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Role of the HSPA9/HSC20 Chaperone Pair in Promoting Directional Human Iron-Sulfur Cluster Exchange Involving Monothiol Glutaredoxin 5

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Abstract

Iron-sulfur clusters are essential cofactors found across all domains of life. Their assembly and transfer are accomplished by highly conserved protein complexes and partners. In eukaryotes a [2Fe-2S] cluster is first assembled in the mitochondria on the iron-sulfur cluster scaffold protein ISCU in tandem with iron, sulfide, and electron donors. Current models suggest that a chaperone pair interacts with a cluster-bound ISCU to facilitate cluster transfer to a monothiol glutaredoxin. In humans this protein is glutaredoxin 5 (GLRX5) and the cluster can then be exchanged with a variety of target apo proteins. By use of circular dichroism spectroscopy, the kinetics of cluster exchange reactivity has been evaluated for human GLRX5 with a variety of cluster donor and acceptor partners, and the role of chaperones determined for several of these. In contrast to the prokaryotic model, where heat-shock type chaperone proteins HscA and HscB are required for successful and efficient transfer of a [2Fe-2S] cluster from the ISCU scaffold to a monothiol glutaredoxin. However, in the human system the chaperone homologues, HSPA9 and HSC20, are not necessary for human ISCU to promote cluster transfer to GLRX5, and appear to promote the reverse transfer. Cluster exchange with the human iron-sulfur cluster carrier protein NFU1 and ferredoxins (FDX's), and the role of chaperones, has also been evaluated, demonstrating in certain cases control over the directionality of cluster transfer. In contrast to other prokaryotic and eukaryotic organisms, NFU1 is identified as a more likely physiological donor of [2Fe-2S] cluster to human GLRX5 than ISCU.

Graphical abstract

Cluster exchange chemistry for human glutaredoxin 5 (GLRX5) reveals distinct behavior from bacterial homologues with chaperones inhibiting rather than promoting cluster transfer from the iron-sulfur cluster scaffold protein ISCU1, and controlling the directionality of transfer. Human iron-sulfur cluster carrier protein NFU1 is identified as the most likely donor to GLRX5.

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INTRODUCTION

First characterized by EPR and Mössbauer spectroscopy in the 1960s, iron-sulfur clusters have emerged as a leading class of highly conserved, essential cofactors for all life on earth [1]. The three most common types of clusters contain [2Fe-2S], [3Fe-4S], and [4Fe-4S] cores, while additional variants exist for more specialized roles [2]. Found in iron-sulfur proteins, these simple complexes allow organisms to perform numerous biochemical tasks, including nucleic acid processing and repair, cellular sensors, electron transfer and catalysis [3–7].

Glutaredoxins are small, constitutive proteins that are found across all domains of life. They may be divided into two categories (dithiol and monothiol [8]), but both subcategories utilize the tripeptide glutathione, even though they serve different cellular functions. Dithiol glutaredoxins are primarily involved in regulating the redox potential of the cell, in concert with their thioredoxin counterparts; but instead of directly rescuing oxidized cysteines, the dithiol glutaredoxins utilize glutathione to accept the oxidative damage from the target protein before being rescued by glutathione reductase [8]. Monothiol glutaredoxins comprise the other category and are capable of binding Fe-S clusters [9]. The bridging clusters are ligated by a Cys ligand from the conserved CGFS site of each of the two component monomers [9], which are complemented by two other Cys ligands from two exogenous glutaredoxin-bound glutathione molecules (one per monomer) [10]. The structural features are illustrated by the crystal structure of human glutaredoxin 5 (GLRX5), which displays the bridging-cluster (Figure 1).

Monothiol GLRX5 is a mitochondrial Fe-S protein that is required for Fe-S protein activity downstream from assembly [11] and is believed to serve as an intermediate carrier that delivers cluster to a variety of apo protein targets [11, 12]. Monothiol glutaredoxins have been shown to exchange cluster with target proteins in a variety of organisms, including *Arabidopsis thaliana* and *Azotobacter vinelandii* [11, 13]. However, GLRX5 has only recently been studied with regards to direct cluster transfer to target apo proteins *in vitro*.

