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Human Frataxin Is an Allosteric Switch That Activates the Fe−S Cluster Biosynthetic Complex[†]

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ABSTRACT: Cellular depletion of the human protein frataxin is correlated with the neurodegenerative disease Friedreich's ataxia and results in the inactivation of Fe−S cluster proteins. Most researchers agree that frataxin functions in the biogenesis of Fe−S clusters, but its precise role in this process is unclear. Here we provide in vitro evidence that human frataxin binds to a Nfs1, Isd11, and Isu2 complex to generate the four-component core machinery for Fe−S cluster biosynthesis. Frataxin binding dramatically changes the K_M for cysteine from 0.59 to 0.011 mM and the catalytic efficiency (k_{cat}/K_M) of the cysteine desulfurase from 25 to 7900 M^{-1} s^{-1}. Oxidizing conditions diminish the levels of both complex formation and frataxin-based activation, whereas ferrous iron further stimulates cysteine desulfurase activity. Together, these results indicate human frataxin functions with Fe^{2+} as an allosteric activator that triggers sulfur delivery and Fe−S cluster assembly. We propose a model in which cellular frataxin levels regulate human Fe−S cluster biosynthesis that has implications for mitochondrial dysfunction, oxidative stress response, and both neurodegenerative and cardiovascular disease.

Iron−sulfur (Fe−S) clusters are essential cofactors that are required for biochemical reactions and processes in all life forms. In vitro, iron and sulfide can be used to chemically synthesize Fe−S clusters or activate Fe−S apoproteins (*1*, *2*). In vivo, Fe−S clusters are synthesized by biosynthetic pathways that are required to circumvent the toxicity and indiscriminant reactivity of "free" iron and sulfide. Fe−S clusters are synthesized in prokaryotes using the nitrogen fixation (NIF), iron−sulfur cluster assembly (ISC), and mobilization of sulfur (SUF) pathways (*3*). In eukaryotes, the biosynthesis of Fe−S clusters occurs in the matrix space of the mitochondria and includes at least a dozen proteins (*4*, *5*). Defects in the human Fe−S cluster biosynthesis pathway are associated with cardiomyopathy and neurodegenerative ataxia and contribute to genomic instability, the development of cancer, and aging (*5*−*7*).

Many of the required proteins for Fe−S cluster biosynthesis have been identified, and their general biochemical function has been elucidated. Human Isu2 (homologue of bacterial IscU) is a 14 kDa monomer (*8*) that provides a scaffold for building [Fe_2S_2] and, possibly, [Fe_4S_4] clusters. Clusters are synthesized on Isu2 through interactions with proteins that mediate sulfur, iron, and electron transfer. Sulfur atoms are provided by the 94 kDa homodimeric cysteine desulfurase Nfs1, which catalyzes the pyridoxal 5′-phosphate-dependent breakdown of cysteine to alanine (*9*, *10*). During Nfs1 catalysis, a cysteine on a flexible loop attacks a substrate−PLP[1] adduct to generate a persulfide species. The resulting sulfane sulfur can then be transferred in a rate-limiting step to the scaffold protein for Fe−S biosynthesis or, in vitro, can be reductively cleaved by redox agents to form sulfide ion (*10*, *11*). It has been proposed that iron atoms are donated by both frataxin (Fxn) (*12*−*14*) and IscA (*15*). Electrons may be provided by a ferredoxin (*16*, *17*). Finally, chaperone proteins interact with Isu and assist in delivering intact Fe−S clusters to their apo targets (*18*). ATP and GTP are also required during some stage of Fe−S cluster biogenesis (*19*).

The role of Fxn in this Fe−S cluster assembly process remains elusive. In vivo studies support a role for the 14 kDa monomeric Fxn in Fe−S cluster assembly (*20*, *21*), possibly as an iron chaperone. An iron detoxification and storage role has also been proposed (*22*, *23*) and refuted (*24*, *25*). Recently, Pastore and coworkers provided evidence that the *Escherichia coli* Fxn homologue functions as a negative regulator for Fe−S cluster assembly (*26*). Such a function for eukaryotic Fxn is difficult to reconcile with genetic and biochemical data, which suggest Fxn facilitates rather than inhibits Fe−S cluster biosynthesis (*12*, *20*, *21*, *27*, *28*). Further kinetic experiments that address the effect of human frataxin on Fe−S cluster biosynthesis are required to resolve this issue.

A network of protein−protein interactions appears to be critical for Fe−S cluster biosynthesis (*5*, *29*). Fxn, Isu, and Nfs1 physically interact in pull-down experiments using yeast (*28*) and human (*30*) mitochondrial extracts. Iron-dependent Fxn−Isu interactions are further supported by genetic studies in yeast (*31*) and biophysical experiments with recombinant human proteins (*12*). Isu−Nfs1 interactions are defined by a crystal structure of the homologous *E. coli* IscU−IscS complex (*32*). In eukaryotes, an additional protein, Isd11, is also known to form a complex with Nfs1 and is vital for Nfs1 function (*33*−*35*). Deletion of genes encoding Nfs1, Isd11, Isu1, or Fxn in *Saccharomyces cerevisiae* produces similar phenotypes that include defects in Fe−S cluster

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[1]Abbreviations: ABC, ammonium bicarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FRDA, Friedreich's ataxia; Fxn, frataxin; GTP, guanosine 5′-triphosphate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; PLP, pyridoxal 5′-phosphate; SD, Nfs1 and Isd11 protein complex; SDF, Nfs1, Isd11, and Fxn protein complex; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SDU, Nfs1, Isd11, and Isu2 protein complex; SDUF, Nfs1, Isd11, Isu2, and frataxin protein complex; TCEP, tris(2-carboxyethyl)phosphine; Tris, tris(hydroxymethyl)-aminomethane.

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proteins (*33*, *36*). These data hint at a multiprotein complex that functions as a Fe−S cluster assembly machine. Here in vitro evidence that the foundation for the human Fe−S cluster assembly machine is made up of a Nfs1, Isd11, Isu2, and Fxn protein complex is presented. In addition, kinetic data that support a role for human Fxn as an allosteric switch that activates this complex for Fe−S cluster biosynthesis are provided.

EXPERIMENTAL PROCEDURES

Protein Purification. Plasmids containing human Nfs1 (Δ1−55) and Isd11 (pZM4) were generously provided by S. Leimkühler (*37*). The Nfs1 and Isd11 plasmids were transformed into *E. coli* strain BL21(DE3) for coexpression. Cells were grown at 37 °C until they reached an OD of 0.6; the temperature was decreased to 16 °C, and protein expression was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested 16 h later and transferred to a temperature-controlled (10−15 °C) anaerobic glovebox (mBraun). Cells were resuspended in buffer A [50 mM Tris (pH 8.0)] with 500 mM NaCl, 5 mM imidazole, and 100 μM pyridoxal 5′-phosphate (PLP) and ruptured by sonication (Branson sonifier 450). Soluble material was loaded onto a Ni-NTA column (16/13, GBiosciences) and eluted with a linear gradient from 5 to 250 mM imidazole. Yellow fractions were concentrated, combined with 100 μM PLP, 5 mM dithiothreitol (DTT), and 2 mM EDTA, and loaded onto a Sephacryl S300 column (26/60, GE) equilibrated in buffer A with 250 mM NaCl. Fractions containing Nfs1 (47 kDa) and Isd11 (11 kDa) were >95% pure as determined by SDS−PAGE. Protein concentrations were estimated by the Bradford method (*38*) or using an extinction coefficient of 42670 M^{-1} cm^{-1} at 280 nm (*39*). The yield of Nfs1 and Isd11 was 3 mg/L of cells.

