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Effector Role Reversal during Evolution: The Case of Frataxin in Fe−S Cluster Biosynthesis

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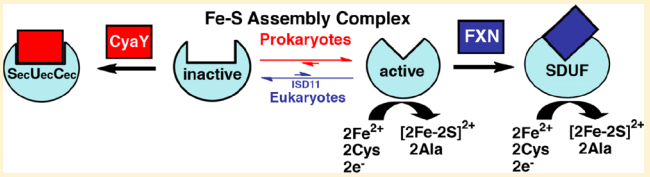
ABSTRACT: Human frataxin (FXN) has been intensively studied since the discovery that the FXN gene is associated with the neurodegenerative disease Friedreich's ataxia. Human FXN is a component of the NFS1-ISD11-ISCU2-FXN (SDUF) core Fe−S assembly complex and activates the cysteine desulfurase and Fe−S cluster biosynthesis reactions. In contrast, the *Escherichia coli* FXN homologue CyaY inhibits Fe−S cluster biosynthesis. To resolve this discrepancy, enzyme kinetic experiments were performed for the human and *E. coli* systems in which analogous cysteine desulfurase, Fe−S assembly scaffold, and frataxin components were interchanged. Surprisingly, our results reveal that activation or inhibition by the frataxin homologue is determined by which cysteine desulfurase is present and not by the identity of the frataxin homologue. These data are consistent with a model in which the frataxin-less Fe−S assembly complex exists as a mixture of functional and nonfunctional states, which are stabilized by binding of frataxin homologues. Intriguingly, this appears to be an unusual example in which modifications to an enzyme during evolution inverts or reverses the mode of control imparted by a regulatory molecule.



Iron−sulfur (Fe−S) clusters are protein cofactors that are essential for most living organisms. Fe−S clusters primarily function in electron transfer but also participate in substrate binding and activation, protein stabilization, regulation of gene expression or enzyme activity, radical generation, and as a sulfur donor.[1−3] These biological functions require the Fe−S cluster to vary from solvent accessible to completely buried and to be accommodated in a diverse set of protein scaffolds.[4] Despite this diversity, conserved metallochaperone proteins recognize and insert Fe−S clusters into apo Fe−S proteins through mechanisms that remain poorly understood. This assembly system must also control the reactivity of the building blocks for Fe−S clusters, ferrous iron and sulfide, to reduce toxicity and cell death. In humans, ferrous iron is prone to undergo Fenton chemistry: reaction with hydrogen peroxide to generate highly reactive hydroxyl radicals that damage proteins, lipids, and DNA, whereas sulfide is more toxic than cyanide, is a potent inhibitor of cytochrome *c* oxidase, and targets olfactory nerves, the eyes, and the brain.[5] Defects in these metallochaperone systems have profound effects on iron metabolism and result in mitochondrial dysfunction and human disease.[6−8]

In prokaryotes, three different Fe−S assembly systems have been characterized: the nitrogen fixation (NIF), iron−sulfur cluster assembly (ISC), and mobilization of sulfur (SUF) pathways.[9−13] *Escherichia coli* contains both ISC and SUF systems and appears to use the ISC pathway under "normal" growth conditions and the SUF system under conditions of iron limitation or oxidative stress.[14] The ISC operon is controlled by IscR, a Fe−S cluster binding transcriptional repressor, and contains genes for a cysteine desulfurase (IscS), scaffold protein (IscU), ferredoxin (Fdx), IscA, molecular chaperones (hscA and hscB), and IscX (also known as yfhj and ORF3). The scaffold protein IscU (in this article the *E. coli* homologue is referred to as $IscU_{ec}$, or U_{ec}) is the central protein in Fe−S cluster biosynthesis and is responsible for the assembly of [2Fe−2S] and [4Fe−4S] clusters.[15] IscS (*E. coli* homologue referred to as $IscS_{ec}$ or S_{ec}) catalyzes the PLP-dependent breakdown of cysteine to alanine and provides the inorganic sulfur for Fe−S clusters.[16,17] Fdx likely provides electrons for cluster assembly and IscA may be an iron donor or alternate scaffold.[18−20] The function of IscX is unknown, but it appears to compete with CyaY for binding to IscS.[21−23] Once the cluster is formed, the chaperones hscA and hscB facilitate the transfer of intact Fe−S clusters to apo target proteins.[24−26] A homologue of human frataxin (*E. coli* homologue referred to as CyaY or as C_{ec} in protein complexes in this article) is not part of the ISC operon but has been shown to bind to the $IscS_{ec}$−$IscU_{ec}$ ($S_{ec}U_{ec}$) complex[21,27,28] and inhibit Fe−S cluster assembly.[29]

A similar ISC Fe−S cluster assembly pathway functions in the matrix space of eukaryotic mitochondria.[8,30] The Fe−S assembly proteins are encoded by nuclear DNA and often contain a targeting sequence that is cleaved upon import into mitochondria. For the human pathway, the cysteine desulfurase NFS1 (59% identical to $IscS_{ec}$), which forms a eukaryotic-specific functional complex with ISD11,[31−33] the scaffold protein ISCU2 (also known as Isu2; 70% identical to $IscU_{ec}$), and FXN (20% identical to CyaY) form a NFS1-ISD11-ISCU2-FXN (SDUF) complex[34,35] that is analogous to the $IscS_{ec}$-

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$IscU_{ec}$-CyaY ($S_{ec}U_{ec}C_{ec}$) complex. Recent *in vitro* studies indicate that FXN is an allosteric activator of Fe−S cluster assembly that increases the catalytic efficiency (k_{cat}/K_M) for the cysteine desulfurase and the rate of Fe−S cluster biosynthesis.[34] Intriguingly, *in vitro* data indicate *E. coli* CyaY and human FXN have opposing regulator functions in Fe−S cluster biosynthesis.[29,34] Different physiological functions may also be supported by different *in vivo* phenotypes for *E. coli* and mouse model systems lacking frataxin.[36,37] However, both CyaY and FXN can at least partially complement strains of yeast lacking the frataxin homologue Yfh1,[38,39] which implies similar functions.

