess of holo ISA over apo Fd was used in cluster transfer reactions, with overall concentrations of Fd and ISA of 1500 and 100 nM, respectively. Following Fe−S cluster transfer from holo ISA to apo Fd, the concentration of holo Fd increased 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 ∼4 mM, and the velocity of reduction of cytochrome *c* by DTT was 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 ISA to apo Fd was obtained from eq 1 following the determination of k_{obs}. Control experiments showed no measurable reduction in the level 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 native ISA and Fd are shown to be similar to those for Cys derivatives of ISA. In fact, the latter are slightly more active. For example, the second-order rate constant (k_2) for the complex of native *Sp* ISA and *Sp* Fd is 170 M^{-1} min^{-1} (25 °C), which is almost equal to that of the complex formed between C72A *Sp* ISA and *Sp* Fd, but smaller than that of the complex formed between C136A *Sp* ISA or C138A *Sp* ISA and *Sp* Fd. Furthermore, the yield (shown in Figure 1) of Fe−S cluster transfer from native ISA or Cys to Ala mutant ISA is quite different. At 25 °C, the yield of Fe−S cluster transfer from native ISA is >90%, but only 35% from C72A *Sp* ISA, 65% from C136A *Sp* ISA, and 35% from C138A *Sp* ISA. The same results were obtained by using chromatographic separation. These observations indicate that the stability of the ISA-bound Fe−S cluster may influence both the rate constant for and the yield of Fe−S cluster transfer. At 4 °C, both the rate constants and the yields of Fe−S cluster transfer from native ISA are significantly different from those determined for the Cys to Ala derivatives. In Table 1, the rate constant for Fe−S cluster transfer from native ISA to *Sp* Fd is 72 M^{-1} min^{-1}, and is lower than that determined for cluster transfer from the cysteine derivatives of ISA (120 M^{-1} min^{-1} for C72A ISA, 135 M^{-1} min^{-1} for C136A ISA, and 131 M^{-1} min^{-1} for C138A ISA). The yield of Fe−S cluster transfer from native ISA or cysteine mutant proteins is between 35 and 15%. The yield from native ISA at 4 °C is very low (25%) in comparison to the yield of 95% obtained at 25 °C. The influence of temperature on $[2Fe\text{-}2S]^{2+}$ cluster transfer will be discussed later. Cross-species Fe−S cluster transfer was also examined, with *Hs* Fd displaying rate constants for Fe−S cluster transfer similar to those of *Sp* Fd (169 vs 170 M^{-1} min^{-1} at 25 °C and 67 vs 72 M^{-1} min^{-1} at 4 °C, respectively). That is, the identity of the apo Fd appears not to be the critical factor for accepting Fe−S clusters.

