Published in final edited form as:

Metallomics. 2016 December 07; 8(12): 1283–1293. doi:10.1039/c6mt00193a.

Mapping Cellular Fe-S Cluster Uptake and Exchange Reactions - Divergent Pathways for Iron-Sulfur Cluster Delivery to Human Ferredoxins

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Abstract

Ferredoxins are protein mediators of biological electron-transfer reactions and typically contain either [2Fe–2S] or [4Fe–4S] clusters. Two ferredoxin homologues have been identified in the human genome, Fdx1 and Fdx2, that share 43% identity and 69% similarity in protein sequence and both bind [2Fe–2S] clusters. Despite the high similarity, the two ferredoxins play very specific roles in distinct physiological pathways and cannot replace each other in function. Both eukaryotic and prokaryotic ferredoxins and homologues have been reported to receive their Fe-S cluster from scaffold/delivery proteins such as IscU, Isa, glutaredoxins, and Nfu. However, the preferred and physiologically relevant pathway for receiving the [2Fe–2S] cluster by ferredoxins is subject to speculation and is not clearly identified. In this work, we report on *in vitro* UV-visible (UV-vis) circular dichroism studies of [2Fe–2S] cluster transfer to the ferredoxins from a variety of partners. The results reveal rapid and quantitative transfer to both ferredoxins from several donor proteins (IscU, Isa1, Grx2, and Grx3). Transfer from Isa1 to Fdx2 was also observed to be faster than that of IscU to Fdx2, suggesting that Fdx2 could receive its cluster from Isa1 instead of IscU. Several other transfer combinations were also investigated and the results suggest a complex, but kinetically detailed map for cellular cluster trafficking. This is the first step toward building a network map for all of the possible iron-sulfur cluster transfer pathways in the mitochondria and cytosol, providing insights on the most likely cellular pathways and possible redundancies in these pathways.

Graphical abstract

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
See DOI: 10.1039/x0xx00000x

Mapping of [2Fe-2S] cluster transfer to cellular Fdx's from several donor Proteins suggest an approach to mapping cellular cluster trafficking.

Introduction

Fe-S proteins constitute one of the largest classes of proteins, with varied functions that include electron transport, regulation of gene expression, substrate binding and activation, and radical generation.³ For this reason, iron-sulfur cluster biosynthetic pathways and machinery are conserved across all kingdoms of life.⁵ The last decade has resulted in significant advances in the biochemical characterization of the mitochondrial ISC assembly machinery, which is essential not only for the biogenesis of mitochondrial, but also for cytosolic and nuclear Fe-S proteins. Various models for mitochondrial iron-sulfur cluster biosynthesis and trafficking have been considered, such as the assembly of a core [2Fe-2S] cluster on the IscU scaffold protein, with subsequent transfer to intermediate proteins such as glutaredoxin 5 (Grx5),^{5, 7} prior to direct transfer to other [2Fe-2S] cluster proteins such as ferredoxins, or to the scaffold proteins Isa1/2, where assembly of a [4Fe-4S] cluster has been proposed.^{8, 9} Subsequently, [4Fe-4S] clusters may be transferred to final acceptor apo proteins by intermediate carriers such as Nfu or BOLA3^{10–12}, although the former may be more relevant to [2Fe-2S] cluster transfer.^{2, 5, 7} There is also a requirement for Fe-S cluster transport to the cytosol, most likely by ABC-type transporters such as ABCB7 in mammals and Atm1 in yeast.^{13, 14} While pair wise cluster transfer reactions of several key proteins in mitochondrial Fe-S cluster assembly have been established individually, the inter-relatedness of the cluster-transfer reactions and the possible existence of discrete networks of pathways for specific metabolic roles remains unclear. In this report we describe the results of studies designed to map inter-protein cluster transfer pathways for assembly and trafficking, including IscU, Isa, glutaredoxins, Nfu, and ferredoxins.

The role of scaffold proteins such as human IscU (*Hs* IscU) is well known. Fe-S clusters are transiently assembled and then delivered to recipient proteins. The gene for hIscU encodes two isoforms by alternative splicing that result in a mitochondrial and a cytosolic form of the protein, both of which are active in Fe-S cluster assembly.¹⁵ Though the primary role of IscU is established to be biogenesis of Fe-S cluster via proteins such as frataxin, IscS and HscA/HscB-type chaperones, there is extensive literature supporting its versatile role in

efficiently delivering Fe-S clusters to apo target proteins in both mitochondria and the cytosol.^{4, 16–22} Successful [2Fe-2S] cluster transfer from hIscU to both human ferredoxin 1 and ferredoxin 2,²³ and to human Grx2 without the need of chaperones has also been shown.⁴ Cluster transfer has also been reported from *A. vinelandii* IscU to apo Grx5 in the presence of HscA/HscB/Mg-ATP, which in turn transfers cluster to ferredoxin.²⁴

Another scaffold protein that has been identified in the yeast mitochondrial ISC assembly machinery is Isa with two homologues, Isa1 and Isa2. However, their molecular function remains unknown. A recent study in yeast has shown that both Isa1 and Isa2 bind Fe *in vivo*.²⁵ They are related to the ubiquitous “A-type” ISC assembly proteins from bacteria and other eukaryotes²⁶ and interact with the ISC assembly protein Iba57.²⁷ Grx5 has also been shown to bind and transfer cluster to Isa1 and Isa2.^{8, 9} The need for careful evaluation of kinetic data and careful appraisal of exchange reactions is highlighted by the fast, efficient and quantitative cluster transfer reported from yeast cytosolic monothiol glutaredoxin to an A-type protein (Nif-IscA), even though this process is not physiologically relevant, as there is no evidence for an A-type Fe-S protein (such as Isa) in the yeast cytosol.²⁸ *E. coli* IscA has been reported to transfer a [2Fe-2S] cluster to *E. coli* apo ferredoxin.¹⁶ Therefore, IscA may be an alternative scaffold protein for Fe-S cluster assembly and transfer,^{29, 30} or it may serve as an iron-delivery protein.^{31, 32}