To resolve this discrepancy and better understand the role of frataxin in Fe−S cluster biosynthesis, enzyme kinetic experiments were performed for the human and *E. coli* systems in which analogous components were interchanged. Surprisingly, our results reveal that the cysteine desulfurase component, $IscS_{ec}$/NFS1-ISD11, rather than CyaY/FXN or $IscU_{ec}$/ISCU2 dictates the functional differences in how the frataxin homologues modulate Fe−S cluster assembly.

EXPERIMENTAL PROCEDURES

Preparation of Human Recombinant Proteins. Human NFS1 (Δ1−55) and ISD11 were coexpressed in *Escherichia coli* strain BL21(DE3) as previously described.[34,40] Cells were lysed by French press in 50 mM Tris pH 8.0, 500 mM NaCl, and 5 mM imidiazole. The NFS1-ISD11 (SD) complex was purified as previously described,[34] except that a cation exchange column was added between the Ni-NTA and S300 gel filtration chromatography steps. The yellow SD fractions from the Ni-NTA column were combined with 100 μM pyridoxal 5′-phosphate (PLP), 5 mM dithiothreitol (DTT), and 2 mM ethylenediaminetetracetic acid (EDTA), diluted 3-fold with 50 mM Tris pH 8.0, loaded onto a cation exchange column (16/14, POROS 50HS), and eluted with a linear gradient from 0 to 1000 mM NaCl. Human ISCU2 (Δ1−35) was purified as previously described,[34] except the fractions for the cation exchange column were eluted with a linear gradient from 0 to 1000 mM NaCl in 50 mM Tris, pH 7.5 buffer, and a S100 rather than a S300 gel filtration column was used. Human FXN (Δ1−55) was purified as previously described,[34] except a S100 rather than a S300 column was used. The Bradford method[41] or extinction coefficients of 42 670, 8250, and 26 030 M^{-1} cm^{-1} at 280 nm[42] were used to estimate the protein concentration of SD, ISCU2, and FXN, respectively.

Preparation of *E. coli* Recombinant Proteins. A plasmid containing the $IscS_{ec}$ gene[29] was transformed into BL21(DE3) *E. coli* cells and grown at 37 °C. Protein expression was induced with 0.5 mM IPTG when the OD_{600} was ∼0.6. Cells were harvested 5 h later and lysed by sonication in 50 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidiazole, and 100 μM PLP. After centrifugation, the supernatant was loaded on a Ni-NTA column (16/13, GBiosciences) and eluted with a linear gradient from 5 to 250 mM imidazole. Yellow fractions were concentrated and further purified on a Sephacryl S300 column (26/60, GE Healthcare) equilibrated in 50 mM HEPES pH 8.0, 250 mM NaCl. Plasmids that contained either $IscU_{ec}$ or CyaY genes[29] were transformed into BL21(DE3) cells and grown at 37 °C. Protein expression was induced with 0.5 mM IPTG when the OD_{600} was ∼0.6. Cells were harvested 3.5 h later and lysed by sonication in 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT. The supernatant was loaded onto a GST column and eluted with a linear gradient from 0 to 7 mM glutathione. The fractions that bound the column were concentrated and dialyzed into 50 mM Tris pH 8.0, 0.5 mM EDTA, and 1 mM DTT and then incubated at room temperature overnight with TEV protease. The tag cleavage product was loaded onto the GST column and the flow through was further purified on a S100 gel filtration column equilibrated in 50 mM HEPES pH 7.5, 150 mM NaCl. The Bradford method[41] or extinction coefficients of 41 370, 11 460, and 28 990 M^{-1} cm^{-1} at 280 nm[42] were used to estimate the protein concentration of $IscS_{ec}$, $IscU_{ec}$, and CyaY, respectively.

Titrations To Favor Protein Complex Formation. Cysteine desulfurase assays were used to determine reaction stoichiometries to favor protein complexes for different combinations of human and *E. coli* proteins. Cysteine desulfurase assays (see below for details) were initiated with 100 μM L-cysteine and included 0.5 μM of the cysteine desulfurase (SD or $IscS_{ec}$) and increasing amounts of the scaffold protein (ISCU2 or $IscU_{ec}$). Once the saturating number of equivalents of the scaffold protein were determined (Table 1), additional titrations were performed with increasing amounts of frataxin (FXN or CyaY).

Table 1. Protein Complexes and Reaction Stoichiometries

name	components	protein ratios
SDU	human NFS1/ISD11 and ISCU2	1:3
SDU_{ec}	human NFS1/ISD11; *E. coli* $IscU_{ec}$	1:3
SDUF	human NFS1/ISD11, ISCU2, and FXN	1:3:3
$SDU_{ec}F$	human NFS1/ISD11; *E. coli* $IscU_{ec}$; human FXN	1:3:200
$SDUC_{ec}$	human NFS1/ISD11 and ISCU2; *E. coli* CyaY	1:3:80
$SDU_{ec}C_{ec}$	human NFS1/ISD11; *E. coli* $IscU_{ec}$ and CyaY	1:3:30
$S_{ec}U_{ec}$	*E. coli* IscS and $IscU_{ec}$	1:5
$S_{ec}U$	*E. coli* IscS; human ISCU2	1:5
$S_{ec}U_{ec}C_{ec}$	*E. coli* IscS, $IscU_{ec}$, and CyaY	1:5:10
$S_{ec}UC_{ec}$	*E.coli* IscS; human ISCU2; *E. coli* CyaY	1:5:10
$S_{ec}U_{ec}F$	*E. coli* IscS and $IscU_{ec}$; human FXN	1:5:15
$S_{ec}UF$	*E. coli* IscS; human ISCU2 and FXN	1:5:15