The human Isu2 (Δ1−35) and Fxn (Δ1−55) genes were codon-optimized for *E. coli*, synthesized by IDT, and subcloned into pET11a vectors. The resulting plasmids were transformed into *E. coli* BL21(DE3) cells, which were grown at 37 °C. Protein expression was induced at an OD_{600} of 0.6 with 0.5 mM IPTG. Cells were harvested 5 h later and sonicated in buffer B [50 mM Tris (pH 7.5)]. For Isu2, the soluble fraction was applied to an anion exchange column (26/22, POROS 50HQ, Applied Biosystems), and the flow-through was collected (Isu2 does not bind) and loaded onto a cation exchange column (16/14, POROS 50HS, Applied Biosystems). Isu2 was eluted with a linear gradient from 0 to 400 mM NaCl in buffer B. Isu2 fractions (14 kDa) were concentrated and further purified with a Sephacryl S300 (26/60) column equilibrated in buffer C (50 mM HEPES and 150 mM NaCl). An extinction coefficient of 8250 M^{-1} cm^{-1} at 280 nm (*39*) was used to estimate the protein concentration. This procedure yielded 4 mg of >95% pure Isu2/L of cells.

For Fxn, the soluble fraction after sonication was loaded onto an anion exchange column (26/22, POROS 50HQ) and eluted with a linear gradient from 0 to 800 mM NaCl in buffer B. The monomeric Fxn fractions were collected and further purified on a S300 gel filtration column equilibrated in buffer C. The fractions containing full-length (residues 56−210) and truncated (residues 82−210) Fxn were collected and further purified by anion exchange chromatography using a linear gradient from 120 to 250 mM NaCl in buffer B. Full-length Fxn and truncated Fxn were collected separately and confirmed by N-terminal sequencing. Truncated Fxn (14 kDa) was used for all experiments. An extinction coefficient of 26030 M^{-1} cm^{-1} at 280 nm (*39*) was used to estimate the protein concentration. This procedure yielded 6 mg of >95% pure truncated Fxn/L of cells.

Cysteine Desulfurase Activity Measurements. Sulfide production for the Nfs1 and Isd11 complex (SD) was measured using slight modifications to a methylene blue assay described by Leimkühler and co-workers (*37*, *40*). Assay mixtures in a total volume of 0.8 mL contained 1 μM SD, 10 μM PLP, 2 mM DTT, 250 mM NaCl, and 50 mM Tris (pH 8.0). The reactions were initiated by addition of L-cysteine (0.1−1.0 mM) and the mixtures incubated at 37 °C. Sulfide production was linear for the first 30 min, and an incubation time of 10 min was chosen to generate sufficient product for straightforward detection. Assays were quenched by 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. After being incubated for 20 min to allow for methylene blue synthesis, the samples were centrifuged (5 min at 12000*g*), and the absorbance at 670 nm was converted to a sulfide concentration using a standard curve.

Similarly, cysteine desulfurase activities were measured for SD after a 30 min anaerobic incubation with 3 equiv of Isu2 or Fxn. In a separate experiment, 3 equiv of Isu2 and Fxn were incubated with SD for 30 min prior to initiation of the reaction with L-cysteine (2.5−200 μM). Michaelis−Menten kinetics for the cysteine desulfurase reaction were also examined for the protein components in the presence of 10 equiv of $Fe(NH_4)_2(SO_4)_2$. Reaction rates as a function of cysteine concentration were fit to the Michaelis−Menten equation using KaleidaGraph (Synergy Software).

The cysteine desulfurase activity was also determined at a "physiological" (0.1 mM) cysteine concentration with 1 μM SD alone, and with 3 equiv of Isu2 and/or Fxn. For the Fe titration, the proteins were diluted by a factor of 2 and 0−5 μM $Fe(NH_4)_2(SO_4)_2$ was added. To assess if other metal ions also stimulated cysteine desulfurase activity, we repeated the assay of SD with 3 equiv of both Isu2 and Fxn in the presence of 10 equiv of $FeCl_3$, $Zn(OAc)_2$, $CoSO_4$, $NiCl_2$, $MgCl_2$, $CuSO_4$, $Ca(OAc)_2$, or $MnCl_2$.

Fe−S Cluster Formation Assay. Assay mixtures in 0.25 mL contained 8 μM SD, 24 μM Isu2, 100 μM $Fe(NH_4)_2(SO_4)_2$, 3 mM DTT, and 0−16 μM Fxn in 50 mM Tris (pH 7.4) with 250 mM NaCl. The assay mixture was incubated in an anaerobic glovebox for 30 min before the reaction was initiated with 0.1 mM L-cysteine. Fe−S cluster formation was monitored at 456 nm for 90 min. Units are defined as the amount of Isu2 required to produce 1 μmol of Fe−S cluster per minute at 25 °C. An extinction coefficient of 5.8 mM^{-1} cm^{-1} was used for $[Fe_2S_2]$ cluster absorption (*41*). The rate of Fe−S cluster formation was fit to first-order kinetics by using Agilent UV−visible ChemStation software.

Protein Complex Determination. Assays included 40 μM SD, 120 μM Isu2, 120 μM Fxn, 10 mM DTT, 10 mM EDTA, 0.8 mM $Fe(NH_4)_2(SO_4)_2$, 50 mM NaCl, and 100 mM HEPES (pH 7.5). Samples were incubated for 30 min in an anaerobic glovebox and evaluated for protein complex formation using blue native gels (*42*) that contained 6.5% separating and 5% stacking layers. Titration experiments with Fxn and Isu2 were performed using the same procedure except the titrant concentration was varied from 5 to 120 μM. The amount of protein on the native gel was estimated by densitometry; gels were scanned with a Typhoon Trio Imager and analyzed with ImageQuant. The slower migrating band was cut out of the native gel and dehydrated with 66% acetonitrile in 25 mM ammonium bicarbonate (ABC) (pH 8). The protein was extracted by incubation with 0.1% SDS and 10 mM ABC (pH 8) for 30 min at room temperature and mixed with an equal volume of 66% acetonitrile

Table 1: Rate Constants for Human Nfs1 Activity with Isu2 and Fxn

complex	k_{cat} (min^{-1})	K_M^{cys} (mM)	k_{cat}/K_M (M^{-1} s^{-1})
SD	1.9 ± 0.1	0.34 ± 0.06	93
SD with Fxn	1.7 ± 0.1	0.33 ± 0.06	86
SD with Isu2	0.89 ± 0.04	0.59 ± 0.05	25
SD with Isu2 and Fxn	5.2 ± 0.4	0.011 ± 0.003	7900
SD with Isu2, Fxn, and Fe^{2+}	10.7 ± 1.0	0.017 ± 0.005	10500

and 25 mM ABC (pH 8). The solution was concentrated with a speed-vac, and the proteins were analyzed by 14% SDS−PAGE.