The current model of mitochondrial cluster assembly begins with the iron-sulfur cluster scaffold protein ISCU in conjunction with sulfide, iron, and electron donors [14–16]. ISCU is a highly conserved scaffold on which nascent [2Fe-2S] clusters are assembled [17] and, in the case of bacteria and yeast, subsequently delivered to a target monothiol glutaredoxin 5 with the aid of heat-shock type chaperone proteins, pairing chaperone proteins DnaJ/HscB/Jac1/Hsc20 and DnaK/Hsp70/HscA/Ssq1/HSPA9, respectively. These interact with the holo ISCU-type host to stabilize a conformational change and enable cluster transfer to the target apo proteins [11, 18]. In yeast, the HSPA9 homolog Ssq1 was shown to interact with both ISCU and GLRX5, facilitating cluster transfer by complex formation and suggesting the use of chaperones in cluster transfer to be conserved across domains [19].

HSPA9 is the human homolog of HscA, a member of the Hsp70 family [20], which has been similarly implicated in Fe-S cluster synthesis and found to bind to the disordered form of ISCU [21, 22]. HSPA9 also serves multiple functions, including protection from oxidation, cell cycle regulation, and the import of cytosolic proteins into mitochondria [23–26]. Holo GLRX5 can deliver cluster directly to downstream target [2Fe-2S] proteins, such as ferredoxins 1 and 2. GLRX5 has also been suggested to deliver its cluster to the iron-sulfur cluster scaffold/maturation proteins IscA1 and IscA2 [27, 28]. Additionally, diseases associated with mutations in GLRX5 all display hyperglycinemia, as well as other symptoms, indicative of lowered lipoate synthase (LIAS) activity in addition to generalized faults in Fe-S cluster assembly [29, 30].

We have previously reported on the transfer of a [2Fe-2S] cluster from a [2Fe-2S](GS)₄ complex to various target apo proteins, including ISCU and glutaredoxin 3 (GLRX3) [31], and general mapping of cluster transfer to and from mitochondrial and cytosolic iron-sulfur proteins. Herein we report the addition of GLRX5 to the human mitochondrial trafficking map [32]. The glutathione-complexed species may serve as a labile storage pool that can donate and accept cluster, export it from mitochondria via the human mitochondrial export protein ABCB7, and sequester iron to mitigate cellular iron toxicity [33]. GLRX5 utilizes two glutathione molecules to ligate a bridging Fe-S cluster [34], so the relationship between GLRX5, glutathione, and the glutathione-complexed cluster was further investigated to better understand the role of both GLRX5 and the cluster complex in mitochondrial cluster transfer.

The importance of GLRX5 in human metabolism is emphasized by the deleterious effects that faulty GLRX5 biosynthesis or expression has on cellular health. Deletion of the gene for GLRX5 in zebrafish is lethal, displaying anemia and iron overload [35]. In humans, two disorders are associated with GLRX5. The first is a result of a defect in intron splicing and yields symptoms characteristic of sideroblastic anemia (SA), including liver iron overload and cirrhosis [35]. The other derives from a single codon deletion, mutant K51del, and results in variant nonketonic hyperglycinemia [36]. As noted earlier, hyperglycinemia most likely arises from a flawed glycine cleavage machinery that results from lower LIAS activity [37], which in turn is responsible for the biosynthesis of the essential lipoate cofactor for this pathway [38]. In this work we focus on the process by which GLRX5 exchanges its [2Fe-2S] cluster with partner proteins, to better understand the role of human GLRX5 in mitochondrial cluster chemistry and the functional role of the HSPA9 and HSC20

chaperones in mediating cluster exchange with human ISCU and human iron-sulfur cluster carrier protein NFU1.