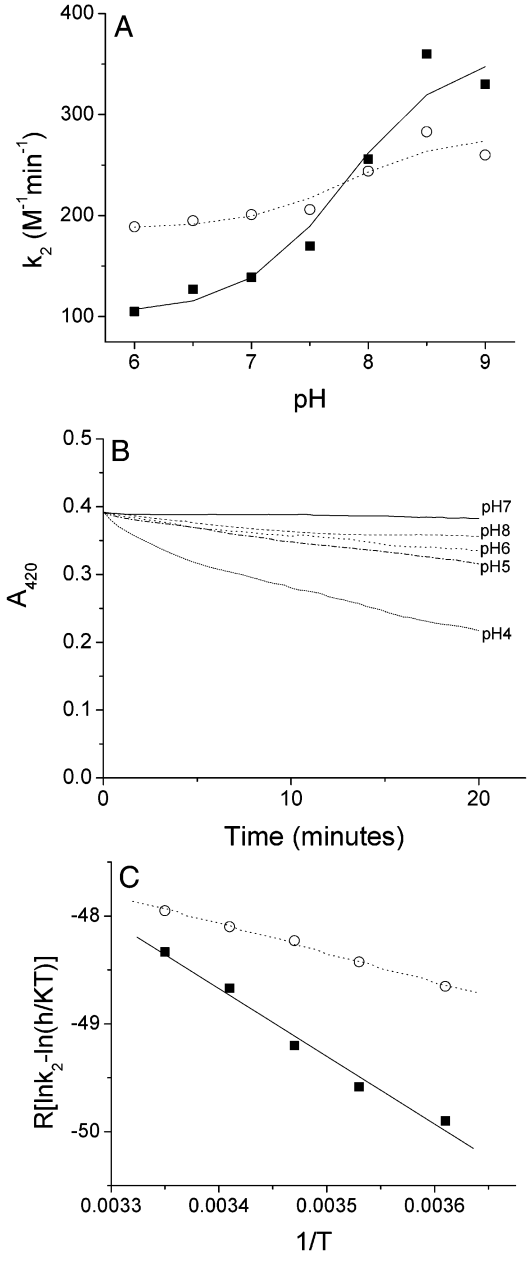

FIGURE 2: (A) pH dependence of Fe−S cluster transfer from wild type holo *Sp* ISA (■) or C136A holo *Sp* ISA (○) to apo *Sp* Fd at room temperature. (B) Influence of pH on cluster stability for native *Sp* ISA determined through the time dependence of cluster absorbance with pH. Spectra were recorded in 50 mM buffer (Tris-HCl for pH 8, phosphate for pH 7, Bis-Tris for pH 6, acetate for pH 5, and formate for pH 4) and 50 mM NaCl. (C) Plots (ln k_2 vs 1/*T*) for Fe−S cluster transfer from wild type holo *Sp* ISA (■) or holo C136A *Sp* ISA (○) to apo *Sp* Fd. Enthalpic and entropic components (ΔH^* and ΔS^*, respectively) were obtained from the slope and intercept.

Influence of pH on [2Fe-2S]$^{2+}$ Cluster Transfer. Fe−S cluster transfer from holo *Sp* ISA to apo *Sp* Fd was examined at various pH values. For native ISA, k_2 exhibited a pH dependence (Figure 2, top) that could be fit to a standard equation (eq 3) to yield the ionization constant for a titratable residue with a pK_a of 7.8. 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, both native ISA and C136A ISA were found to precipitate and the yield of Fe−S cluster transfer was less than 10%, making it difficult to determine rate

Table 2: Dependence of Cluster Transfer on Temperature

(A) Temperature Dependence of k_2 for Cluster Transfer from *Sp* ISA to Apo *Sp* Fd

native ISA−apo Fd		C136A ISA−apo Fd	
temperature (°C)	k_2 (M^{-1} min^{-1}) (yield, %)	temperature (°C)	k_2 (M^{-1} min^{-1}) (yield, %)
25	170 (95)	25	206 (64)
20	141 (85)	20	188 (60)
15	106 (55)	15	173 (60)
10	86 (32)	10	154 (32)
4	72 (25)	4	135 (30)

(B) Activation Parameters for Fe−S Cluster Transfer from *Sp* ISA

ISA−apo Fd complex	ΔH^* (kcal mol^{-1})	ΔS^* (cal K^{-1} mol^{-1})	ΔG^* (kcal mol^{-1})[a]
Sp ISA−*Sp* Fd	6.3 ± 1.2	-27.3 ± 2.8	14.4 ± 1.6
Sp C136A ISA−*Sp* Fd	2.7 ± 0.3	-38.9 ± 3.2	14.3 ± 1.5

(C) Activation Parameters for Fe−S Cluster Transfer from *Hs* ISU (*19*)

ISU−Fd complex	ΔH^* (kcal mol^{-1})	ΔS^* (cal K^{-1} mol^{-1})	ΔG^* (kcal mol^{-1})[a]
Hs ISU−*Hs* Fd	5.5 ± 1.0	-27.7 ± 3.1	13.7 ± 1.8
Hs D37A ISU−*Hs* Fd	1.1 ± 0.2	-46.8 ± 5.2	15.0 ± 1.4

[a] Calculated for 298 K.

constants with reasonable accuracy. For C136A ISA, k_2 displayed a similar pH dependence (Figure 2, top). It is important to note that these pH variations in 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. Figure 2 also shows the pH dependence of cluster stability, with only a modest change in cluster degradation observed at pH 6.

Influence of Temperature on [2Fe-2S]$^{2+}$ Cluster Transfer. Fe−S cluster transfer from native ISA to apo Fd shows a more pronounced temperature dependence than transfer to C136A ISA (Table 2), reflecting the greater activation entropy for the latter. Data were readily fit to a standard Eyring equation (eq b) (as shown in Figure 2, bottom), and ΔH^* for native ISA was determined to be 6.3 kcal/mol, relative to a value of 2.7 kcal/mol for C136A ISA (Table 2), and ΔS^* determined to be -27.3 cal K^{-1} mol^{-1} for native, relative to a value of -38.9 cal K^{-1} mol^{-1} for C136A ISA.