Protein Complex Isolation. SD was incubated with 3 equiv of Isu2 and Fxn, 2 equiv of PLP, 5 mM tris(2-carboxyethyl)phosphine (TCEP) or DTT, and 50 mM Tris (pH 7.4) with 50 mM NaCl buffer for 30 min in an anaerobic glovebox. The Nfs1, Isd11, and Isu2 complex (SDU) and the Nfs1, Isd11, Isu2, and Fxn complex (SDUF) were loaded onto a S200 gel filtration column and compared to molecular mass standards to estimate the molecular mass. Molecular mass standards (Bio-Rad) included bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1.4 kDa). SDU and SDUF were also further purified on a cation exchange column and eluted with a linear gradient from 150 to 1000 mM NaCl in buffer B. Protein complexes were confirmed by SDS−PAGE.

RESULTS

Isu2 and Frataxin Stimulate Nfs1 and Isd11 Cysteine Desulfurase Activity. Human recombinant Fxn and Isu2 were assayed for their ability to alter the kinetics of the cysteine desulfurase for Fe−S cluster biosynthesis. In this assay, cysteine was converted to alanine and the resulting persulfide intermediate was reduced with DTT and detected as sulfide. In the absence of additional proteins, purified human recombinant SD exhibited Michaelis−Menten kinetics for sulfide production with a Michaelis constant (K_M) of 0.34 mM for cysteine and a k_{cat} of 1.9 min^{-1} (Table 1). The resulting catalytic efficiency (k_{cat}/K_M) of 93 M^{-1} s^{-1} for recombinant human SD was nearly identical to a previously reported value (*37*) of 96 M^{-1} s^{-1}. The kinetic parameters k_{cat} and K_M were not significantly changed upon addition of Fxn to SD. In contrast, the k_{cat} was lowered by a factor of 2 and the K_M nearly doubled upon addition of Isu2 to SD (Table 1).

Both the k_{cat} and the K_M were dramatically altered by the addition of Fxn to SD in the presence of Isu2, which resulted in an increase in the catalytic efficiency from 25 to 7900 M^{-1} s^{-1} (Table 1). This increase in catalytic efficiency was due to a nearly 6-fold faster k_{cat} for sulfide production and a change in K_M for cysteine from 0.59 to 0.011 mM. Because the physiological concentration of cysteine in eukaryotic cells is thought to be 0.1 mM (*43*−*45*), we determined cysteine desulfurase activities at this concentration to mimic cellular conditions. The sulfide production activity for SD was similar when either Isu2 or Fxn was individually added but dramatically increased when both were present (Figure 1A). Together, these data indicate that Fxn greatly stimulates the cysteine desulfurase in an Isu2-dependent manner, facilitating catalysis at physiological cysteine concentrations.

Given the iron dependence of Fxn−Isu interactions (*12*, *28*, *46*), the ferrous iron dependence of the kinetics of sulfide production was explored. Iron had little effect on the cysteine desulfurase activities of SD, SD with Isu2, and SD with Fxn (Figure 1A). In contrast, iron stimulated the activity of SD when both Isu2 and Fxn were present; kinetic analysis revealed that this increase in activity was due to a doubling of the k_{cat} rate rather than a significant change in the K_M (Table 1). A reaction mixture containing SD, Isu2, and Fxn was titrated with iron, and the maximal activity was achieved after the addition of 1 equiv of iron (Figure 1B). A similar SD, Isu2, and Fxn reaction was not significantly stimulated by ferric iron and other first row transition metals, with the possible exception of Mn^{2+} (Figure 2). Together, these data indicated that both Isu2 and Fxn are necessary for the activation of the SD cysteine desulfurase, which can be further stimulated by 1 equiv of ferrous iron.

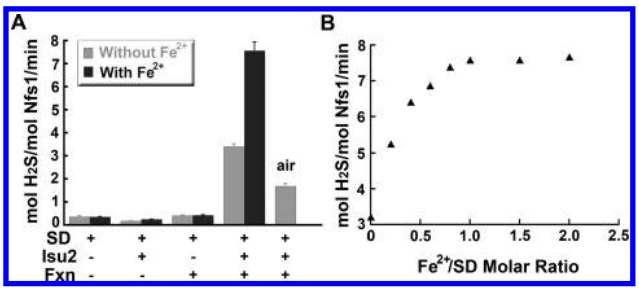

FIGURE 1: Frataxin and ferrous iron stimulate cysteine desulfurase activity. (A) Rates of SD sulfide production were determined in the presence of 3 equiv of Isu2, 3 equiv of Fxn, and either 0 or 10 equiv of ferrous iron. Equivalents are calculated on the basis of the SD concentration. For the oxidized sample, SD was incubated with Isu2 and Fxn for 30 min in air, and then the reaction was initiated with cysteine and DTT. Error bars are for three independent measurements. (B) Rates of sulfide production were determined for SD in the presence of 3 equiv of Isu2 and Fxn and increasing amounts of ferrous iron.

Human Frataxin Accelerates Fe−S Cluster Assembly. Fe−S cluster biosynthesis rates were determined by following the increase in absorbance at 456 nm, commonly used to monitor $[Fe_2S_2]$ cluster formation (*41*), in the presence of 0.1 mM cysteine and 0.1 mM Fe^{2+}. An assay containing SD with Isu2 exhibited a specific activity of 0.0027 unit/mg, whereas addition of Fxn increased the rate by a factor of 25 (Figure 3A). Titration of Fxn into a reaction mixture containing SD and Isu2 revealed that the specific activity increased linearly with added Fxn and was maximal after the addition of 1 equiv of Fxn and SD (Figure 3B). These results revealed that Fxn stimulates formation of Fe−S clusters on Isu2 and indicated that Fxn functions as an activator for human Fe−S cluster biosynthesis.

Human Nfs1, Isd11, Isu2, and Fxn Form a Multiprotein Complex. Because Isu2 and Fxn together dramatically changed the kinetic parameters of the cysteine desulfurase (Table 1), we hypothesized that Nfs1, Isd11, Isu2, and Fxn may form a

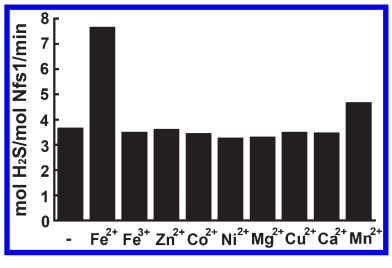

FIGURE 2: Stimulation of cysteine desulfurase activity by metal ions. Rates of sulfide production were determined for SD in the presence of 3 equiv of Isu2 and Fxn and 10 equiv of metal ions.