Cysteine Desulfurase Activity Measurements. Assay mixtures (800 μL) contained 0.5 μM cysteine desulfurase (SD or $IscS_{ec}$), 10 μM PLP, 2 mM DTT, 5 μM $Fe(NH_4)_2(SO_4)_2$, and increasing or saturating amounts (Table 1) of the scaffold protein (ISCU2 or $IscU_{ec}$) and frataxin (FXN or CyaY) in 50 mM Tris pH 8.0, and 250 mM NaCl.[34,40,43] Samples were incubated in an anaerobic glovebox (10−14 °C) for 30 min. The cysteine desulfurase reactions were initiated by addition of L-cysteine (0.01−1 mM) at 37 °C and quenched after 10 min by the addition of 100 μL of 20 mM *N,N*-dimethyl-*p*-phenylenediamine in 7.2 N HCl and 100 μL of 30 mM $FeCl_3$ in 1.2 N HCl, which also initiated the conversion of sulfide to methylene blue. After a 20 min incubation at 37 °C, the absorption at 670 nm due to methylene blue formation was measured and compared with a Na_2S standard curve to quantitate sulfide production. Cysteine desulfurase reactions were also performed for mixed species Fe−S assembly complexes with 0 to 80 equiv of $Fe(NH_4)_2(SO_4)_2$ and 100 μM L-cysteine. Units are defined as μmol sulfide/μmol cysteine desulfurase per minute at 37 °C. Rates were fit to the Michaelis−Menten equation using KaleidaGraph (Synergy Software).

Fe−S Cluster Formation. Assay mixtures contained 8 μM of the cysteine desulfurase (SD or $IscS_{ec}$), 5 mM DTT, 200 μM

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$Fe(NH_4)_2(SO_4)_2$, 100 μM L-cysteine, 50 mM Tris pH 8.0, and 250 mM NaCl in a total volume of 0.2 mL. In addition, the number of equivalents (relative to the cysteine desulfurase) for the scaffold protein (ISCU2 or $IscU_{ec}$) and frataxin (FXN or CyaY) required to saturate the activity (above; Table 1) were included in the assay mixture. The scaffold protein was incubated with 5 mM DTT in 50 mM Tris pH 8, 250 mM NaCl in an anaerobic glovebox for 30 min prior to mixing with the remaining assay components in an anaerobic cuvette and then incubated at 10 °C for 30 min in the cuvette holder. The reaction was initiated by injecting L-cysteine to a final concentration of 100 μM with a gastight syringe. Fe–S cluster formation was monitored at 456 nm at 10 °C, and then the first 3000 s were fit as first-order kinetics using KaleidaGraph. The rate was converted to the activity using an extinction coefficient of 5.8 mM^{-1} cm^{-1} at 456 nm for [2Fe–2S] cluster absorbance.[15] Units are defined as the μmol of [2Fe–2S] cluster/μmol cysteine desulfurase per minute at 10 °C.

RESULTS

Ratio of Protein Complex Components for Activity Measurements. The objectives of this study were to provide insight into functional differences in frataxin-mediated regulation of Fe–S cluster biosynthesis for prokaryotes and eukaryotes by measuring cysteine desulfurase and Fe–S formation activities of mixed species protein complexes. The sulfur generated by the cysteine desulfurase reaction can be directly measured or can be used as a component of the Fe–S formation reaction. Titrations were performed to determine the relative ratios of the different components for maximum activity. Previous experiments revealed that adding 3 equiv of ISCU2 minimized the cysteine desulfurase activity of the NFS1-ISD11 (SD) complex (Table 1), likely through formation of a NFS1-ISD11-ISCU2 (SDU) species.[34] Likewise, adding 3 equiv of FXN to SDU maximized the cysteine desulfurase activity by forming a NFS1-ISD11-ISCU2-FXN (SDUF) complex.[34] A similar strategy was used here to determine and then compensate for weaker binding of *E. coli* proteins to the human assembly complex and human proteins to the *E. coli* assembly complex.

First, protein reaction ratios were determined for the human SD complexes by measuring cysteine desulfurase activities with increasing amounts of the scaffold protein, either ISCU2 or $IscU_{ec}$, and frataxin, either FXN or CyaY. The cysteine desulfurase activity of SD decreased with addition of either ISCU2 or $IscU_{ec}$ and minimized after the addition of ~2 equiv/SD (Figure 1A). 3 equiv of ISCU2 or $IscU_{ec}$ was therefore included with SD for kinetic experiments of the SDU or NFS1-ISD11-$IscU_{ec}$ (SDU_{ec}) complexes (Table 1). The binding of frataxin homologues to the SDU complex increased the cysteine desulfurase activity that maximized after the addition of either 3 equiv of FXN or 80 equiv of CyaY (Figure 1B). Similarly, the binding of frataxin homologues to the SDU_{ec} complex increased the cysteine desulfurase activity that maximized after either 200 equiv of FXN or 30 equiv of CyaY (Figure 1C).

Second, protein reaction ratios were determined for $IscS_{ec}$ using similar titrations. The cysteine desulfurase activity of $IscS_{ec}$ slightly decreased with addition of $IscU_{ec}$ and slightly increased with addition of ISCU2 (Figure 1D). This perturbation in $IscS_{ec}$ activity maximized after the addition of 3–5 equiv of $IscU_{ec}$ or ISCU2. To favor complex formation, 5 equiv of $IscU_{ec}$ or ISCU2 was therefore added to $IscS_{ec}$ (Table 1) for all kinetic experiments of $IscS_{ec}$–$IscU_{ec}$ ($S_{ec}U_{ec}$) and $IscS_{ec}$–ISCU2 ($S_{ec}U$) complexes, respectively. Titrations of up to 50 equiv of CyaY or FXN did not significantly change the cysteine desulfurase activity of either the $S_{ec}U_{ec}$ or $S_{ec}U$ complex (data not shown). Therefore, protein complex stoichiometries of 1:5:10 were used for the $S_{ec}U_{ec}C_{ec}$ and $S_{ec}UC_{ec}$ complexes based on the number of equivalents of CyaY previously determined to inhibit Fe–S cluster formation.[29] Similarly, protein complex stoichiometries of 1:5:15 were arbitrarily chosen for the Michaelis–Menten kinetics (below) of the $S_{ec}U_{ec}F$ and $S_{ec}UF$ complexes (Table 1).