Other proteins that may play a vital role in mitochondrial Fe-S cluster assembly and trafficking are glutaredoxins and FeS carrier proteins such as Nfu. Glutaredoxins are widely distributed among all organisms, and have been shown to play an important role in Fe-S protein biogenesis, cellular Fe-S cluster trafficking, and in regulatory and signaling mechanisms for Fe-S target proteins.^{33–35} Human Grx2 (*Hs* Grx2) is primarily a mitochondrial dithiol glutaredoxin that has been implicated in cellular apoptosis and mitochondrial redox regulation.^{36, 37} Human Grx5 (*Hs* Grx5) is a single domain monothiol glutaredoxin localized to the mitochondria³⁸ and is proposed to facilitate the transfer of nascent Fe-S clusters to target proteins during maturation of Fe-S cluster-containing proteins.^{8, 24, 39} It is also suggested to play a significant role in the disease condition sideroblastic anemia.^{40, 41} By contrast, eukaryotic Grx3 (yeast, yGrx3) is a multidomain monothiol glutaredoxin that is localized to the nucleus and/or the cytosol.⁴² In yeast, [2Fe-2S]-bridged Grx3 homodimers are proposed to transport iron from cytosolic pools to iron-dependent enzymes and the mitochondrion, as well as involvement in regulating cellular iron homeostasis by interacting with the Fe-responsive transcription factors (Aft1 and Aft2).^{42–44} Human Nfu may act as an alternative scaffold protein to IscU, possibly involved in binding a [4Fe-4S] cluster,⁴⁵ although the possibility of consecutive transfer of two [2Fe-2S] clusters cannot be excluded. In yeast, Nfu1 may be deleted without obvious consequences¹¹, but in cyanobacteria the deletion of Nfu is lethal, whereas genetic ablation of IscA and SufA has no drastic consequences,^{21, 46} highlighting the need for redundancies in Fe-S cluster trafficking pathways in order to avoid lethal knockouts. The focus of this investigation is on cluster transfer to these two human ferredoxin homologs (FDX1/ *Hs* Fdx1 and FDX2/ *Hs* Fdx2) via various potential cluster donors.

We define endpoint proteins as those where the protein-bound cluster is not transferred to a subsequent partner. Examples include proteins such as LIAS, aconitase, and ferredoxins.

The latter are involved in electron transfer reactions and typically contain [2Fe–2S] cluster, though bacterial ferredoxins also bind [4Fe–4S] clusters.^{47, 48} Two ferredoxin homologues (Fdx1 and Fdx2) have been identified in the human genome⁴⁹ and share 43% identity and 69% similarity in protein sequence.²³ Despite the high similarity, the two ferredoxins are believed to play very specific roles in distinct pathways⁵⁰; however, they are both thought to be required for iron-sulfur cluster biogenesis, so some functional overlap may exist.⁵¹ Fdx2 is equally expressed in the adrenal as in other tissues, and Fdx1 participates more in Cyt P450 reactions.⁵¹ What distinguishes the specific functions of these two ferredoxin proteins remains unclear, despite high similarity in structure and conformation. Both proteins display similar UV-vis and CD spectra in their holo forms²³, consistent with the occurrence of a [2Fe–2S] cluster center in a coordination environment that includes four terminal cysteine ligands and two bridging sulfides in an approximately tetrahedral coordination mode around each iron. Both the ferredoxins and even the prokaryotic homologues of ferredoxin have been reported to receive their Fe-S cluster from all the proteins discussed above such as IscU, Isa, glutaredoxins, and Nfu. However, the preferred and physiologically relevant pathway for receiving the [2Fe-2S] cluster by ferredoxins is subject to speculation and is not clearly identified. In this paper, we begin a process to identify the most likely physiologically relevant trafficking pathways based on evaluation of differences in kinetic rate constants for cluster transfer between pairs of putative protein partners. In addition, we also attempt to outline a [2Fe-2S] transfer pathway map involving several mitochondrial ISC machinery proteins.

Experimental

Materials

PD10 desalting columns were purchased from GE Healthcare. Ferric chloride, sodium sulfide, DTT (Dithiothreitol), TCEP (Tris (2-carboxy-ethyl) phosphine) and L-cysteine were purchased from Fisher. The gene for *Saccharomyces cerevisiae* S288c glutaredoxin 3 (Grx3) in pET28b(+) vector was ordered from GenScript. Primers (Forward: TAAGCACATATGCCGGTCATCGAAATTAATGAT and Reverse: TGCTTAAAGCTTTTAGGATTGCAGTGC GTTGTG) were purchased from Integrated DNA Technologies, Inc.

Protein expression and purification

Purification of human IscU (*Hs* IscU) and *Thermatoga maritima* (*Tm* Nifs) was performed as previously reported.^{52–54} The expression vector for human ferredoxin-1 (*Hs* Fdx1) was kindly provided by J. Markley and protein was expressed and purified according to literature procedures.⁵⁵ Purification for human ferredoxin-2 (*Hs* Fdx2) was performed as previously reported.²³ The ferredoxins were purified as holo proteins and were then subsequently converted to apo forms by treatment with 100 mM EDTA, 5mM DTT and 8M urea in a buffered solution, pH 7.5. To isolate the apo ferredoxins, the colorless solution was passed through a PD-10 column to remove the EDTA, DTT and urea. Apo protein concentration was determined by use of the Bradford assay, as described at the end of the protein expression and purification section of the experimental.