Influence of Viscosity on [2Fe-2S]$^{2+}$ Cluster Transfer. Fe−S cluster transfer from holo *Sp* ISA to apo *Sp* Fd was examined as a function of solution viscosity. Both 20 and 40% (w/v) sucrose were applied to study the influence of viscogen and yielded k_2 values of 148 and 142 M^{-1} min^{-1}, respectively. These values are not significantly different from that with 0% sucrose (170 M^{-1} min^{-1}). Such viscosity studies show that diffusion is not the rate-limiting step, which is most likely the process of Fe−S cluster transfer between ISA and Fd. ISA and Fd have been previously shown to form a complex through chemical cross-linking experiments (*20*), and this complex is most likely the key intermediate in the process of Fe−S cluster transfer. Studies of Fe−S cluster transfer from holo C136A *Sp* ISA to apo *Sp* Fd show a similar pattern of behavior, with k_2 values of ~206 (0% sucrose), 186 (20% sucrose), and 175 M^{-1} min^{-1} (40% sucrose).

NADPH- and Fd Reductase-Mediated Fe−S Cluster Transfer. To determine the role redox chemistry plays cluster transfer rates, the reaction was carried out under solution conditions that included catalytic amounts of NADPH, Fd reductase, and holo Fd. No enhancement of the cluster transfer rate constant was observed. In fact, the rate constant (k_2) for NADPH- and Fd reductase-mediated Fe−S cluster transfer from holo ISA to apo Fd was determined to be 156 M^{-1} min^{-1} and found to be slightly lower than that for the reaction without NADPH and Fd reductase (170 M^{-1} min^{-1}). After the first 10 min of reaction, the yield from each set of conditions (with and without addition of NADPH and Fd reductase) was ~18%. However, after 20 min, the yield of the NADPH- and Fd reductase-mediated Fe−S cluster transfer was only 42%, and significantly lower than the yield (45%) of the reaction without NADPH and Fd reductase. The cluster transfer reaction appeared to slow after 10 min, most likely as a result of Fd reduction by NADPH and Fd reductase. There is most likely an equilibrium electron transfer reaction between Fd and the cluster in ISA. Since the Fe−S clusters in ISA are reductively labile, this would result in degradation of the ISA-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 10 min, the concentration of holo Fd was low, and reduction of ISA by reduced Fd was slow. After 10 min, the increasing concentration of holo Fd facilitated catalytic reduction and degradation of holo ISA and slowed the second-order cluster reaction to apo Fd. This resulted in a lower value of k_2 for the NADPH- and Fd reductase-mediated Fe−S cluster transfer. 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 3) is as expected. The pseudo-first-order k_{obs} of 0.037 min^{-1} ($k_2 = 154$ M^{-1} min^{-1}) and overall cluster transfer yields were again found to be lower than observed in the absence of reduced Fd.

DISCUSSION

ISA type proteins are thought to provide an alternative platform for assembly of [2Fe-2S]$^{2+}$ clusters prior to transfer to target apoproteins (*19*, *20*, *24*), and may complement the previously characterized ISU-mediated cluster assembly pathway. Accordingly, the results of a comparison of the relative effectiveness of each pathway may provide significant mechanistic insight into the function of this class of protein that is likely to be of value in understanding other eukaryotic and bacterial ISA proteins. To this end, we have carried out a rigorous kinetic analysis of the chemistry of cluster transfer from representative eukaryotic ISA type proteins to an apo Fd target. It is important to note, however, that Fe−S clusters can often be assembled in vitro with no accessory proteins, and so the intracellular roles for ISA and ISU should perhaps be viewed less in the context of accelerating the reaction and more in the context of serving to avoid toxicity problems associated with free iron and sulfide.

Cluster Transfer Yield and Kinetic Parameters for ISA-Mediated Reconstitution of Fd. ISA type proteins are basic