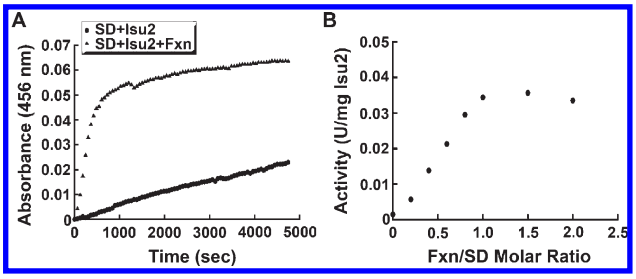

FIGURE 3: Frataxin accelerates the rate of Fe−S cluster biosynthesis. (A) Fe−S cluster formation was monitored by an increase in absorbance at 456 nm as a function of time. Assays included SD with 3 equiv of Isu2 and SD with 3 equiv of both Isu2 and Fxn. (B) The rate of Fe−S cluster biosynthesis was determined as a function of added Fxn.

multiprotein complex. To test this hypothesis, we incubated SD, Isu2, and Fxn with 5−10 mM DTT in an anaerobic glovebox for 30 min and then tested for protein−protein interactions using native gel electrophoresis (Figure 4A). Addition of all four proteins resulted in the appearance of a slower migrating band compared to Fxn on the native gel that was consistent with formation of a protein complex. The slower migrating band was dependent upon the presence of each of the protein components (Figure 4A). This protein complex formed in the presence of 10 mM EDTA and the addition of ferrous iron had no obvious affect on band intensity. Cutting this band out of the native gel and analyzing with SDS−PAGE revealed the presence of all four added proteins (Figure 4B). This slower migrating band on the native gel was therefore associated with a noncovalent Nfs1, Isd11, Isu2, and Fxn protein complex (SDUF) that was stable in the presence of metal chelators.

Next, protein titration experiments were performed to probe the requirements for complex formation and provide an indication of the subunit stoichiometry. First, 0−3 equiv of Fxn was added to a mixture of SD and Isu2, followed by native gel electrophoresis to monitor SDUF formation (Figure 5A). The slower migrating band was not observed in the absence of Fxn, increased in intensity with added Fxn, and was maximal after the addition of 1 equiv of Fxn and SD (Figure 5B). Similarly, Isu2 was titrated into a mixture of SD and Fxn (Figure 6A). The slower migrating band was not formed in the absence of Isu2, increased with added Isu2, and was maximal after the addition of 2 equiv of Isu2 and SD (Figure 6B). These experiments further supported SD, Isu2, and Fxn as components of the protein complex associated with the slower migrating band and placed an upper limit on the stoichiometry at one Fxn and two Isu subunits for each Nfs1.

Oxidizing Conditions Inhibit Complex Formation. The redox requirements for complex formation and frataxin-dependent activation were determined. First, a native gel assay was used to determine that in the absence of a reducing agent (DTT or TCEP) the intensity of the slower migrating band was diminished (Figure 4A, lane 8). Second, a SD sample was incubated with Isu2 and Fxn in air for 30 min; DTT and cysteine were added, and the cysteine desulfurase activity was measured. This air-oxidized sample exhibited diminished cysteine desulfurase activity compared to the equivalent anaerobic sample with all four components (Figure 1A). Together, the data indicated a correlation between SDUF formation and activation of the Fe−S cluster assembly machinery that was influenced by redox conditions.

Nfs1, Isd11, Isu2, and Fxn Form ~150−180 kDa Complexes. Analytical gel filtration chromatography was used to

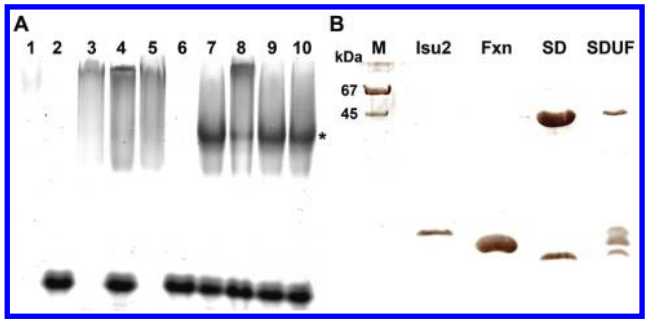

FIGURE 4: Nfs1 or Isd11, Isu2, and Fxn form a multiprotein complex. (A) Native gel showing protein requirements for complex formation: lane 1, Isu2 (Isu2 has an estimated pI of 8.9 and is difficult to observe under the native gel conditions); lane 2, Fxn; lane 3, SD; lane 4, SD with Fxn; lane 5, SD with Isu2; lane 6, Isu2 with Fxn; lane 7, SD with Isu2 and Fxn; lane 8, SD with Isu2 and Fxn without DTT; lane 9, SD with Isu2, Fxn, and EDTA; lane 10, SD with Isu2, Fxn, and Fe^{2+}. An asterisk and a diamond mark the positions of the slower migrating band and Fxn, respectively. (B) SDS−PAGE gel showing the protein components of the slower migrating band. The band from panel A was extracted and analyzed by SDS−PAGE.

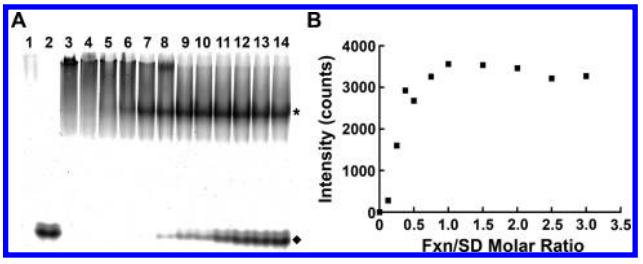

FIGURE 5: Fxn induces formation of SDUF. (A) Native gel showing the formation of SDUF with added Fxn: lane 1, Isu2; lane 2, Fxn; lane 3, SD; lanes 4−14, SD with Isu2 and 0−3 equiv of Fxn. An asterisk and a diamond mark the positions of SDUF and Fxn, respectively. (B) One equivalent of Fxn is required to form SDUF. Densitometry was used to quantitate the SDUF band from panel A.

estimate the oligomeric state and molecular mass of the human proteins and complexes. SD, Isu2, and Fxn exhibited retention times consistent with estimated molecular masses of 150, 17, and 17 kDa, respectively (Figure 7A). Gel filtration chromatographic analysis of SD incubated with both Isu2 and Fxn revealed an apparent 180 kDa species (Figure 7A). SDS−PAGE analysis revealed the presence of Nfs1, Isd11, Isu2, and Fxn (Figure 7B), which was consistent with SDUF. Interestingly, gel filtration analysis of a SD sample incubated with Isu2 resulted in a slightly different retention time and an estimated molecular mass of 170 kDa (Figure 7A). SDS−PAGE analysis of the 170 kDa fractions revealed the presence of Nfs1, Isd11, and Isu2, consistent with SDU. In contrast, a similar experiment with SD incubated with Fxn provided no evidence of SDF (Nfs1, Isd11, and Fxn protein complex). The formation of SDU and not SDF was consistent with the perturbation of the SD kinetic parameters upon addition of Isu2 or Fxn (Table 1). No higher-molecular mass species or aggregate, such as oligomeric Fxn, was observed in these experiments. These gel filtration results were consistent with human Nfs1, Isd11, and Isu2 assembling into SDU and Nfs1, Isd11, Isu2, and Fxn assembling into SDUF in which Fxn binding depends on the presence of Isu2.