A construct of human Grx2 (comprising residues 56-161) with a tobacco etch virus cleavable N-terminal His₆ tag in expression vector pNic-Bsa4 was kindly provided by Drs. Kavanagh, Muller-Knapp and Oppermann and protein was expressed and purified as previously reported.⁴ *Schizosaccharomyces pombe* Isa1 protein was expressed and purified as previously reported.⁵⁶ Expression and purification of human Nfu were performed from BL21 (DE3) competent cells as previously described.^{57, 58} The yeast Grx3 (Q03835, residues 36-285) open reading frame containing both the Trx-like and Grx-like domains but lacking the first 35 amino acids (1–35)⁵⁹ was cloned into pET28b(+) between HindIII and NdeI restriction sites and expressed in *Escherichia coli* BL21 (DE3).¹ The transformed cells were grown in 10 mL of Luria Bertani (LB) broth media containing kanamycin (50 µM) at 37 °C overnight and then transferred to 3 L of fresh LB media containing kanamycin and incubated at 37 °C until the OD₆₀₀ reached 0.8.

Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (300 µM) and the cell culture incubated at 30 °C for 10 h. Cell pellets were collected by centrifugation at 4330 g for 15 min, suspended in 30 mL of buffer containing 50 mM Hepes, 100 mM NaCl, pH 7.5, and lysed by use of a dismembranator. The lysate was centrifuged at 28,928 g for 30 min, the supernatant subsequently applied to a Ni-NTA column, and the protein eluted with buffer containing 50 mM Hepes, 100 mM NaCl, 0.25 M imidazole, pH 7.5, prior to analysis on a 12% SDS-PAGE gel that was then visualized by Coomassie Blue staining. Dialysis was carried out at 4 °C against 50 mM Hepes, 100 mM NaCl, pH 7.5. Protein concentration was determined by use of the Bradford assay.

***In vitro* reconstitution of apo proteins**

In vitro reconstitution of Grx2 and Grx3 was performed as previously reported,⁴ where 200 µM apo protein was incubated with 2 µM *Tm* Nifs, 5 mM DTT, and 3 mM GSH, argon-purged for 30 min, and then made up to 0.6 mM in Fe²⁺ and 0.6 mM in L-cysteine. Following incubation for 2 h, the reaction mixture was concentrated and passed through a PD10 desalting column to remove excess DTT, GSH, Fe²⁺, S²⁻ and L-cysteine, and the UV-vis spectrum was obtained for both apo and holo Grx2 and Grx3. Reconstitution of human Nfu was performed as previously described², by the same method used for reconstitution of glutaredoxins, but in the absence of GSH. For IscU and Isa1,²⁰ 200 µM solutions of apo proteins were incubated with 50 mM DTT and 8 M urea, argon purged for 30 min, and then made up to 1 mM in Fe²⁺ and 1 mM in S²⁻. After incubation for 1 h, the reaction mixture was concentrated and passed through a PD10 desalting column, equilibrated with 50 mM Hepes, 100 mM NaCl, pH 7.5, to remove excess DTT, urea, Fe²⁺ and S²⁻, and the UV-vis spectrum was obtained for both apo and holo IscU and Isa1.

Following reconstitution, total protein concentration was determined by use of the Bradford assay. Holo protein concentration was determined by extinction coefficients and confirmed through iron quantitation.

Iron quantitation^{20, 60}

Holo protein (50 µM, 200 µL) was acidified by concentrated HCl (60 µL) and heated to 100 °C for 15 min. The resulting suspension was centrifuged at 14,000 rpm for 2 min and

the supernatant (100 μ L) was diluted with Tris-HCl (0.5 M, 1.3 mL, pH 8.5). Solutions of sodium ascorbate (0.1 mL, 5%) and bathophenanthroline-disulfonate (0.4 mL, 0.1%) were sequentially added to the neutralized reaction solution with mixing between each addition. The solution was incubated at 25 °C for 1 h and iron was quantitated by measuring the absorbance at 535 nm on a UV-vis spectrophotometer and calculated from a calibration curve made with 0.01–0.30 mM FeCl₃ standard solutions. Subsequent analysis of the pellet revealed no bound iron.

Cluster transfer experiment by circular dichroism

CD spectra were recorded using a Jasco J815 spectropolarimeter. The time course of cluster transfer from holo iron-sulfur cluster proteins to apo proteins was monitored under anaerobic conditions at 25°C using UV-vis CD spectroscopy in small volume 1-cm quartz cuvettes. CD scans from 600 – 300 nm were collected to analyze signature peaks from cluster-bound protein at a scan rate of 200 nm/min with a 2 min interval between accumulations. Reactions were carried out in 50 mM HEPES, 100 mM NaCl, pH 7.5 and were prepared by degassing a mixture of apo protein in 5 mM DTT. The reaction was transferred to an anaerobic cuvette via a gas tight syringe and was initiated via the addition of degassed holo protein. The concentration of [2Fe-2S] cluster for each holo protein was determined via iron quantitation following reconstitution and are specified for each of the transfer reactions analyzed in the figure legends. Data were processed by use of JASCO Spectramanager II Analysis software and analyzed using the chemical kinetics and equilibria program DynaFit by BioKin.⁶¹ The deconvolution function from Spectramanager II analysis software was used for analysis of bands in the spectra that contained overlapping Lorentzian curves having the same full width at half maximum value that accurately distinguishes the peak positions for each band.