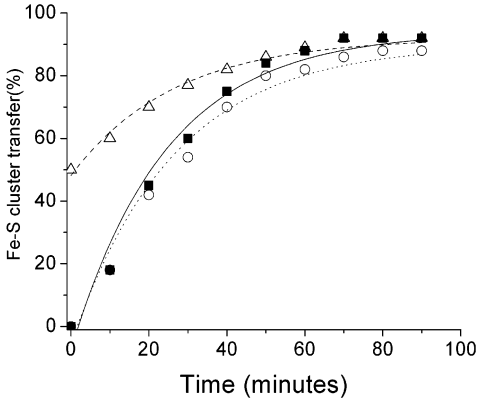

FIGURE 3: NADPH- and Fd reductase-mediated Fe−S cluster transfer with no initial holo Fd (○). The reaction mixture contained holo ISA (243 μM), apo Fd (16 μM), Fd reductase (48 nM), and NADPH (120 μM). NADPH- and Fd reductase-mediated Fe−S cluster transfer with 1 equiv of holo Fd added (△). The reaction mixture contained holo ISA (243 μM), apo Fd (8 μM), holo Fd (8 μM), Fd reductase (48 nM), and NADPH (120 μM). A control reaction (■), where the reaction mixture contained holo ISA (243 μM), apo Fd (16 μM), and neither Fd reductase nor NADPH, represented Fe−S cluster transfer from holo ISA to Fd without mediation by NADPH and Fd reductase. Reaction progress was assessed by use of the cytochrome *c* assay at 25 °C. A plot of the yield (percent) 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 with 1 molar equiv 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_{obs} for cluster transfer are 0.038 min^{-1} (○, $k_2 \sim 156$ M^{-1} min^{-1}), 0.037 min^{-1} (△, $k_2 \sim 154$ M^{-1} min^{-1}), and 0.04 min^{-1} (■, $k_2 \sim 170$ M^{-1} min^{-1}).

(pI ∼8−9), and Fd's are acidic (pI ∼4−5). They can be readily separated by native polyacrylamide gel electrophoresis, and reconstitution of apo Fd from holo ISA can be followed by native PAGE. Holo and apo ISA proteins remain in the loading lane, while apo and holo Fd are well-resolved following Coomassie staining (Figure 1). Holo Fd and holo ISA can also be distinguished from the apoprotein by use of established iron staining techniques. In larger-scale reactions, the product holo Fd can be readily isolated by chromatographic separation. As previously noted, the yield of the holoprotein following addition of wild type holo ISA to apo Fd (better than 90%) is significantly greater than that obtained by addition of Cys to Ala mutant ISA (∼65% for C136A ISA and ∼35% for C72A and C138A ISA). This most likely reflects the more labile Fe−S clusters in the Cys mutant proteins that give rise to a significant low yield of Fe−S cluster transfer to apo Fd over the shorter time course of the reaction. Control experiments carried out in the absence of ISA, but with addition of an equivalent concentration of iron and sulfide, yielded negligible yields of holo Fd under conditions analogous to those used for ISA-mediated reconstitution.

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

Table 3: Comparison of Second-Order Rate Constants (k_2) for Fe−S Cluster Transfer from ISA or ISU to Apo Fd[a]

ISU−apo Fd complex	k_2 (M^{-1} min^{-1})	ISA−apo Fd complex	k_2 (M^{-1} min^{-1})
Hs ISU−*Hs* Fd	540 ± 23 (25 °C)	*Sp* ISA−*Sp* Fd	170 ± 8 (25 °C)
	238 ± 18 (4 °C)		72 ± 6 (4 °C)
Sp ISU−*Hs* Fd	237 ± 19 (4 °C)	*Sp* ISA−*Hs* Fd	169 ± 8 (25 °C)
			67 ± 6 (4 °C)
Hs ISU−*Sp* Fd	294 ± 21 (25 °C)		
	94 ± 6 (4 °C)		
Sp ISU−*Sp* Fd	80 ± 5 (4 °C)		

[a] All data obtained with the Cyt *c* assay. Solution conditions for ISU reactions have been defined previously, but are comparable to those used for ISA reactions.

Cluster transfer from native *Sp* ISA to apo Fd was found to be slower than that from Cys derivatives of ISA. This result reflects the enhanced lability of the Cys72, Cys136, and Cys138 derivative proteins, relative to native, that most likely facilitates the process of Fe−S cluster transfer. Our previous investigations of ISU-mediated Fe−S cluster transfer to apo Fd had shown similar results (*19*, *21*). The D37A derivatives of both *Hs* and *Sp* ISU were shown to have lower rate constants (k_2) than the native ISU since the Fe−S clusters in D37A ISU were more stable than those of the native form (*19*, *21*).

The second-order rate constants for cluster transfer from native *Sp* ISA to apo *Sp* Fd were found to be comparable to that for *Sp* ISU transfer (k_2 values of 80 and 72 M^{-1} min^{-1}, respectively, at 4 °C). Both *Sp* ISA and *Sp* ISU exhibited significantly lower k_2 values than the *Hs* homologues with a k_2 of 238 M^{-1} min^{-1} for cluster transfer from *Hs* ISU to apo *Hs* Fd at 4 °C. Furthermore, while we previously found that the rate constants for cluster transfer from ISU depended on the source of the Fd target (*Hs* or *Sp*) rather than the ISU, the dependence on Fd was not found for ISA-mediated cluster transfer (Table 3). In fact, the cross reaction of *Sp* ISA with *Hs* apo Fd yielded a rate constant ($k_2 \sim 170$ M^{-1} min^{-1} at 25 °C) that was similar to that of *Sp* apo Fd obtained under similar conditions. These data demonstrate that the identity of the apo Fd does not affect the rates of cluster transfer from ISA to apo Fd. This in turn indicates that the binding site for ISA on apo Fd is most likely different than that for ISU.