MATERIALS and METHODS

Purchased Materials

The gene for *Homo sapiens* glutaredoxin 5 (GLRX5), located in the pET28b(+) vector between the NdeI and HindIII restriction sites and lacking the first 31 amino acids (1–31) that correspond to the mitochondrial targeting sequence [27], was ordered from GenScript. PD-10 desalting columns were purchased from GE Healthcare. Ferric chloride, sodium sulfide and L-cysteine were obtained from Fisher.

Cloning and expression of GLRX5

The expression vector carrying the gene for GLRX5 was transformed and expressed in *Escherichia coli* BL21(DE3). Transformed cells were grown in 7 mL of Luria-Bertani (LB) broth media containing 30 µg/mL kanamycin and incubated at 37 °C until the OD₆₀₀ reached 0.9. A one-liter LB culture was inoculated with this culture and allowed to grow at 37 °C until the OD₆₀₀ reached 0.9. Protein expression was induced with the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and cultures were incubated at 30 °C for 16 h. Cell pellets were collected by centrifugation at 5000 rpm for 15 min, and then stored at –80 °C.

Purification of GLRX5

Human GLRX5 was purified aerobically at 4 °C. Stored cell pellets were resuspended in 50 mM HEPES, 100 mM NaCl, pH 7.5 buffer. Deoxyribonuclease was added to a concentration of 1 mg/mL and the pellet was lysed with a sonic dismembrator at 4 °C with 10 s pulses and 1 min rest intervals. The lysate was centrifuged at 15,000 rpm for 50 min, and the supernatant was loaded onto a Ni-NTA column. The resin was washed 3x with 50 mM HEPES, 100 mM NaCl, 50 mM imidazole and pH 7.5, and then protein was eluted with 50 mM HEPES, 100 mM NaCl, and 250 mM imidazole, pH 7.5, and subsequently concentrated by Amicon ultrafiltration. Imidazole was removed by use of a PD-10 desalting column and SDS-PAGE was used to determine protein purity. Protein concentration was determined by use of the Bradford assay. Isolated GLRX5 was found to purify with an amber-brown color, indicating at least a portion of the protein purified in the cluster-bound form, and holo concentration was determined by iron quantitation and confirmed by use of reported extinction coefficients for homologs [13].

Purification of secondary proteins

Hs ISCU, *Hs* NFU, *Thermatoga maritima* (*Tm*) NifS, and *Hs* Ferredoxin-2 (FDX2) were all purified as previously described [16, 32, 39, 40]. A construct of human ferredoxin-1 (FDX1) was provided by Dr. Markley (U. Wisconsin), with the protein expressed and purified according to literature procedures [41]. *Hs* HSPA9 was purified as previously described [20].

Human HSC20 was purified aerobically at 4 °C. Stored cell pellets were resuspended in 50 mM HEPES, 10 mM NaCl, pH 7.5 buffer. Deoxyribonuclease was added to a concentration

of 1 mg/mL and the pellet was lysed with a sonic dismembrator. The lysate was centrifuged at 15,000 rpm for 50 min, and the supernatant was loaded onto a Ni-NTA column. The resin was washed 3x with 50 mM HEPES, 100 mM NaCl, 50 mM imidazole and pH 7.5, then the protein was eluted with 50 mM HEPES, 100 mM NaCl, 250 mM imidazole and pH 7.5, and subsequently concentrated by Amicon ultrafiltration. Imidazole was removed via a PD-10 desalting column and SDS-PAGE was used to determine protein purity. Protein concentration for HSC20 was determined from the 280 nm absorbance.

UV-vis and CD characterization of GLRX5

Samples of as-purified and chemically reconstituted GLRX5 were added anaerobically to degassed buffer. UV-vis scans were taken from 800 to 200 nm. Spectral features included a shoulder at 330 nm and peaks in the 400–500 nm range (Fig. S1), indicative of a [2Fe-2S] species [42]. Anaerobic samples of GLRX5 were also obtained in the 300–600 nm range by use of a Jasco CD instrument. Cluster-bound GLRX5 displays prominent negative features at 350 nm and 412 nm, with a prominent positive feature at 450 nm.