A second-order rate constant for cluster transfer was obtained via a method similar to the iron-sulfur cluster transfer method of Johnson and colleagues.^{28, 62} Proteins were mixed in a 1:1 ratio (20–100 μ M) and the peak at 330 nm for IscU, 370 nm for Isa1, 460 nm for Grx3, 365 nm for Grx2, 445 nm for Fdx1 and 440 nm for Fdx2 was monitored by CD. The data were converted to percent cluster transfer. The percentage of cluster transferred was determined from a set of control spectra of the holo proteins at a series of known concentrations recorded after reconstitution, and the plot of percent cluster transfer versus time was analyzed using DynaFit program⁶¹ to determine the second-order rate constants for the various reactions by best-fit simulation to second-order kinetics.

Results

The sensitivity and intensity of UV-vis CD spectra of the iron-sulfur cluster in a chiral protein environment makes this technique the method of choice for monitoring the kinetics of cluster uptake or cluster transfer. The CD profiles for the cluster center for each of the proteins in this work, *Hs* IscU, *S. pombe* Isa1, *Hs* Grx2, *S. cerevisiae* Grx3, *Hs* Nfu, *Hs* Fdx1 and *Hs* Fdx2, are distinct and readily distinguished from each other, thereby providing a convenient and quantitative measure of cluster transfer between these proteins by measuring changes in ϵ values at specific wavelengths. Previous work in our laboratory has

already characterized some transfer reactions between these proteins^{2, 4} and a stable glutathione-complexed [2Fe-2S] cluster (Table S1).^{1, 63}

Cluster transfer from holo ([2Fe-2S]) IscU to apo ferredoxins (Fdx1 and Fdx2)

UV-vis CD spectroscopy was used to evaluate both, cluster transfer and demonstrate the kinetics from human IscU to human apo ferredoxins (Fdx1 and Fdx2). The time course for cluster transfer from holo [2Fe-2S] IscU to apo ferredoxins was monitored at room temperature using a 1:1 donor:acceptor cluster stoichiometry and is shown in Fig. 1. While these transfer reactions were not carried out in the preferred pseudo first-order kinetics range, as a result of limitations from detection sensitivity and protein solubility, we have developed a method to provide estimates for second-order rate constants in cluster transfer reactions, similar to previously published literature.^{18, 28, 62} We have shown in test cases that the apparent second-order rate constant obtained in this manner agrees with the actual rate constant determined from more traditional concentration dependence measurements.^{1, 2} In each of the transfer reactions studied, transfer occurred over the course of an hour under the solution conditions used and is quantitative as seen by the change in CD ϵ values for the ferredoxins. The transfer from holo IscU to apo Fdx1 was about 4-fold faster with an apparent second-order rate constant of $8700 \pm 590 \text{ M}^{-1} \text{ min}^{-1}$, as compared to apo Fdx2 which displayed an apparent second-order rate constant of $2400 \pm 1400 \text{ M}^{-1} \text{ min}^{-1}$ for the transfer.

This kinetic data was obtained by monitoring cluster transfer every 2 min for a total of 60 min for each reaction. For the slower reaction, holo IscU to apo Fdx2, the data was also collected for a longer time (up to 3 h); however, transfer was complete within the first hour. Rate constants were determined by continuously monitoring the time dependent reaction at 445 nm for Fdx1 and 440 nm for Fdx2, and accounting for the cluster concentration in each reaction. These rate-constants for cluster transfer are in the physiologically relevant range and are significantly faster than the previously reported transfer from *Azotobacter vinelandii* HscA/HscB/ATP mediated $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound IscU to apo Fdx ($800 \text{ M}^{-1} \text{ min}^{-1}$)¹⁸ but slower than $[\text{Fe}_2\text{S}_2]^{2+}$ cluster-bound GrxS14 to apo plant-type ferredoxin ($20000 \text{ M}^{-1} \text{ min}^{-1}$).⁶⁴ The cluster transfer is apparently unidirectional, as the reverse reactions involving holo ferredoxins and apo IscU with the same concentration of cluster-bound ferredoxins showed no change in the CD spectrum after an hour of reaction (data not shown). Also, control experiments involving holo IscU and no apo ferredoxin did not show cluster breakdown over a period of an hour under anaerobic conditions as monitored by UV-vis spectroscopy (data not shown).¹ This indicates that the cluster was transferred as an intact species from holo IscU to apo ferredoxins and not via cluster degradation and reassembly on the target apo ferredoxins, consistent with our prior assessment of such transfers in earlier Mossbauer experiments.²⁰

Cluster transfer from holo ([2Fe-2S]) Isa1 to apo ferredoxins (Fdx1 and Fdx2)

Isa has been implicated both as an alternative scaffold protein for Fe-S cluster assembly and transfer,^{29, 30} or as an iron-delivery protein.^{31, 32} Bacterial IscA has been shown to form a [2Fe-2S] complex and to transfer cluster to apo ferredoxin.^{16, 65} Also *S. pombe* Isa1 has been shown to transfer cluster to both, human and *S. pombe* ferredoxin with very similar

rate constants.⁶⁶ In this report, we have investigated the ability of *S. pombe* Isa1 to transfer [2Fe-2S] cluster to both *Hs* Fdx1 and *Hs* Fdx2 to identify any kinetic preference for an acceptor protein, and also to compare the efficiency of transfer relative to that seen for IscU to ferredoxins.