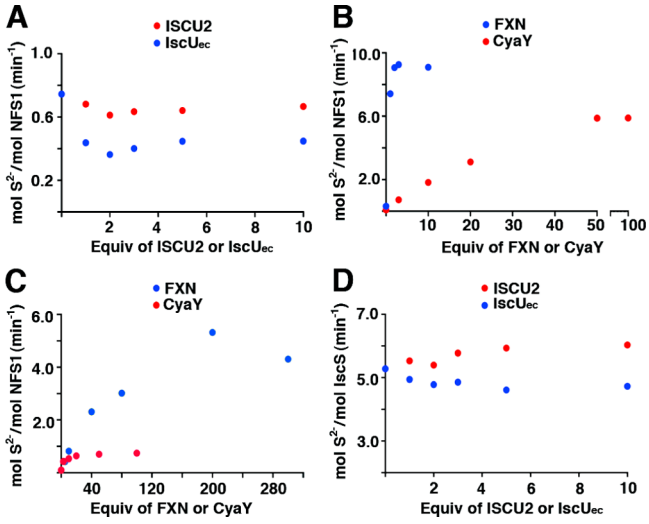

Figure 1. Determination of protein ratios that favor complex formation. Cysteine desulfurase activities were measured for (A) the human SD complex with increasing amounts of ISCU2 or $IscU_{ec}$, and for (B) the SDU and (C) SDU_{ec} complexes with increasing amounts of FXN or CyaY. (D) The cysteine desulfurase activity was measured for *E. coli* IscS with increasing amounts of ISCU2 or $IscU_{ec}$.

$IscU_{ec}$ and CyaY Stimulate the Cysteine Desulfurase Activity of the Human SD Complex. Saturating amounts of ISCU2 or $IscU_{ec}$ and FXN or CyaY determined above were added to the SD complex, and the rates of the cysteine desulfurase reaction were measured as a function of the L-cysteine concentration. The human SD complex exhibited a k_{cat}/K_M of 660 M^{-1} s^{-1} for the cysteine desulfurase reaction (Table 2), which is slightly higher (primarily due to a lower K_M for L-cysteine) than previously reported values of ~100 M^{-1} s^{-1}.[34,40] The addition of ISCU2 or $IscU_{ec}$ and formation of the SDU or SDU_{ec} complex slightly decreased the k_{cat} (Figure 2 and Table 2). The binding of FXN to form the SDUF and $SDU_{ec}F$ complexes stimulated the k_{cat} for the cysteine desulfurase reaction by ~7-fold relative to the SDU and SDU_{ec} complexes (Figure 2). Similarly, CyaY binding and formation of the $SDUC_{ec}$ and $SDU_{ec}C_{ec}$ complexes resulted in a 4–6-fold increase in the k_{cat} relative to SDU and SDU_{ec} (Figure 2). These experiments show that $IscU_{ec}$ can functionally substitute for ISCU2 and that CyaY can replace FXN as an activator of the cysteine desulfurase reaction for the human Fe–S assembly complex.

Iron-Dependent Stimulation of Cysteine Desulfurase Reaction for SD Complexes. Cysteine desulfurase activities were determined for SD complexes as a function of added iron. From 0 to 80 equiv of Fe(II) was added to the SDUF, $SDUC_{ec}$, $SDU_{ec}F$, and $SDU_{ec}C_{ec}$ complexes, and the cysteine desulfurase activity was measured (Figure 3A). The activity maximized after

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Table 2. Kinetic Data for Fe−S Assembly Complexes

name	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	Fe−S cluster assembly activity (min^{-1})
SD	1.5 ± 0.1	0.038 ± 0.012	660 ± 200	
SDU	1.0 ± 0.1	0.028 ± 0.008	600 ± 180	0.26 ± 0.22
SDUF	6.7 ± 0.4	0.011 ± 0.004	9700 ± 3900	
SDUF + Fe	15.4 ± 1.1	0.015 ± 0.006	16800 ± 6300	30.39 ± 1.18
$SDUC_{ec}$	5.7 ± 0.4	0.027 ± 0.01	3500 ± 1300	
$SDUC_{ec}$ + Fe	6.7 ± 0.2	0.016 ± 0.003	7100 ± 1400	3.94 ± 0.48
SDU_{ec}	0.59 ± 0.02	0.018 ± 0.003	550 ± 120	1.30 ± 0.32
$SDU_{ec}F$	4.2 ± 0.1	0.009 ± 0.002	7900 ± 2000	
$SDU_{ec}F$ + Fe	6.1 ± 0.3	0.012 ± 0.003	8300 ± 2300	25.54 ± 1.17
$SDU_{ec}C_{ec}$	2.1 ± 0.1	0.012 ± 0.003	2900 ± 840	
$SDU_{ec}C_{ec}$ + Fe	1.9 ± 0.1	0.011 ± 0.002	2800 ± 480	1.21 ± 0.19
S_{ec}	7.5 ± 0.1	0.017 ± 0.002	7300 ± 860	
$S_{ec}U_{ec}$	4.7 ± 0.3	0.019 ± 0.006	4100 ± 1400	12.23 ± 0.59
$S_{ec}U_{ec}C_{ec}$	5.8 ± 0.2	0.025 ± 0.003	3800 ± 440	
$S_{ec}U_{ec}C_{ec}$ + Fe	4.2 ± 0.1	0.020 ± 0.004	3500 ± 680	0.79 ± 1.76
$S_{ec}U_{ec}F$	5.2 ± 0.1	0.017 ± 0.002	5100 ± 870	
$S_{ec}U_{ec}F$ + Fe	4.5 ± 0.2	0.024 ± 0.004	3200 ± 940	4.56 ± 0.40
$S_{ec}U$	6.5 ± 0.2	0.019 ± 0.003	5800 ± 1000	14.63 ± 0.95
$S_{ec}UC_{ec}$	7.4 ± 0.1	0.016 ± 0.002	7600 ± 900	
$S_{ec}UC_{ec}$ + Fe	7.3 ± 0.3	0.017 ± 0.004	7100 ± 1600	0.88 ± 0.64
$S_{ec}UF$	7.6 ± 0.3	0.020 ± 0.006	6300 ± 1600	
$S_{ec}UF$ + Fe	6.9 ± 0.1	0.016 ± 0.002	7200 ± 890	3.62 ± 0.24