Chemical reconstitution of apo proteins

GLRX5 and NFU1 were chemically reconstituted according to established protocols, where a solution of 1 mL protein, 2 μ M *Tm* NifS, 5 mM DTT, and 3 mM GSH underwent multiple rounds of argon-purging [43]. To this anaerobic solution, FeCl₃ and L-cysteine were added to 0.8 mM each, and the solution stirred under anaerobic conditions for 1 h. ISCU was chemically reconstituted according to previous literature using Na₂S in place of L-cysteine, and without *Tm* NifS added to the reaction mixture [44]. To remove excess DTT, FeCl₃, and L-cysteine/Na₂S, the solution was passed through a PD-10 desalting column equilibrated with 50 mM HEPES, and 100 mM NaCl, pH 7.5. The concentration of holo protein was determined both by iron quantitation and use of extinction coefficients [13, 45], while total protein concentration was determined via the Bradford assay.

Iron quantitation of holo proteins

Iron quantitation was performed according to protocol [46], where holo proteins were acidified by the addition of 60 μ L of 12M HCl and heated to 100 °C for 15 min. Samples were centrifuged and the supernatant diluted into 0.5 M, pH 8.5 Tris buffer. 100 μ L of 5% fresh sodium ascorbate and 400 μ L of 0.1% bathophenanthroline disulfonate were added to the samples before incubation in the dark for 1 h at 25 °C. Sample absorbance at 535 nm was measured and iron quantified through the use of a calibration curve of 1 μ M to 100 μ M FeCl₃ solutions.

Conversion of holo to apo GLRX5

GLRX5 partially purifies in the cluster-bound form with an amber-brown color. Conversion to apo GLRX5 was performed according to literature procedures, where 1 mL of protein was incubated with 100 mM EDTA, 8 M Urea, and 5 mM DTT, pH 7.5 and heated to 45 °C for 1 h [47]. Subsequently, reagents were removed by passing the solution through a PD-10 desalting column equilibrated with 50 mM HEPES, 100 mM NaCl, pH 7.5 buffer and the

absence of residual holo protein confirmed by UV-vis, while the Bradford assay was used to determine protein concentration.

Cluster stability of GLRX5

Glutathione has been shown to extract Fe-S clusters from certain [2Fe-2S] cluster containing proteins, including GLRX3 [27, 31]. Therefore, to confirm both previous work as well as study the effect of excess glutathione on cluster-bound GLRX5, cluster extraction was attempted. Degassed holo GLRX5 was incubated with varying concentrations of glutathione and UV-vis scans were taken over the period of one hour, but no extraction was observed, even at a 3 mM excess of glutathione.

Cluster transfer monitored by circular dichroism

CD spectra were recorded on a Jasco J-815 CD spectrometer at a range of 600–300 nm with a scan rate of 200 nm/min at 25 °C. A solution of 40 μM apo protein, 5 mM DTT, and 3 mM GSH in 50 mM HEPES, 100 mM NaCl, pH 7.5, was thawed under vacuum and injected into a quartz 1 cm anaerobic cuvette, with additional reagents noted below as needed. Holo protein was injected to a final concentration of 40 μM to initiate cluster transfer.

For cluster transfers involving the presence of the HSPA9/Hsc20 chaperone pair, HSPA9 and Hsc20 were added anaerobically with the apo protein to a final concentration of 20 μM each and ATP and MgCl₂ added to final concentrations of 40 mM and 2 mM, respectively. Spectra were processed using JASCO Spectramanager II Analysis and Origin 7.0 software packages.