DISCUSSION

Human Fe−S cluster assembly proteins were isolated and conditions discovered for forming previously uncharacterized

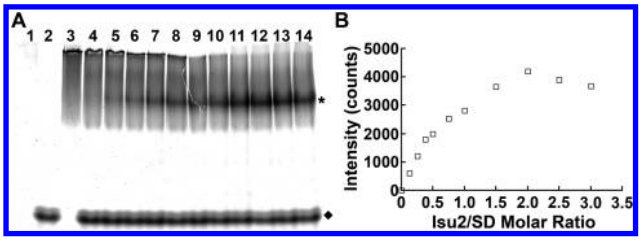

FIGURE 6: Isu2 induces formation of SDUF. (A) Native gel showing formation of SDUF with added Isu2: lane 1, Isu2; lane 2, Fxn; lane 3, SD; lanes 4−14, SD with Fxn and 0−3 equiv of Isu2. An asterisk and a diamond mark the positions of SDUF and excess Fxn, respectively. (B) Densitometry was used to quantitate the SDUF band from panel A.

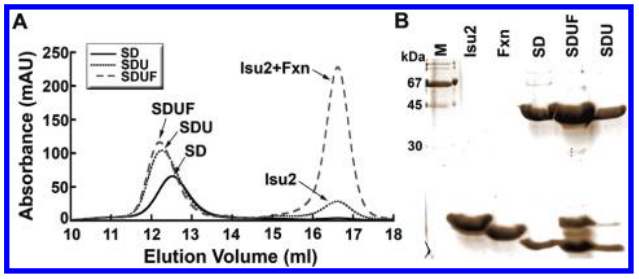

FIGURE 7: Molecular mass estimates and protein components for SD, SDU, and SDUF. (A) Three equivalents of Isu2 was added to SD to generate SDU. Three equivalents of both Isu2 and Fxn was added to SD to form SDUF. Samples were analyzed via anaerobic gel filtration chromatography. (B) SDS−PAGE analysis of Fe−S cluster assembly complexes confirms protein components.

SDU and SDUF comprised of Nfs1, Isd11, and Isu2 and Nfs1, Isd11, Isu2, and Fxn, respectively. Previously, a protein complex was proposed for Fe−S cluster biosynthesis in yeast that included oligomeric rather than monomeric Fxn (*47*). Gel filtration results presented here (Figure 7A) revealed no evidence of oligomeric Fxn and confirmed human Fxn is in the monomeric form. Together, these data suggest that SDU and SDUF have molecular masses of approximately 170−180 kDa, represent distinct functional states for Fe−S cluster biosynthesis (see below), and are the mechanistically relevant species for future study.

SDU is essentially inactive at physiological cysteine concentrations. The concentration of cysteine in mouse liver or brain homogenates is reported to be 0.1 mM (*43*). The concentration of cysteine in rat liver mitochondria was originally undetectable (*48*, *49*), but recent studies revealed a concentration of 0.07 mM (*45*). Kinetic analysis of human SDU (SD and Isu2) using 0.1 mM cysteine revealed very low sulfide production and Fe−S biosynthesis activities (Figures 1A and 3A). The modest activities are explained by the low "physiological" substrate concentration (0.1 mM) compared to the high K_M (0.59 mM) for human SDU. We propose SDU is in the "off" state for Fe−S cluster assembly.

Binding of Fxn to SDU generates an ~180 kDa SDUF species with a possible $\alpha_2\beta_2\gamma_2\delta_2$ stoichiometry. A recent crystal structure revealed that bacterial IscS (Nfs1 homologue) binds to IscU (Isu2 homologue) with an elongated $\alpha_2\beta_2$ stoichiometry (*32*). Titration results indicated each Nfs1 binds a maximum of one Fxn (Figure 5B). If we assume each Nfs1 also binds one Isd11, the resulting $\alpha_2\beta_2\gamma_2$ SDU and $\alpha_2\beta_2\gamma_2\delta_2$ SDUF have calculated molecular masses of 144 and 172 kDa, respectively, which are close to the estimated masses (170 and 180 kDa, respectively) determined by gel filtration chromatography (Figure 7A). The similar retention times for SDU and SDUF are consistent with the elongated architecture for the bacterial IscS−IscU structure, and a model suggesting a more globular IscS−IscU−Fxn species (*32*). Together, these results suggest $\alpha_2\beta_2\gamma_2$ SDU and $\alpha_2\beta_2\gamma_2\delta_2$ SDUF stoichiometries but do not rule out alternate stoichiometries with additional Isd11 or Isu2 molecules.

Fxn binding and stimulation of cysteine desulfurase activity are reminiscent of sulfur acceptor proteins in bacterial systems. Fxn binding to SDU (SD, Isu2, and Fxn) increased the sulfide production activity 20-fold (Figure 1A). Stimulation of cysteine desulfurases by binding partners is well-known. In *E. coli*, IscS activity is stimulated 6-fold by IscU binding (*50*) and SufS activity is increased 50-fold by SufE interactions (*51*). SufE is a sulfur transfer protein (*52*) that is structurally similar to IscU (*53*). In *Bacillus subtilis*, the scaffold apoprotein SufU stimulated SufS activity 40-fold, whereas the cluster-bound SufU stimulated SufS at a much lower level (*54*). Cysteine desulfurases are also stimulated by molecular chaperones involved in Fe−S cluster assembly (*55*) and proteins involved in tRNA (*56*) and molybdopterin (*57*) biosynthesis.

Fxn binding activates SDU, possibly through a conformational change. Fxn binding increased the k_{cat} nearly 6-fold and decreased the K_M for cysteine by more than 50-fold (Table 1). Because there is no evidence that Fxn directly participates in cysteine desulfurase catalysis, we suggest Fxn acts indirectly as an allosteric activator and induces a conformational change that positions the Nfs1 persulfide loop and a conserved cysteine on Isu2 for direct sulfur transfer and Fe−S cluster biosynthesis. In this model (Figure 8), Fxn binding to SDU switches the Nfs1 flexible loop from a nonfunctional to a catalytic conformation, which enhances substrate binding and lowers the K_M for cysteine. We hypothesize that Fxn binding to SDU also induces a conformational change in Isu2 that facilitates the transfer of sulfur from Nfs1 and thereby increases the cysteine turnover number (k_{cat}). The persulfide cleavage or sulfur transfer step is the rate-limiting step in other cysteine desulfurases (*11*). Regardless of the molecular details, the binding of Fxn to SDU dramatically increases the catalytic efficiency of the complex and essentially turns the assembly system "on" for Fe−S cluster biosynthesis at physiological cysteine concentrations.