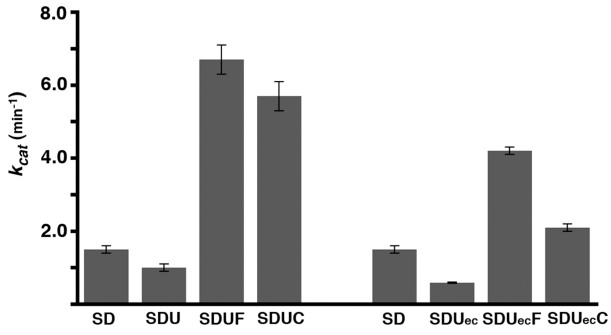

Figure 2. Stimulation of cysteine desulfurase reaction by addition of frataxin homologues. The k_{cat} for the cysteine desulfurase reaction was plotted for complexes of human SD with the scaffold protein (ISCU2 or $IscU_{ec}$) and frataxin (FXN or CyaY).

about 1, 1, and 5 equiv for the SDUF, $SDUC_{ec}$, and $SDU_{ec}F$ complexes, respectively. In contrast, the $SDU_{ec}C_{ec}$ complex did not exhibit a significant Fe-based activation. In addition, the SD complexes were incubated with 10 equiv of ferrous iron and the Michaelis−Menten parameters for the cysteine desulfurase activity were determined (Table 2). Ferrous iron had a large effect on the k_{cat} of the SDUF complex, as previously reported,[34] a moderate effect on the $SDUC_{ec}$ and $SDU_{ec}F$ complexes, and no effect on the $SDU_{ec}C_{ec}$ complex (Figure 3B).

CyaY Can Substitute for FXN in Fe−S Cluster Synthesis by Human SD Complexes. The rate of Fe−S cluster assembly for the SDU, SDU_{ec}, SDUF, $SDUC_{ec}$, $SDU_{ec}F$, and $SDU_{ec}C_{ec}$ complexes were determined by monitoring the increase in absorbance at 456 nm[15] at 10 °C and converting this change in absorbance to an activity measurement (μmol [2Fe−2S]/μmol of complex per minute). In the absence of FXN or CyaY, the SDU and SDU_{ec} complexes exhibited very low or no activity and were similar to a control reaction in which sulfide was substituted for cysteine (Table 2 and Figure 4). The addition of FXN greatly stimulated the Fe−S assembly

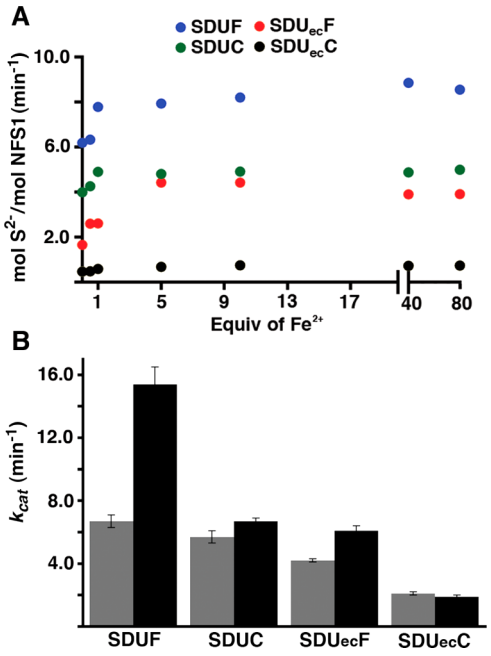

Figure 3. Iron-dependent perturbation of cysteine desulfurase activity for SD complexes. (A) Cysteine desulfurase activity plotted as a function of added ferrous iron. (B) Plot of k_{cat} values for the cysteine desulfurase reaction of SD complexes with (black) and without (gray) 10 equiv of ferrous iron.

activity of SDU and SDU_{ec}. In contrast, addition of CyaY resulted in a very modest or no increase in Fe−S assembly activity of the SDU and SDU_{ec} complexes, respectively. A CyaY-based stimulation was more readily observed when the Fe−S cluster assembly assays were repeated at room temperature (Figure 4A, inset). Thus, *E. coli* CyaY can substitute for human FXN in the human Fe−S assembly complex and strongly stimulate the cysteine desulfurase activity. CyaY also weakly stimulates the Fe−S cluster formation reaction.

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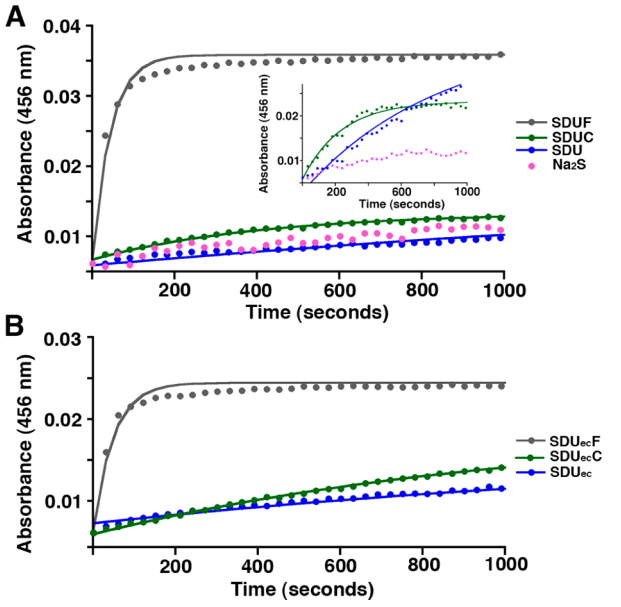

Figure 4. Stimulation of Fe–S cluster assembly activity by frataxin homologues. (A) The Fe–S assembly activities were measured at 10 °C for the Na_2S control and SDU, $SDUC_{ec}$, and SDUF complexes. The inset shows the activities of the SDU, $SDUC_{ec}$, and Na_2S control at 25 °C. (B) The Fe–S assembly activities were measured at 10 °C for the Na_2S control and SDU_{ec}, $SDU_{ec}C_{ec}$, and $SDU_{ec}F$ complexes.