UV-vis CD spectroscopy was used to evaluate both the occurrence and kinetics of cluster transfer from *Sp* Isa1 to human apo ferredoxins (Fdx1 and Fdx2). The time course for cluster transfer from holo [2Fe-2S] Isa1 to apo ferredoxins was monitored at room temperature using a 1:1 donor: acceptor cluster stoichiometry and is shown in Fig. 2. For Fdx1 and Fdx2, transfer occurred over the course of 120 and 60 min respectively, and is quantitative as determined by the change in CD ϵ values for the ferredoxins. The apparent second-order rate constants for transfer from holo Isa1 to apo Fdx1 and apo Fdx2 are $6500 \pm 1970 \text{ M}^{-1} \text{ min}^{-1}$ and $8500 \pm 2380 \text{ M}^{-1} \text{ min}^{-1}$ respectively.

This kinetic data was obtained by monitoring cluster transfer every 2 min for a total of 120 min for each reaction, which were complete within an hour as observed by 100% conversion of the donor CD spectrum to that of the acceptor CD spectrum on the basis of the cluster ϵ values. Rate constants were determined by continuously monitoring the time-dependent reaction at 445 nm for Fdx1 and 440 nm for Fdx2 and considering the cluster concentration for each reaction. Cluster transfer was apparently unidirectional, as the reverse reactions involving holo ferredoxins and apo Isa1, with the same concentration of cluster-bound ferredoxins, showed no change in the CD spectrum after an hour of reaction (data not shown).¹ Also, control experiments involving holo Isa1 in the absence of apo ferredoxin did not show evidence of cluster breakdown over a period of an hour under anaerobic conditions, as monitored by UV-vis spectroscopy (data not shown). This indicates that the cluster was transferred as an intact species from holo Isa1 to apo ferredoxins and not via cluster degradation and reassembly on the target apo ferredoxins. A comparison of cross-species rate constants for cluster transfer to human apo Fdx targets from *S. pombe* Isa1 demonstrated that the identity of the Fdx (Fdx1 or Fdx2) is less critical for promoting cluster transfer.

Cluster transfer from holo ([2Fe-2S]) monothiol and dithiol glutaredoxins to apo ferredoxins (Fdx1 and Fdx2)

Glutaredoxins were initially classified as monothiol or dithiol proteins based on the nature of the active sites (either CxxC or CGFS).⁶⁷ However, comparative genomic analyses led to the identification of several Grx isoforms with diverse active site sequences, as well as fusion proteins comprising multiple Grx domains in addition to a Trx-like domain. Monothiol glutaredoxins such as *A. vinelandii* Grx5 have been shown to transfer intact [2Fe-2S] cluster to apo ferredoxin. Plant chloroplastic monothiol glutaredoxin GrxS14 has also been reported to transfer cluster to apo ferredoxin,⁶⁴ while dithiol glutaredoxin has also been found to be capable of transferring [2Fe-2S] to apo ferredoxin through direct interaction.⁶⁸ Accordingly, in this report, we have investigated the ability of the monothiol *S. cerevisiae* Grx3 and dithiol human Grx2 to transfer [2Fe-2S] cluster to *Hs* Fdx1 and *Hs* Fdx2 in order to identify any preference for an acceptor protein, as seen for IscU to ferredoxins. A similar procedure to that described earlier was used to evaluate both cluster transfer, and the kinetics of cluster

transfer from monothiol *S. cerevisiae* Grx3 and dithiol human Grx2 to human apo ferredoxins (Fdx1 and Fdx2). The time course of cluster transfer from holo [2Fe-2S] glutaredoxins to apo ferredoxins was monitored at room temperature using a 1:1 donor: acceptor cluster stoichiometry and is shown in Figs. 3 and 4. The reactions were performed in the presence of 3 mM glutathione as glutaredoxins use 2 molecules of glutathione as exogenous thiol ligands for their clusters.^{4, 69} Transfer reactions occurred over the course of 60–180 min, and the apparent second-order rate constants for transfer from holo Grx3 to apo Fdx1 and apo Fdx2 determined are $2100 \pm 500 \text{ M}^{-1} \text{ min}^{-1}$, and $695 \pm 138 \text{ M}^{-1} \text{ min}^{-1}$, respectively (Fig. 3). Interestingly, holo Grx2 was found to transfer cluster only to apo Fdx1 with a second order rate constant of $1160 \pm 200 \text{ M}^{-1} \text{ min}^{-1}$ (Fig. 4), but not to apo Fdx2 within a 3 h time period. For cluster transfer from holo Grx3 to apo Fdx2, the reaction was monitored for 4 h and the reaction was about 90 percent complete at the end of 3 h. The rate constant for this reaction is the lowest rate that was measured for any transfer reaction; thereby, a rate constant in the range of $700 \text{ M}^{-1} \text{ min}^{-1}$ appears to set a lower limit for physiologically-relevant cluster exchange activity. The cluster transfer for both of the glutaredoxins is apparently unidirectional, as the reverse reactions involving holo ferredoxins and apo glutaredoxins with the same concentrations of cluster-bound forms of ferredoxins showed no change in the CD spectrum after an hour of reaction (data not shown). Also control experiments with holo glutaredoxins in the absence of apo ferredoxin did not display cluster breakdown over a period of an hour under anaerobic conditions, as monitored by UV-vis spectroscopy (data not shown). This indicates that the cluster was transferred as an intact species from holo glutaredoxins to apo ferredoxins and not via cluster degradation and reassembly on the target apo ferredoxins.