To obtain kinetic data for these transfers the change in CD signal was monitored over time at key wavelengths uniquely characteristic to each Fe-S protein. This data was fit as previously reported where DynaFit software was used to determine the second-order rate constants for each cluster transfer by using a best-fit simulation to second-order kinetics [48]. Background cluster degradation is negligible within the timescale of these experiments and is within the standard errors cited for the experiments.

RESULTS

CD spectroscopy is a powerful tool that enables the monitoring of cluster transfer from donor holo protein to acceptor apo protein because the chiral active site of each holo protein displays a unique CD spectrum (Figs. S2–S5). By monitoring the change in CD signal from the donor species to the acceptor species we gain kinetic insight into the selectivity of mitochondrial pathways for iron-sulfur cluster exchange. By use of such experiments, we have previously developed a preliminary map of cluster transfer pathways for both mitochondrial and cytosolic cluster trafficking [32, 47].

GLRX5 cluster stability and exchange with [2Fe-2S](GS)₄ and glutathione

We have previously shown that a [2Fe-2S] cluster complexed to four glutathione molecules is capable of donating its cluster to a variety of target apo proteins [31, 33]. However, in the case of apo GLRX5, no transfer was observed (Fig. S6), even with a ten-fold excess of the

glutathione-complexed [2Fe-2S](GS)₄ cluster, clearly indicating that GLRX5 cannot accept cluster from this species. This result is consistent with the observation that apo GLRX5 is monomeric [27] and lacks a well-defined site to accept cluster [33], in contrast to GLRX3, which tends to dimerize even in the apo state.

GLRX5 is isolated, at least in part, in the cluster-bound holo state, even under aerobic conditions, supporting the intrinsic stability of holo GLRX5. Excess glutathione has been shown to abstract clusters from a variety of holo proteins, but earlier literature suggested that GLRX5 is resistant to cluster extraction [27, 31, 49], indicating the cluster to be stably bound and resistant to degradation. Consistent with these prior reports we have also found GLRX5 to be stable toward glutathione extraction in the range of 0.25 to 5 mM under anaerobic conditions. Even at the highest concentration of glutathione, both the UV-vis and CD spectra for GLRX5 remained unchanged (data not shown).

Cluster exchange between GLRX5 and ISCU in the absence of chaperones

An important question relating to the human ISCU and GLRX5 proteins is whether or not they follow the chaperone-promoted ISCU to GLRX5 cluster transfer model previously supported by eukaryotic and prokaryotic homologues. In the absence of chaperones a unidirectional transfer of cluster was indeed observed from holo ISCU to apo GLRX5 (Figure 2), with an apparent rate constant of $10,300 \text{ M}^{-1}\text{min}^{-1}$, but the reverse transfer from holo GLRX5 to apo ISCU was not observed (Figure S7), indicating a propensity for directional cluster transfer from ISCU to GLRX5. This stands in marked contrast to the case previously described for the *A. vinelandii* homologs, where it was shown that transfer from IscU and Grx5 required the presence of the HscA/HscB chaperone pair to proceed [11]. It is also noteworthy that in the case of the faster transfers greater deviations are observed in early CD signature from holo donor, relative to the isolated holo protein, because transfer is already occurring.

Impact of HSPA9/HSC20 chaperones on cluster exchange between GLRX5 and ISCU

Interestingly, in the presence of HSPA9 and Hs HSC20, we find holo GLRX5 to transfer cluster to apo ISCU with an apparent rate constant of $7,500 \text{ M}^{-1}\text{min}^{-1}$ (Figure 3). This again runs counter to previous studies with *A. vinelandii*, where no transfer was seen from Grx5 to IscU, even over the course of three hours [11]. A possible cause of this difference may be that the human chaperones are stabilizing a structural state that promotes cluster delivery to ISCU [21] and inhibiting the reverse reaction of cluster transfer from ISCU to GLRX5 (Figure S8). To further test this idea, ISCU-GLRX5 cluster transfer was examined for a substituted derivative of ISCU, D37A that has been shown to stabilize the bound cluster [50] and the preferred structural state of the holo protein [18]. Cluster transfer from holo GLRX5 to apo D37A (Figure 4) was observed with an apparent rate constant of $3,500 \text{ M}^{-1}\text{min}^{-1}$ while the reverse transfer, from holo D37A to apo GLRX5, was not observed (data not shown), consistent with the hypothesis that the direction of cluster transfer between ISCU and GLRX5 depends, at least in part, on the structural state of ISCU.