CyaY and FXN Have Minor Effects on the Cysteine Desulfurase Activity of $IscS_{ec}$ Complexes. Purified recombinant $IscS_{ec}$ exhibited a k_{cat} of 7.5 min^{-1} (Figure 5A and Table 2), which is similar to the previously reported value of 8.5 min^{-1}.[44] Addition of $IscU_{ec}$ diminished the k_{cat} for the cysteine desulfurase reaction by a factor of 1.6, whereas a more moderate decrease in k_{cat} was observed upon ISCU2 addition (Figure 5A). Previously, $IscU_{ec}$ was shown to either have no effect on the activity of $IscS_{ec}$[45] or stimulate the activity 6-fold[46] using different experimental conditions and assays. The k_{cat} for $S_{ec}U_{ec}$ and $S_{ec}U$ were increased slightly upon addition of either CyaY or FXN (Figure 5A and Table 2). The $IscS_{ec}$ cysteine desulfurase activity was slightly decreased by added ferrous iron, consistent with a previous report,[47] which appeared to occur within the first equiv of added iron for the $S_{ec}U_{ec}F$ and $S_{ec}U_{ec}C_{ec}$ complexes and 10 equiv of iron for the $S_{ec}UF$ complex (Figure 5B). Steady state kinetics also revealed the k_{cat} slightly decreased upon addition of 10 equiv of ferrous iron (Figure 5A). The slight decrease in cysteine desulfurase activity upon addition of iron (Figure 5) is opposite of the general Fe-based activation observed for SD complexes (Figure 3). Overall, these results indicate that binding of frataxin homologues and ferrous iron have minor effects on the cysteine desulfurase activity of IscS complexes.

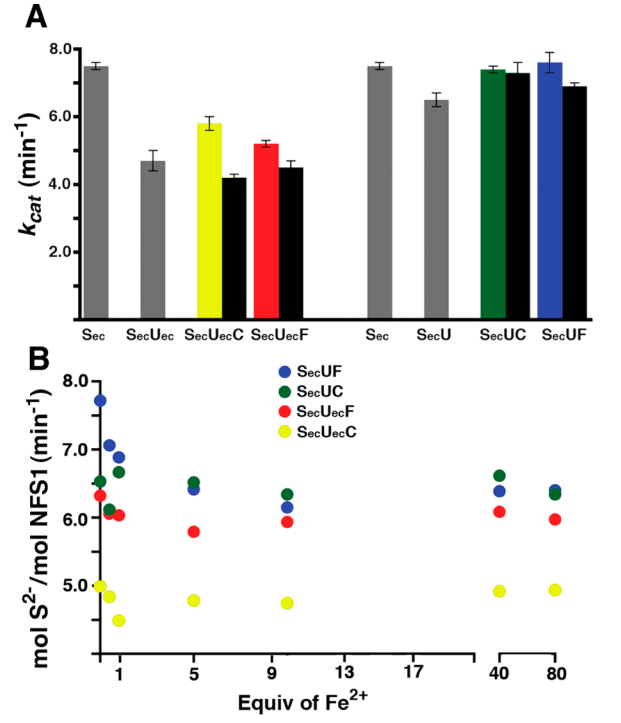

Figure 5. Cysteine desulfurase activities for IscS complexes with and without iron. (A) The k_{cat} for the cysteine desulfurase was plotted for complexes of *E. coli* IscS (S_{ec}) without (gray or colored) and with (black) 10 equiv of ferrous iron. (B) Cysteine desulfurase activities for IscS complexes were measured with 100 μM L-cysteine and increasing amounts of ferrous iron.

CyaY and FXN Inhibit Fe–S Assembly by $IscS_{ec}$ Complexes. The Fe–S cluster assembly rate for the $S_{ec}U_{ec}$ and $S_{ec}U$ complexes were similar to each other (Figure 6) and significantly higher than analogous SDU_{ec} and SDU complexes with NFS1-ISD11 (Figure 4). The addition of 10 equiv of CyaY to the $S_{ec}U_{ec}$ and $S_{ec}U$ complexes completely inhibited Fe–S cluster formation (Figure 6A,B), consistent with previous results.[29] At 2 equiv, CyaY is a slightly more effective inhibitor of the native $S_{ec}U_{ec}$ complex than the mixed species $S_{ec}U$ complex. The addition of FXN to the $S_{ec}U_{ec}$ and $S_{ec}U$ complexes (Figure 6C,D) also lowered the rate and possibly the amount of cluster bound; however, FXN did not completely eliminate Fe–S cluster formation. Thus, bacterial IscS assembly complexes are inhibited in Fe–S cluster formation by the inclusion of either frataxin ortholog.

DISCUSSION

The physiological function of frataxin has been intensively investigated since it was associated with the neurodegenerative disease Friedreich's ataxia (FRDA; reviewed in refs 48 and 49). A decrease in FXN levels in yeast and mouse model systems results in a phenotype that includes loss of Fe–S cluster enzyme activity, accumulation of iron in mitochondria, and susceptibility to oxidative stress.[50−52] In addition, FXN levels are correlated with the age of disease onset for FRDA patients,[53,54] and FXN missense mutations were identified that show FRDA-like phenotypes in mouse[52] and yeast[55] model systems. A role for frataxin as an Fe chaperone and/or donor in Fe–S cluster biosynthesis is often cited due to the frataxin depletion phenotype,[50−52] the ability of frataxin to bind Fe,[56−63] and the acceleration of Fe–S cluster formation by Fe-bound FXN *in vitro*.[58] Recent *in vitro* data as well as data presented here reveal that human FXN binds to the SDU complex and activates both the cysteine desulfurase and Fe–S formation reactions.[34] Moreover, assays using FXN missense mutations exhibit defects in binding and activation that appear to correlate with the severity of clinical progression for FRDA patients.[64,65] The physiological function of CyaY is less clear. In contrast to FXN in eukaryotes, deletion or overexpression of CyaY does not affect cellular growth, iron content, and survival after exposure to H_2O_2.[36,66] Part of the complication for these types of studies is the presence of both the ISC and SUF Fe–S