Cluster transfer from holo ([2Fe-2S]) Isa1 to other potential apo - targets

In addition to its role as a scaffold protein and cluster transfer to ferredoxins, we investigated the role of Isa for transfer to other physiologically-relevant proteins including IscU, Nfu and Grx2, since all these proteins are localized in the mitochondria. For each reaction, transfer occurred over the course of 60 min. The apparent second-order rate constants for transfer from holo Isa1 to apo Grx2, apo IscU and apo Nfu were determined as $3500 \pm 1300 \text{ M}^{-1} \text{ min}^{-1}$, $2900 \pm 600 \text{ M}^{-1} \text{ min}^{-1}$ and $6700 \pm 1560 \text{ M}^{-1} \text{ min}^{-1}$, respectively (Fig. 5). The rate constants were obtained by monitoring cluster transfer every 2 min for a total of 60 min for each reaction. All reactions were complete within an hour, as observed by 100% conversion of the donor CD spectrum to that of the acceptor CD spectrum on the basis of the cluster ϵ values. The rate constants were determined by continuously monitoring the time dependent reaction at 370 nm for Grx2, 380 nm for IscU and 365 nm for Nfu and the cluster concentration for each reaction. Cluster transfer was found to be apparently unidirectional, as the reverse reactions involving holo Grx2, or holo IscU or holo Nfu and apo Isa1 with the same concentrations of cluster bound forms showed no change in the CD spectrum after an hour of reaction (data not shown). Also control experiments involving holo Isa1 and no apo Grx2 or IscU or Nfu, did not show cluster breakdown over a period of an hour under anaerobic conditions as monitored by UV-vis spectroscopy (data not shown). Therefore, this indicates that the cluster was transferred as an intact species from holo Isa1. Overall, this data is of interest because it highlights alternative physiological roles for Isa1. The results described in this report enable us to advance models of cluster assembly and delivery from a

kinetic perspective, and address questions of physiological relevance concerning redundancy in Fe-S biogenesis pathways and evolutionary protein conservation in a quantitative manner.

Discussion

While the mitochondrial ISC assembly machinery has been characterized by cell-based and biochemical assays in recent years, the understanding of such reactions, in terms of fundamental molecular mechanisms, remains lacking. Following the initial assembly of Fe-S cluster on a scaffold protein such as IscU; subsequent cluster transfer pathways to other protein partners, as well as a molecular understanding of cluster exchange chemistry need to be addressed. Recent studies have shown that this is a complex and highly interconnected process that may involve several distinct proteins at each stage. Lill and coworkers have proposed that the entire process can currently be subdivided into two major steps; first the release and transfer of the IscU-bound Fe-S cluster to intermediate proteins that transiently bind the cluster, and second the apo protein-specific Fe-S cluster insertion into the target proteins.⁷⁰ However, as shown by this report and several other published works,^{1, 2, 4, 24, 28, 35, 39, 64} there are several key players involved in the Fe-S cluster biosynthesis pathway and their roles in Fe-S cluster trafficking can range from highly specific to promiscuous in terms of possible putative partners.

The results presented here serve to map out cluster transfer pathways by determination of kinetic rate constants between putative partner pairs (Fig. 6), where natural cluster exchange reactions are expected to show higher levels of reactivity. IscU has been shown to transfer [2Fe-2S] clusters to ferredoxins;^{18, 23, 64} however, this is the first report to compare the relative rate constants for transfer to Fdx1 or Fdx2. A significant difference is observed in the rate constants for cluster uptake between Fdx1 and Fdx2, indicating that IscU can efficiently transfer cluster to both human ferredoxins. Transfer to Fdx1 compared to Fdx2 is kinetically preferred, which could be indicative of a model where Fdx2 could receive its cluster either from an alternative protein partner, or through a pathway involving multiple proteins. Alternatively, the kinetic preference could reflect the difference in physiological needs, since the proteins may perform different functions,^{50, 51} or suggest a molecular affinity based on the divergent evolution of the two ferredoxins. However, knowledge of the cellular concentrations and protein-protein binding affinities would confirm the kinetic preference at the cellular level. Johnson et al have shown that cluster transfer from *A. vinelandii* IscU to ferredoxin via Grx5 in the presence of HscA/HscB/MgATP in the most kinetically and physiologically relevant pathway with a rate constant of $2100 \text{ M}^{-1}\text{min}^{-1}$.²⁴ However, we have seen relatively efficient rates of transfer in the absence of chaperones, suggesting differences in these pathways between species, since we have investigated human IscU and human ferredoxins.

Isa1 on the other hand shows no kinetic preference between the two ferredoxins. The transfer from Isa1 to Fdx2 is significantly faster than that of IscU to Fdx2 based on rate constants, suggesting a possible redundant function for Isa1 as scaffold that can complete transfer to the ferredoxins when IscU is compromised, since Isa1 is not required for maturation of ferredoxin in yeast.²⁵ Alternatively, the fact that humans have two ferredoxin proteins, unlike yeast, with potentially two distinct functions does establish a possible role

for Isa1 in aiding the maturation of one of the two homologues. There is a kinetic preference based on second-order rate constants, where Fdx2 could receive its cluster from Isa1 instead of IscU. Isa1 can receive its cluster from either the labile pool of [2Fe-2S](GS)₄ complex¹ or possibly from glutaredoxins²⁸ with subsequent transfer to Fdx2. However, thus far we have not observed any transfer to Isa1 from the various proteins we have worked with in this report. While this may indicate a requirement for chaperones to mediate cluster transfer to Isa1, typically the absence of chaperones does not completely eliminate the transfer reaction. *In vivo* studies of the human Isa proteins could confirm the hypothesis of potential transfer to [2Fe-2S] targets, such as the ferredoxins, especially when the levels of IscU are low.