Cluster exchange between GLRX5 and NFU1

NFU1 is a mitochondrial Fe-S protein that has recently become the focus of increased attention, having been implicated in multiple mitochondrial dysfunctions syndrome (MMDS) while its precise role(s) in mitochondrial cluster chemistry is an evolving area of study [44, 51, 52]. Recently, we have established several cluster transfers to and from NFU1, supporting a role as an alternative cluster carrier/trafficking protein that could also mediate cluster exchange with GLRX5 [31, 32, 66 – 69]. Bidirectional cluster transfer was observed, although the observed rate constants for each transfer direction were significantly different, as well as distinctive influences from chaperones. Figure 5 shows the transfer from GLRX5 to NFU1 with an apparent, and relatively small rate constant of $950 \text{ M}^{-1}\text{min}^{-1}$. No cluster transfer was observed in the presence of HSPA9 and HSC20 chaperones (Fig. S9), however, given the modest rate in the absence of cluster it appears that chaperones have a minimal impact on cluster transfer from GLRX5 to NFU1.

By contrast, we have previously shown the reverse transfer from NFU1 to GLRX5 to be much faster, with a rate constant of $35,100 \text{ M}^{-1}\text{min}^{-1}$ [66], where it was also observed that chaperones did not have a significant impact on cluster exchange between NFU1 and GLRX5 with a rate constant of $28,500 \text{ M}^{-1}\text{min}^{-1}$ and within error limits of the transfer without chaperones present (Table 1).

Cluster exchange between GLRX5 and ferredoxins 1 and 2

Ferredoxin 1 and ferredoxin 2 have been established as mitochondrial [2Fe-2S]-bound proteins that as electron carriers and assist in heme- and steroidogenesis [53]. On the cluster trafficking pathways in the cell, both ferredoxins can be viewed as terminal cluster acceptors [32]. To better understand GLRX5's potential to deliver cluster directly to target [2Fe-2S] apo proteins, transfers to and from ferredoxin 1 and ferredoxin 2 with GLRX5 were attempted. Successful transfers from holo GLRX5 to either apo ferredoxin 1 (Figure 6) or apo ferredoxin 2 (Figure 7) were seen, with apparent rate constants of $2,000 \text{ M}^{-1}\text{min}^{-1}$ and $650 \text{ M}^{-1}\text{min}^{-1}$, respectively. No transfer from either holo ferredoxin to apo GLRX5 was observed (data not shown), consistent with their role as terminal cluster acceptors in the mitochondria.

DISCUSSION

Much of the available information concerning GLRX5 in eukaryotes derives from investigations of yeast mutants and disease characterization in humans [36, 54], while direct studies of the human GLRX5 are few in number, relative to other relevant proteins. In previous reports on the related GLRX3 protein we have reported on cluster transfer reactivity from holo ISCU to apo GLRX3 in the absence of the HSPA9/HSC20 chaperone pair, resulting in a rapid unidirectional transfer [47]. This differs from the observation for *A. vinelandii* Grx5 where these chaperones are required for efficient, unidirectional transfer from IscU to apo Grx5 [11]. In a similar manner to GLRX3, transfer from human holo ISCU to apo GLRX5 occurs rapidly in the absence of HSPA9 and HSC20 (Figure 2), indicating that the eukaryotic system for cluster assembly on ISCU and its transfer may differ significantly from the prokaryotic model, where chaperones are required for significant

transfer. Remarkably, no transfer was observed from holo ISCU to apo GLRX5 in the presence of HSPA9 and HSC20 (Figure S8), but instead allowed cluster transfer from holo GLRX5 to apo ISCU (Figure 3). In contrast to this, the *A. vinelandii* transfer rate was reduced to a mere $30 \text{ M}^{-1}\text{min}^{-1}$ when chaperones were absent.