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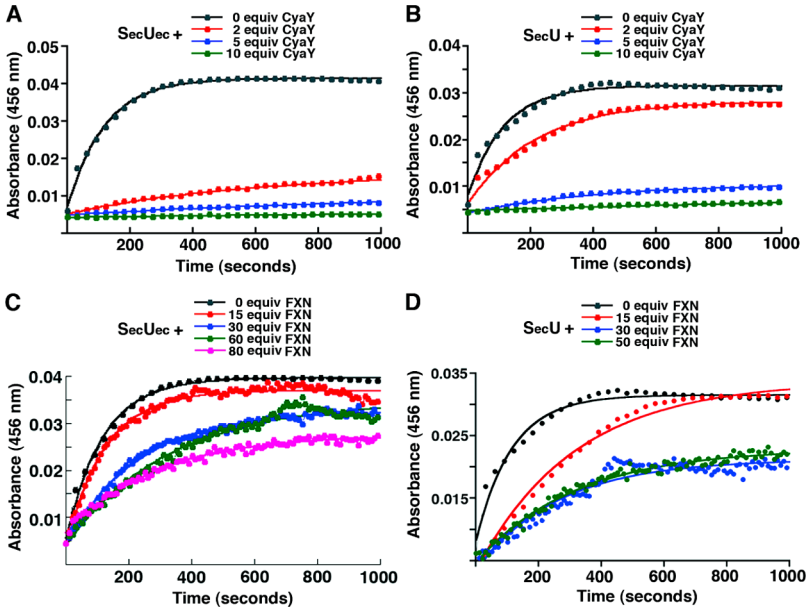

Figure 6. Frataxin-based inhibition of Fe−S cluster formation for IscS complexes. The rate of Fe−S cluster formation were measured for the $S_{ec}U_{ec}$ complex with increasing amounts of (A) CyaY and (C) FXN and for the $S_{ec}U$ complex with increasing amounts of (B) CyaY and (D) FXN.

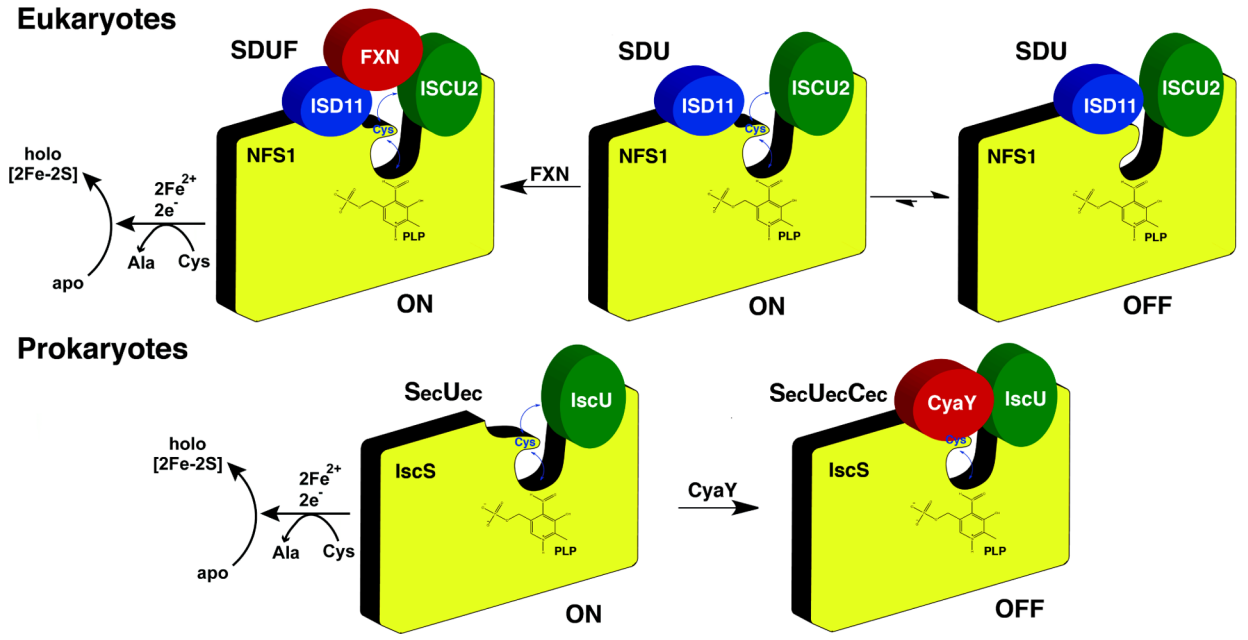

Figure 7. Working model for frataxin regulation of Fe−S cluster biosynthesis. In eukaryotes, a pre-equilibrium model is proposed in the absence of FXN in which a cysteine desulfurase and Fe−S cluster assembly deficient form is favored over a functional form of the SDU complex. FXN binding stabilizes the functional form and promotes sulfur transfer from NFS1 to ISCU2 and Fe−S cluster synthesis activities. In contrast, the prokaryotic $S_{ec}U_{ec}$ complex that lacks CyaY exhibits cysteine desulfurase and Fe−S assembly activities. CyaY binding may induce a conformational change in the $S_{ec}U_{ec}$ complex that does not significantly affect the cysteine desulfurase activity, but abolishes Fe−S cluster synthesis. The "ON" and "OFF" labels indicate the Fe−S assembly activity of the respective complexes, the blue arrows from the PLP to the mobile Cys loop indicate the presence of cysteine desulfurase activity, and the blue arrows from the Cys loop to the scaffold protein (shown in green) indicate interprotein sulfur transfer. The displayed model represents half of the expected dimeric Fe−S assembly complexes.

assembly systems *in E. coli*. If the physiological role of CyaY were to inhibit Fe−S cluster formation by the ISC system, then one would expect overexpression of CyaY in the absence of the SUF system to give a loss of Fe−S cluster phenotype. To the best of our knowledge this experiment has not been reported. Unfortunately, the complicated phenotype for human FXN depletion, the lack of a phenotype for CyaY depletion, and the opposing in vitro functions in Fe−S cluster assembly have contributed to the general confusion over the physiological role of this protein.