Although [2Fe-2S] cluster transfer from Grx3 to ferredoxins may not be physiologically relevant, because Grx3 is nuclear or cytosolic,⁷¹ whereas ferredoxins are localized in the mitochondria, this observation for distinct and preferential kinetic cluster transfer from Grx3 to ferredoxins could complement Fe-S cluster trafficking between mitochondrial monothiol glutaredoxins, such as Grx5, and ferredoxins, and it clearly supports the proposal that monothiol glutaredoxins play an important role in Fe-S cluster trafficking. Transfer from Grx3 to ferredoxin proteins, although not representing a physiologically possible pathway based on cellular compartmentalization, speaks to potential interactions prior to mitochondrial import and points to areas of consideration that cell studies alone would never have prompted. Furthermore, this transfer reaction, as well as the transfer reactions we have demonstrated from glutaredoxin 3 to ferredoxin proteins, most likely reflects conserved functionality and cluster binding domains across monothiol glutaredoxins. Since glutaredoxin 3 has been shown to functionally compensate for glutaredoxin 5 in cellular studies,^{43, 72–74} it is likely that these proteins can be functionally redundant in *in vitro* assays to represent a conserved transfer pathway, which has already been established by protein-protein interactions and cluster transfer reaction between Grx5 and ferredoxin.²⁴

Grx2 is implicated primarily in cellular apoptosis³⁶ and mitochondrial redox regulation.³⁷ However, there has been no demonstration of a direct link between dithiol Grx2 in cluster trafficking. We were able to demonstrate successful and specific cluster transfer from holo Grx2 to apo Fdx1, supporting a possible role for dithiol glutaredoxins, such as Grx2, in cluster transfer pathways, which has yet to be explored by cellular studies. Even though Fdx1 and Fdx2 share high sequence and structural similarities, there are marked differences in transfer rates and preferences for each of these proteins from various cluster donors, thereby demonstrating not only the redundancy in putative partners for a particular cluster transfer reaction, but also the possibility for the recovery of iron-sulfur cluster biogenesis in the condition of loss of function mutations by compensatory transfer pathways.

The role of DTT in all the transfer reactions has been previously investigated,^{1, 2} since recent studies have suggested formation of Fe-S cluster-associated high molecular weight species (HMWS) when excess DTT is added to the reaction mixtures. These can then facilitate cluster transfer between putative protein partners or formation of [4Fe-4S] cluster on scaffold proteins if excess DTT is added after the formation of [2Fe-2S] clusters.^{75, 76} In our studies, we incubated holo proteins (holo IscU, holo Isa1, holo Grx2, holo Grx3, holo ferredoxins, and [2Fe-2S](GS)₄ complex) with excess DTT. No significant change in the CD

signal of the holo proteins was observed, clearly indicating that under our conditions, DTT is not forming any intermediate Fe-S species.^{1, 2}

We also investigated several other transfer combinations as summarized in Fig. 6 (see also supplementary data, Fig. S1 and S2), thereby proposing a complex, yet kinetically detailed map for cellular cluster trafficking. In addition, we have also outlined a physiological role for the glutathione-complexed [2Fe-2S] cluster in both, mitochondrial and cytosolic cluster trafficking pathways.¹

Conclusions

This report is the first step towards building a network map for all the possible iron-sulfur cluster exchange pathways in mitochondria. Combining this map with all the other available literature will further our understanding and provide key insights into the complex world of global iron-sulfur mobilization. The multitude of cluster exchange pathways and partners could reflect alternative exchange mechanisms that may be utilized under different cellular conditions or under certain cellular protein concentrations. Alternatively, it may reflect the occurrence of non-physiological transfers in a reconstituted *in vitro* model, but speak mainly to evolutionary conservation of iron-sulfur cluster protein families, such as the glutaredoxins. Further studies may resolve the matter, although this would require knowledge not only of relative rate constants, but also of relative physiological concentrations. Additionally, examination of a variety of protein partners will most likely provide insights into more complex cluster exchange reactions, such as the transfer of [4Fe-4S] clusters, and the ability of Fe-S proteins to function in different roles, such as the potential for IscU to bind both [2Fe-2S] and [4Fe-4S] clusters.¹⁹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Marina Bakhtina for her assistance with circular dichroism experiments. This work was supported by a grant from the National Institutes of Health [AI072443]. Christine Wachnowsky was supported by an NIH Chemistry/Biology Interface training grant (T32 GM095450).

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Significance to Metallomics

Herein we take the first steps toward a general mapping of iron-sulfur cluster exchange pathways in mitochondria to provide understanding of the complexity of cellular iron-sulfur mobilization in the mitochondria and cytosol, providing insight on the most likely cellular pathways and possible redundancies. We report on [2Fe-2S] cluster transfer to the ferredoxins from a variety of putative partners. The results reveal rapid, intact, selective and quantitative cluster transfer to both ferredoxins from several donor proteins, and begins the process of providing a kinetically detailed map for cellular cluster trafficking.

Figure 1.

Time course measurements for cluster transfer from the [2Fe-2S]²⁺-bound holo *Hs* IscU to apo *Hs* Fdx1 (20 μM each) (A) and to apo *Hs* Fdx2 (40 μM each) (C) were monitored by UV-vis CD spectroscopy under anaerobic conditions in semi-micro 1 cm cuvettes at room temperature. UV-vis CD spectra were recorded every 2 min for 60 min following addition of the cluster bound forms of hIscU to apo ferredoxins. The kinetics of appearance of the holo ferredoxin CD signal following cluster transfer from holo IscU to apo Fdx1 (B) and to apo Fdx2 (D) was monitored. Cluster transfer reactions were carried out with a 1:1 donor:acceptor cluster stoichiometry. The change in extinction values are based on the initial [2Fe-2S]²⁺ cluster concentration. The apparent second order rate constants obtained for each reaction were determined as (B) $8700 \pm 590 \text{ M}^{-1} \text{ min}^{-1}$ and (D) $2400 \pm 1400 \text{ M}^{-1} \text{ min}^{-1}$ for holo IscU to apo Fdx1 and to apo Fdx2, respectively.