Influence of pH on [2Fe-2S]$^{2+}$ Cluster Transfer. Both ISA- and ISU-mediated Fe−S cluster transfer to apo Fd have been shown to be pH-dependent. The involvement of protonation chemistry may reflect a variety of roles that 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. Figure 2 shows that the rate of cluster degradation of ISA is relatively slow at pH 6 and does not account for the pH dependence of the cluster transfer activity shown in the same figure. That is, the variation in the pH dependence of cluster stability for ISA (*20*) is not consistent with the cluster transfer profile exhibited in Figure 2, and so cluster protonation, which would tend to result in degradation of the Fe−S cofactor, is unlikely in this case. For ISU, we obtained evidence for general base catalysis involving proton removal from the attacking Cys nucleophiles from the apo Fd target. Application of this idea to ISA is supported by the pH profile, which yielded an apparent pK_a of 7.8, slightly higher than

the value of 6.9 found for ISU but consistent with deprotonation of a solvent accessible Cys (*25*), thereby providing a more effective nucleophilic center for facilitating cluster transfer to apo Fd. As for ISU, the possible involvement of a His (through salt bridge formation as a critical element of Fd recognition) cannot be excluded, although the measured pK_a is relatively high for His. As for ISU, the pH profiles do support solvent accessibility and general base catalysis as factors contributing to cluster transfer reactivity. The rather modest difference in reactivity for high and low pH limits most likely reflects the fact that deprotonation of Cys is not a truly rate-limiting step.

Activation Parameters for Cluster Transfer Provide Evidence of a Surface Solvent Accessible Site on Holo ISA. The temperature dependence (Figure 2, bottom) of cluster transfer reactions shows both enthalpic (+ve ΔH^*) and entropic (−ve ΔS^*) barriers (Table 2B). Enthalpic barriers dominate for native ISA, and entropic barriers dominate the reactivity of the Cys derivatives. Inasmuch as enthalpic terms typically reflect bond energies, the decrease in ΔH^* measured for the derivative presumably reflects weaker cluster binding in the ground state of holo C136A ISA. Since the cluster in the C136A derivative is known to be less stabilized than the native protein, the possibility that ΔH^* reflects weaker coordination appears to be reasonable. Furthermore, compared to activation parameters for Fe−S cluster transfer from native *Hs* ISU to apo Fd, both reactions of cluster transfer from wild type proteins are enthalpically dominated and have similar values of entropic change. This provides direct evidence that both reactions from wild type proteins follow similar mechanisms. Cluster transfer from wild type ISA to apo Fd shows a greater enthalpic change. This indicates that binding of Fe−S clusters in ISA is tighter than that in wild type ISU, which results in a lower rate of cluster transfer. All of these results are consistent with our previous findings for ISU (*19*, *21*) and highlight the importance of a solvent accessible cluster in promoting cluster lability and efficient cluster transfer. Importantly, the results from the temperature dependence study also demonstrate that any comparison of rate constants for native versus derivative proteins, or other classes of protein (for example, ISA vs ISU), must be discussed under specific temperature conditions. Clearly, the rate constants for ISA- and ISU-mediated cluster transfer are comparable at 25 °C but not at 4 °C, while ISA and the Cys derivatives show similar rate constants at 25 °C but not at 4 °C.

CONCLUSIONS

Here we report the results from the first detailed kinetic analysis of cluster transfer from ISA type proteins to a Fd target. Significantly, the rates of cluster transfer for ISA and ISU are found to be similar, at physiologically relevant temperatures, when comparing target proteins from the same source species (at least in the case of the Fd target selected for this work). Other factors that contribute to cluster transfer efficiency also appear to be similar, including the importance of cluster lability and solvent accessibility. The one major difference between ISA and ISU cluster transfer to apo Fd appears to be the binding recognition site on the target Fd. Given the very different primary sequences of ISA and ISU protein families, it is likely that Nature has formulated two distinct platforms that ultimately display similar chemical reactivities, differing only in partner recognition mechanisms through surface residues. Further elaboration of such details must await structural characterization of representatives of each protein family.

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BI026939+