Here we used a function replacement approach to understand the factors that mediate the dramatically different in vitro responses to frataxin homologue binding. More specifically, *in vitro* cysteine desulfurase and Fe−S cluster assembly activities were evaluated for protein complexes in which components from the recombinant human Fe−S assembly system: the

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NFS1-ISD11 cysteine desulfurase complex, the scaffold protein ISCU2, and FXN, and the recombinant *E. coli* system composed of the IscS cysteine desulfurase, the scaffold protein $IscU_{ec}$, and CyaY, were interchanged. Surprisingly, our results reveal that activation or inhibition by the frataxin homologue is not controlled by the frataxin homologue but rather dictated by the cysteine desulfurase.

Interestingly, even though $IscS_{ec}$ and NFS1 are almost 60% identical, $IscS_{ec}$ exhibited a k_{cat}/K_M for the cysteine desulfurase activity that is 10−70-fold higher than that of the SD complex and was largely unchanged by the addition of $IscU_{ec}$ and CyaY.[34,40] In contrast, the low activity of the SD complex could be rescued by the addition of ISCU2 and FXN; the SDUF complex had a k_{cat}/K_M that is about double that of $IscS_{ec}$ alone or as a $S_{ec}U_{ec}$ or $S_{ec}U_{ec}C_{ec}$ complex. The $S_{ec}U_{ec}$ and SDU complexes also exhibit striking differences in their ability to facilitate Fe−S cluster formation. The $S_{ec}U_{ec}$ complex is highly active in the Fe−S cluster assembly assay, whereas the SDU complex exhibits little ability to mediate this reaction in the absence of frataxin. These differences were not due to the identity of the scaffold protein: interchanging the $IscU_{ec}$ and ISCU2 proteins did not substantially alter the higher rate of the complexes containing S_{ec} or the substantially inhibited rates of complexes containing SD (Figures 4 and 6). Moreover, addition of CyaY or FXN to these reactions inhibited Fe−S assembly by S_{ec}-containing complexes and activated Fe−S assembly by SD-containing complexes (Figures 4 and 6), albeit inhibition of the *E. coli* complex by CyaY was more efficient than by FXN and activation of the human complex by FXN was more efficient than by CyaY.

Our data therefore suggest that intrinsic features of $IscS_{ec}$ and human SD cysteine desulfurase components control both the basal activity and the effect of binding the frataxin homologues CyaY and FXN. In both cases, binding of the effector molecule switches the activity, either from "off" to "on" or from "on" to "off". In this sense, NFS1 behaves less like its ortholog IscS and more like its paralogs, such as SufS, which are characterized by modest activity that can be increased or activated by accessory proteins.[67−69] Evolutionary innovations in cysteine desulfurases from eukaryotes that control the basal activity and intrinsic features of frataxin homologue regulation are not known; binding of ISD11 and conserved amino acid changes in eukaryotes near the active site could play important roles in this process. Precise molecular details that regulate these features are currently under investigation.

Our data are consistent with FXN and CyaY binding playing a role in modulating Fe−S cluster formation by trapping an "on" conformation of SDU or inducing an "off" conformation of $S_{ec}U_{ec}$ (Figure 7). This model is consistent with the fact that SDU forms a stable complex with FXN,[34,35] and $S_{ec}U_{ec}$ forms a stable complex with CyaY.[21,27,28] The fact that FXN and CyaY can functionally replace each other *in vitro*, albeit at lowered efficiencies, is consistent with the formation of a complex with a common structure and with the ability of both of these proteins to suppress defects caused by deletion of YFH1, which encodes the *S. cerevisiae* frataxin homologue.[38,39] These modifications to the cysteine desulfurase may function as part of a protein-level regulatory mechanism. Future experiments will be aimed at providing additional mechanistic insight into the differences between the human SD complex and $IscS_{ec}$ and how these differences control Fe−S cluster assembly.

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Notes

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ABBREVIATIONS

C_{ec}, *E. coli* frataxin homologue CyaY; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; FRDA, Friedreich's ataxia; FXN, human frataxin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl *β*-D-1-thiogalactopyranoside; ISC, iron−sulfur cluster assembly pathway; $IscS_{ec}$, the *E. coli* cysteine desulfurase IscS; $IscU_{ec}$, the *E. coli* scaffold protein IscU; NIF, nitrogen fixation Fe−S cluster assembly pathway; PLP, pyridoxal-5′-phosphate; SD, protein complex composed of human NFS1 and ISD11; SDU, protein complex composed of human NFS1, ISD11, and ISCU2; SDU_{ec}, protein complex composed of human NFS1 and ISD11 with *E. coli* IscU; SDUF, protein complex composed of human NFS1, ISD11, ISCU2, and FXN; $SDU_{ec}F$, protein complex composed of human NFS1, ISD11, and FXN with *E. coli* Iscu2; $SDUC_{ec}$, protein complex composed of human NFS1, ISD11, and ISCU2 with *E. coli* CyaY; $SDU_{ec}C_{ec}$, protein complex composed of human NFS1 and ISD11 with *E. coli* IscU and CyaY; S_{ec}, *E. coli* IscS; $S_{ec}U_{ec}$, protein complex composed of *E. coli* IscS and IscU; $S_{ec}U$, protein complex composed of *E. coli* IscS with human ISCU2; $S_{ec}U_{ec}C_{ec}$, protein complex composed of *E. coli* IscS, IscU, and CyaY; $S_{ec}UC_{ec}$, protein complex composed of *E. coli* IscS and CyaY with human ISCU2; $S_{ec}U_{ec}F$, protein complex composed of *E. coli* IscS and IscU and human frataxin; $S_{ec}UF$, protein complex composed of *E. coli* IscS with human ISCU2 and frataxin; SUF, sulfur mobilization Fe−S cluster assembly pathway; Tris, tris(hydroxymethyl)-aminomethane; U_{ec}, *E. coli* IscU.

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