Iron enhances the ability of Fxn to activate the cysteine desulfurase. The addition of ferrous iron to SDUF doubled the k_{cat} but had minimal effects on the K_M for cysteine (Table 1). The maximum stimulation was observed after the addition of 1 equiv of iron (Figure 1B). Previously, 1 equiv of iron was also shown to be required for Fxn and Isu interactions (*12*, *46*). Iron does not appear to be required for the formation of SDUF (Figure 4A, lanes 9 and 10), but a role for iron in complex stabilization has not been evaluated. The addition of ferric iron or most of the first row transition metals did not significantly increase the cysteine desulfurase activity (Figure 2). The one exception is Mn^{2+}, which slightly activated (128% of the control) the cysteine desulfurase activity and is a good mimic of ferrous iron. This result is consistent with Fe^{2+}, and possibly Mn^{2+}, functioning in conjunction with Fxn to accelerate the sulfur transfer step in catalysis, possibly by inducing a conformational change in Isu2.

The addition of Fxn to SDU accelerated the rate of Fe−S cluster biosynthesis 25-fold at physiological cysteine concentrations. In this experiment (Figure 3A), the absorbance maximized after a calculated 12 μM $[Fe_2S_2]$ cluster was formed, which is approximately the concentration (8 μM) of SDUF. This calculation is based on the assumption that the absorbance at 456 nm is due to $[Fe_2S_2]$ clusters with extinction coefficients of 5.8 mM^{-1} cm^{-1}

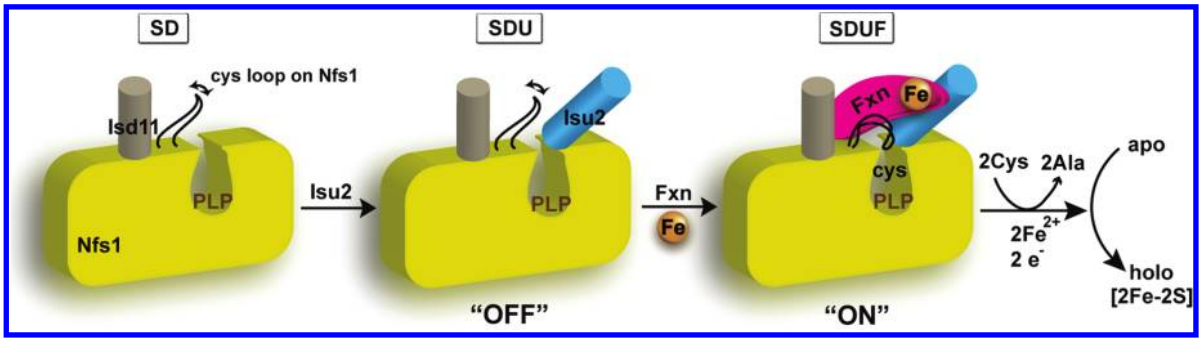

FIGURE 8: Working model for Fxn activation of the Fe−S cluster assembly complex. The Nfs1 flexible loop is stabilized in a nonfunctional conformation for SD and SDU. Fxn binding favors the catalytic loop conformation, enhances substrate binding, and accelerates persulfide bond formation. Fxn binding and Fe binding also induce a conformational change in Isu2 that accelerates the transfer of sulfur from Nfs1 to Isu2 for Fe−S cluster biosynthesis.

(*41*); this $[Fe_2S_2]$ cluster assignment should be viewed as tentative until it is supported by additional spectroscopic studies. Strikingly, addition of Fxn to SDU results in mirrored increases in the rate of Fe−S cluster biosynthesis (Figure 3B) and the amount of SDUF formation (Figure 5B), with both exhibiting a maximum effect after the addition of 1 equiv of Fxn. These data strongly support a model in which Fxn is an activating component of the core Fe−S cluster assembly machine (Figure 8).

An allosteric activator role for Fxn in Fe−S cluster biosynthesis is consistent with most previous studies. This newly discovered function for Fxn is consistent with in vivo experiments that show Fxn depletion results in the loss of activity for Fe−S cluster enzymes (*20*, *21*). Enzyme assay results (Figure 3) indicate that SDU is still able to synthesize Fe−S clusters in the absence of Fxn, albeit at a much lower rate. This explains the residual level of Fe−S enzyme activity in Fxn-depleted yeast mitochondria (*27*). A role for Fxn as an allosteric activator for Fe−S cluster biosynthesis is also consistent with the neurodegenerative and cardiovascular impairment in Friedreich's ataxia (FRDA). FRDA patients with low frataxin levels would be expected to contain only residual or "unactivated" levels of Fe−S cluster biosynthesis, have depleted Fe−S clusters in their respiratory chain, and exhibit mitochondrial dysfunction. Because heart and brain tissue are especially rich in mitochondria, these tissues are particularly susceptible to Fxn deficiencies and Fe−S cluster defects. This allosteric activator role for human Fxn is in contrast to studies of the *E. coli* homologue that suggest Fxn functions as an inhibitor for Fe−S cluster biosynthesis (*26*). An allosteric activator function for Fxn does not necessarily rule out other functions, such as mediating iron delivery for Fe−S cluster synthesis.

Fxn levels in vivo may be used to regulate the Fe−S cluster assembly activity in response to environmental stimuli. The results presented here clearly indicate Fxn is an activating component for the SDUF Fe−S cluster assembly complex. What is unclear is if Fxn protein levels are controlled by environmental cues as a mechanism for regulating Fe−S cluster biosynthesis. Fxn protein levels are known to vary by a factor of > 3 in normal individuals (*58*). Two possible environmental stimuli are iron and oxidative stress. Iron regulates frataxin expression (*59*) and stimulates Fe−S cluster biosynthesis in a Fxn-dependent manner (Figure 1B). Oxidative stress results in chemical modification of Fxn (*60*), whereas oxidizing conditions weaken SDUF formation (Figure 4A, lane 8) and frataxin-based activation (Figure 1A). Additional experiments are required to determine if these or other environmental stimuli are part of a regulatory mechanism that uses Fxn to modulate Fe−S cluster biosynthesis.

Here we establish that a four-protein component complex that includes Nfs1, Isd11, Isu2, and Fxn is capable of synthesizing Fe−S clusters. Future studies are required to clarify if this SDUF interacts transiently with additional biosynthetic components, such as the electron donor (ferredoxin) and iron donor machinery, or if an even larger assembly complex is formed. Additional studies are also required to determine if the molecular chaperones, which facilitate transfer of the Fe−S cluster to apo target scaffolds, interact with an intact SDUF, a subcomplex such as SDU, or dissociated holo-Isu2. In addition, few details are currently available for the mechanisms of human $[Fe_2S_2]$ and $[Fe_4S_4]$ cluster assembly, target recognition, and cluster transfer. The results presented here provide strong evidence that SDU and SDUF represent the off and on states for Fe−S cluster biosynthesis, respectively, lead to testable hypotheses for cellular regulation of Fe−S cluster assembly, and provide a foundation for addressing these mechanistic questions.