Figure 2.

Time course measurements of cluster transfer from the $[2Fe-2S]^{2+}$ -bound holo *S. pombe* Isa1 to apo *Hs* Fdx1 (40 μ M each) (A) and to apo *Hs* Fdx2 (35 μ M each) (C) were monitored by UV-vis CD spectroscopy under anaerobic conditions in semi-micro 1 cm cuvettes at room temperature. UV-vis CD spectra were recorded every 2 min for 120 min following addition of the cluster bound forms of Isa1 to apo ferredoxins. The kinetics of appearance of the holo ferredoxin CD signal following cluster transfer from holo Isa1 to apo Fdx1 (B) and to apo Fdx2 (D) was monitored. Cluster transfer reactions were carried out with a 1:1 donor:acceptor cluster stoichiometry. The change in extinction values are based on the initial $[2Fe-2S]^{2+}$ cluster concentration. The apparent second order rate constants obtained for each reaction were determined as (B) $6500 \pm 1970 M^{-1} min^{-1}$ and (D) $8500 \pm 2380 M^{-1} min^{-1}$ for holo Isa1 to apo Fdx1 and to apo Fdx2, respectively.

Figure 3.

Time course measurements of cluster transfer from the [2Fe-2S]²⁺-bound holo *S. cerevisiae* Grx3 to apo *Hs* Fdx1 (40 μ M each) (A) and to apo *Hs* Fdx2 (40 μ M each) (C) were monitored by UV-vis CD spectroscopy under anaerobic conditions in semi-micro 1 cm cuvettes at room temperature. UV-vis CD spectra were recorded every 2 min for 60–180 min following addition of the cluster bound form of Grx3 to apo ferredoxins. The kinetics of appearance of the holo ferredoxin CD signal following cluster transfer from holo Grx3 to apo Fdx1 (B) and to apo Fdx2 (D) was monitored. Cluster transfer reactions were carried out with a 1:1 donor:acceptor cluster stoichiometry in presence of 3 mM GSH. The change in extinction values are based on the initial [2Fe-2S]²⁺ cluster concentration. The apparent second order rate constants obtained for each reaction were determined as (B) 2100 ± 500 M⁻¹ min⁻¹ and (D) 695 ± 138 M⁻¹ min⁻¹ for holo Grx3 to apo Fdx1 and to apo Fdx2, respectively.

Figure 4.

Time course measurement of cluster transfer from $[2Fe-2S]^{2+}$ -bound holo human Grx2 to apo *Hs* Fdx1 (30 μM each). (A) was monitored by UV-vis CD spectroscopy under anaerobic conditions in semi-micro 1 cm cuvettes at room temperature. UV-vis CD spectra were recorded every 2 min for 60 min following addition of the cluster bound form of Grx2 to apo Fdx1. The kinetics of appearance of the holo ferredoxin CD signal following the cluster transfer from holo Grx2 to apo Fdx1 (B) was monitored. The cluster transfer reactions were carried out with a 1:1 donor:acceptor cluster stoichiometry in presence of 3 mM GSH. The change in extinction values are based on the initial $[2Fe-2S]^{2+}$ cluster concentration. The apparent second order rate constants obtained (B) was $1160 \pm 200 M^{-1} min^{-1}$.

Figure 5.

Time course measurements of cluster transfer from [2Fe-2S]²⁺-bound holo *S. pombe* Isa1 to apo *Hs* Grx2 (50 μ M each) (A), to apo *Hs* IscU (75 μ M each) (C) and to apo *Hs* Nfu (40 μ M each) (E) were monitored by UV-vis CD spectroscopy under anaerobic conditions in semi-micro 1 cm cuvettes at room temperature. UV-vis CD spectra were recorded every 2 min for 60 min following addition of the cluster bound forms of Isa1 to apo ferredoxins. The kinetics of appearance of the holo Grx2 CD signal following cluster transfer from holo Isa1 to apo Grx2 (B), to apo IscU (D) and to apo Nfu (F) was monitored. Cluster transfer reactions were carried out with a 1:1 donor:acceptor cluster stoichiometry. The change in extinction values are based on the initial [2Fe-2S]²⁺ cluster concentration. The apparent second order rate constants obtained for each reaction were determined as (B) $3500 \pm 1300 \text{ M}^{-1} \text{ min}^{-1}$, (D) $2900 \pm 600 \text{ M}^{-1} \text{ min}^{-1}$ and (F) $6700 \pm 1560 \text{ M}^{-1} \text{ min}^{-1}$ for holo Isa1 to apo Grx2, to apo IscU and to apo Nfu respectively.

Figure 6.

Schematic proposal for cluster uptake and transfer pathways. Kinetic rate constants for iron-sulfur cluster uptake from the $[2\text{Fe-2S}](\text{GS})_4$ complex^{1, 2} and for transfer between proteins have been determined, except for holo *Hs* IscU to apo *Hs* Grx2 and vice versa, which has been previously shown to occur.⁴ The $[2\text{Fe-2S}](\text{GS})_4$ complex is believed to be exported from the mitochondria via the ABCB7 transporter.⁶ Following export to the cytosol, the cluster can then be taken directly up by the cytosolic proteins.