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REFERENCES

1. Beinert, H., Holm, R. H., and Münck, E. (1997) Iron-sulfur clusters: Nature's modular, multipurpose structures. *Science 277*, 653–659.
2. Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. (2005) Structure, function, and formation of biological iron-sulfur clusters. *Annu. Rev. Biochem. 74*, 247–281.
3. Py, B., and Barras, F. (2010) Building Fe-S proteins: Bacterial strategies. *Nat. Rev. Microbiol. 8*, 436–446.
4. Rouault, T. A., and Tong, W.-H. (2008) Iron-sulfur cluster biogenesis and human disease. *Trends Genet. 24*, 398–407.
5. Lill, R. (2009) Function and biogenesis of iron-sulphur proteins. *Nature 460*, 831–838.
6. Ye, H., and Rouault, T. A. (2010) Human iron-sulfur cluster assembly, cellular iron homeostasis and disease. *Biochemistry 49*, 4945–4956.
7. Veatch, J. R., McMurray, M. A., Nelson, Z. W., and Gottschling, D. E. (2009) Mitochondrial dysfunction leads to nuclear genome instability via an iron-sulfur cluster defect. *Cell 137*, 1247–1258.
8. Foster, M. W., Mansy, S. S., Hwang, J., Penner-Hahn, J. E., Surerus, K. K., and Cowan, J. A. (2000) A Mutant Human IscU Protein Contains a Stable $[2Fe2S]^{2+}$ Center of Possible Functional Significance. *J. Am. Chem. Soc. 122*, 6805–6806.
9. Zheng, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. U.S.A. 90*, 2754–2758.

10. Zheng, L., White, R. H., Cash, V. L., and Dean, D. R. (1994) Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. *Biochemistry 33*, 4714–4720.
11. Behshad, E., Parkin, S. E., and Bollinger, J. M. (2004) Mechanism of cysteine desulfurase Slr0387 from *Synechocystis* sp. PCC 6803: Kinetic analysis of cleavage of the persulfide intermediate by chemical reductants. *Biochemistry 43*, 12220–12226.
12. Yoon, T., and Cowan, J. (2003) Iron-sulfur cluster biosynthesis. Characterization of frataxin as an iron donor for assembly of [2Fe-2S] clusters in ISU-type proteins. *J. Am. Chem. Soc. 125*, 6078–6084.
13. Layer, G., Ollagnier de Choudens, S., Sanakis, Y., and Fontecave, M. (2006) Iron-sulfur cluster biosynthesis: Characterization of *Escherichia coli* CYaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. *J. Biol. Chem. 281*, 16256–16263.
14. Cook, J. D., Bencze, K. Z., Jankovic, A. D., Crater, A. K., Busch, C. N., Bradley, P. B., Stemmler, A. J., Spaller, M. R., and Stemmler, T. L. (2006) Monomeric yeast frataxin is an iron-binding protein. *Biochemistry 45*, 7767–7777.
15. Ding, H., Clark, R. J., and Ding, B. (2004) IscA mediates iron delivery for assembly of iron-sulfur clusters in IscU under the limited accessible free iron conditions. *J. Biol. Chem. 279*, 37499–37504.
16. Lange, H., Kaut, A., Kispal, G., and Lill, R. (2000) A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins. *Proc. Natl. Acad. Sci. U.S.A. 97*, 1050–1055.
17. Sheftel, A. D., Stehling, O., Pierik, A. J., Elsässer, H.-P., Mühlenhoff, U., Webert, H., Hobler, A., Hannemann, F., Bernhardt, R., and Lill, R. (2010) Humans possess two mitochondrial ferredoxins, Fdx1 and Fdx2, with distinct roles in steroidogenesis, heme, and Fe/S cluster biosynthesis. *Proc. Natl. Acad. Sci. U.S.A. 107*, 11775–11780.
18. Hoff, K. G., Silberg, J. J., and Vickery, L. (2000) Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 97*, 7790–7795.
19. Amutha, B., Gordon, D. M., Gu, Y., Lyver, E. R., Dancis, A., and Pain, D. (2007) GTP is required for iron-sulfur cluster biogenesis in mitochondria. *J. Biol. Chem. 283*, 1362–1371.
20. Muhlenhoff, U., Richhardt, N., Ristow, M., Kispal, G., and Lill, R. (2002) The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. *Hum. Mol. Genet. 11*, 2025–2036.
21. Stehling, O., Elsässer, H., Brückel, B., Muhlenhoff, U., and Lill, R. (2004) Iron-sulfur protein maturation in human cells: Evidence for a function of frataxin. *Hum. Mol. Genet. 13*, 3007–3015.
22. Park, S., Gakh, O., O'Neill, H., Mangravita, A., Nichol, H., Ferreira, G., and Isaya, G. (2003) Yeast frataxin sequentially chaperones and stores iron by coupling protein assembly with iron oxidation. *J. Biol. Chem. 278*, 31340–31351.
23. Cavadini, P., O'Neill, H., Benada, O., and Isaya, G. (2002) Assembly and iron-binding properties of human frataxin, the protein deficient in Friedreich ataxia. *Hum. Mol. Genet. 11*, 217–227.
24. Aloria, K., Schilke, B., Andrew, A. J., and Craig, E. A. (2004) Iron-induced oligomerization of yeast frataxin homologue Yfh1 is dispensable in vivo. *EMBO Rep. 5*, 1096–1101.
25. Seguin, A., Sutak, R., Bulteau, A.-L., Garcia-Serres, R., Oddou, J.-L., Lefevre, S., Santos, R., Dancis, A., Camadro, J.-M., Latour, J.-M., and Lesuisse, E. (2010) Evidence that yeast frataxin is not an iron storage protein in vivo. *Biochim. Biophys. Acta 1802*, 531–538.
26. Adinolfi, S., Iannuzzi, C., Prischi, F., Pastore, C., Iametti, S., Martin, S., Bonomi, F., and Pastore, A. (2009) Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS. *Nat. Struct. Mol. Biol. 16*, 390–396.
27. Duby, G., Foury, F., Ramazzotti, A., Herrmann, J., and Lutz, T. (2002) A non-essential function for yeast frataxin in iron-sulfur cluster assembly. *Hum. Mol. Genet. 11*, 2635–2643.
28. Gerber, J., Muhlenhoff, U., and Lill, R. (2003) An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. *EMBO Rep. 4*, 906–911.
29. Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S.-I., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002) Network of protein-protein interactions among iron-sulfur cluster assembly proteins in *Escherichia coli*. *J. Biochem. 131*, 713–719.
30. Shan, Y., Napoli, E., and Cortopassi, G. A. (2007) Mitochondrial frataxin interacts with ISD11 of the NFS1/ISCU complex and multiple mitochondrial chaperones. *Hum. Mol. Genet. 16*, 929–941.
31. Ramazzotti, A., Vanmansart, V., and Foury, F. (2004) Mitochondrial functional interactions between frataxin and Isu1p, the iron-sulfur cluster scaffold protein, in *Saccharomyces cerevisiae*. *FEBS Lett. 557*, 215–220.
32. Shi, R., Proteau, A., Villarroya, M., Moukadiri, I., Zhang, L., Trempe, J.-F., Matte, A., Armengod, M. E., and Cygler, M. (2010) Structural basis for Fe-S cluster assembly and tRNA thiolation mediated by IscS protein-protein interactions. *PLoS Biol. 8*, e1000354.
33. Adam, A. C., Bornhövd, C., Prokisch, H., Neupert, W., and Hell, K. (2006) The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria. *EMBO J. 25*, 174–183.
34. Shi, Y., Ghosh, M., Tong, W.-H., and Rouault, T. A. (2009) Human ISD11 is essential for both iron-sulfur cluster assembly and maintenance of normal cellular iron homeostasis. *Hum. Mol. Genet. 18*, 3014–3025.
35. Wiedemann, N., Urzica, E., Guiard, B., Müller, H., Lohaus, C., Meyer, H. E., Ryan, M. T., Meisinger, C., Muhlenhoff, U., Lill, R., and Pfanner, N. (2006) Essential role of Isd11 in mitochondrial iron-sulfur cluster synthesis on Isu scaffold proteins. *EMBO J. 25*, 184–195.
36. Muhlenhoff, U., Gerber, J., Richhardt, N., and Lill, R. (2003) Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *EMBO J. 22*, 4815–4825.
37. Marelja, Z., Stöcklein, W., Nimtz, M., and Leimkühler, S. (2008) A novel role for human Nfs1 in the cytoplasm: Nfs1 acts as a sulfur donor for MOCS3, a protein involved in molybdenum cofactor biosynthesis. *J. Biol. Chem. 283*, 25178–25185.
38. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.
39. Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182*, 319–326.
40. Siegel, L. M. (1965) A direct microdetermination for sulfide. *Anal. Biochem. 11*, 126–132.
41. Agar, J., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. (2000) IscU as a scaffold for iron-sulfur cluster biosynthesis: Sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry 39*, 7856–7862.
42. Schägger, H. (2001) Blue-native gels to isolate protein complexes from mitochondria. *Methods Cell Biol. 65*, 231–244.
43. Furne, J., Saeed, A., and Levitt, M. D. (2008) Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am. J. Physiol. 295*, R1479–R1485.
44. Ohmori, S., Nawata, Y., Kiyono, K., Murata, H., Tsuboi, S., Ikeda, M., Akagi, R., Morohashi, K. I., and Ono, B. (1999) *Saccharomyces cerevisiae* cultured under aerobic and anaerobic conditions: Air-level oxygen stress and protection against stress. *Biochim. Biophys. Acta 1472*, 587–594.
45. Ross-Inta, C., Tsai, C.-Y., and Giulivi, C. (2008) The mitochondrial pool of free amino acids reflects the composition of mitochondrial DNA-encoded proteins: Indication of a post-translational quality control for protein synthesis. *Biosci. Rep. 28*, 239–249.
46. Kondapalli, K., Kok, N., Dancis, A., and Stemmler, T. L. (2008) *Drosophila* Frataxin: An Iron Chaperone during Cellular Fe-S Cluster Bioassembly. *Biochemistry 47*, 6917–6927.
47. Li, H., Gakh, O., Smith, D., and Isaya, G. (2009) Oligomeric yeast frataxin drives assembly of core machinery for mitochondrial iron-sulfur cluster synthesis. *J. Biol. Chem. 284*, 21971–21980.
48. Truman, D. E. S., and Korner, A. (1962) Incorporation of amino acids into the protein of isolated mitochondria. A search for optimum conditions and a relationship to oxidative phosphorylation. *Biochem. J. 83*, 588–596.
49. Baird, G. D. (1964) The release of amino acids from rat-liver mitochondrial extract. *Biochim. Biophys. Acta 93*, 293–303.
50. Kato, S.-I., Mihara, H., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: Implications for the mechanism of iron-sulfur cluster assembly. *Proc. Natl. Acad. Sci. U.S.A. 99*, 5948–5952.
51. Loiseau, L., Ollagnier de Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J. Biol. Chem. 278*, 38352–38359.
52. Layer, G., Gaddam, S. A., Ayala-Castro, C. N., Ollagnier de Choudens, S., Lascoux, D., Fontecave, M., and Outten, F. W. (2007) SufE transfers sulfur from SufS to SufB for iron-sulfur cluster assembly. *J. Biol. Chem. 282*, 13342–13350.
53. Goldsmith-Fischman, S., Kuzin, A., Edstrom, W. C., Benach, J., Shastry, R., Xiao, R., Acton, T. B., Honig, B., Montelione, G. T., and Hunt, J. F. (2004) The SufE sulfur-acceptor protein contains a conserved core structure that mediates interdomain interactions in a variety of redox protein complexes. *J. Mol. Biol. 344*, 549–565.

54. Albrecht, A. G., Netz, D. J. A., Miethke, M., Pierik, A. J., Burghaus, O., Peuckert, F., Lill, R., and Marahiel, M. A. (2010) SufU is an essential iron-sulfur cluster scaffold protein in *Bacillus subtilis*. *J. Bacteriol. 192*, 1643–1651.
55. Dutkiewicz, R., Marszalek, J., Schilke, B., Craig, E. A., Lill, R., and Muhlenhoff, U. (2006) The Hsp70 chaperone Ssq1p is dispensable for iron-sulfur cluster formation on the scaffold protein Isu1p. *J. Biol. Chem. 281*, 7801–7808.
56. Kambampati, R., and Lauhon, C. T. (1999) IscS is a sulfurtransferase for the in vitro biosynthesis of 4-thiouridine in *Escherichia coli* tRNA. *Biochemistry 38*, 16561–16568.
57. Zhang, W., Urban, A., Mihara, H., Leimkühler, S., Kurihara, T., and Esaki, N. (2010) IscS functions as a primary sulfur-donating enzyme by interacting specifically with MoeB and MoaD in the biosynthesis of molybdopterin in *Escherichia coli*. *J. Biol. Chem. 285*, 2302–2308.
58. Boehm, T., Scheiber-Mojdehkar, B., Kluge, B., Goldenberg, H., Laccone, F., and Sturm, B. (2010) Variations of frataxin protein levels in normal individuals. *Neurol Sci.* (in press).
59. Li, K., Besse, E., Ha, D., Kovtunovych, G., and Rouault, T. A. (2008) Iron-dependent regulation of frataxin expression: Implications for treatment of Friedreich ataxia. *Hum. Mol. Genet. 17*, 2265–2273.
60. Correia, A., Ow, S., Wright, P., and Gomes, C. (2009) The conserved Trp155 in human frataxin as a hotspot for oxidative stress related chemical modifications. *Biochem. Biophys. Res. Commun. 390*, 1007–1011.