Previously we have reported that *Tm* DnaK stabilizes the cluster-bound form of *Hs* ISCU and inhibits cluster transfer from holo ISCU to apo targets [55]. For eukaryotic ISCU the chaperones may serve to regulate, rather than facilitate, cluster transfer to target apo proteins, as the transfer from holo GLRX5 to apo ISCU (Figure 3) indicates the chaperones to increase ISCU's propensity to bind Fe-S clusters. The D37A mutant of ISCU has been found to stabilize a cluster-bound form of the protein [18, 50], and so the unidirectional transfer from holo GLRX5 to apo ISCU D37A (Figure 4) also supports this idea. Although there has been extensive investigation of cluster assembly and transfer involving ISCU, the use of constructs from different organisms and systems clouds the relevancy of such findings. Despite the high level of homology for ISCU across domains, differences have emerged [17].

For *A. vinelandii* IscU and Grx5 it has previously been reported that chaperones promote a 22-fold enhancement of rate constant for Fe-S cluster transfer from IscU to ferredoxin [56] and 670-fold for Grx5 [57], although for *E. coli* homologs cluster transfer from IscU to apo ferredoxin was increased only 5–10 fold [58, 59]. In this work we demonstrate an inhibitory role for chaperones in promoting cluster exchange from human ISCU1 to GLRX5 (Figure S8), but a stimulatory role in promoting the reverse transfer from GLRX5 to ISCU1 (Figure 3). While the components of the Fe-S cluster biosynthesis pathway and the corresponding chaperones are highly conserved, these differences in rate enhancement suggest organism-specific control over the process, and control of the directionality of cluster transfer.

Ferredoxins 1 and 2 have been established as terminal mitochondrial Fe-S cluster acceptors, including transfer from the cytosolic glutaredoxin GLRX3 [32]. In support of this transfer capability, GLRX5 is also able to unidirectionally transfer cluster to both FDX1 and FDX2 (Figures 6 and 7), supporting their position as terminal cluster targets. For GLRX5, the difference in transfer rate to either ferredoxin is minor, showing little preference for either target. The rates are also relatively low in comparison to other exchange reactions that we have examined, and so GLRX5 would not be expected to serve as a primary cluster donor to ferredoxins, but consistent with the idea that there is considerable redundancy in mitochondrial cluster transfer pathways, allowing for multiple routes for Fe-S clusters to reach their final destination [32].

While the $[2\text{Fe-2S}](\text{GS})_4$ cluster complex has been shown to donate cluster to a variety of relevant mitochondrial and cytosolic Fe-S proteins, including Grx3 from *S. cerevisiae* [31], nevertheless, GLRX5 does not receive cluster from the complex (Figure S6), most likely as a result of its monomeric form in the apo state [27]. As detailed in Table 1, both ISCU and NFU1 are more likely physiological donors [31], although the negative impact of chaperones indicates NFU1 to be the more likely donor. This is also consistent with our previous studies of cluster delivery to cytosolic GLRX3, where redundancy was also observed, but with a clear preference for NFU1 [47].

CONCLUSIONS

This work is an extension of our previous efforts to map physiologically relevant cellular pathways through which Fe-S clusters are transferred in both the mitochondria and cytosol [32, 47]. These pathways begin primarily with ISCU, and so understanding the role and mechanism ISCU and its partners play in cluster assembly and delivery is essential to better grasp the pathogenicity associated with faulty Fe-S proteins [6]. In showing that GLRX5, along with GLRX3, can receive cluster from ISCU without chaperones, we highlight the differences and limitations in applying models from one system to others. There is a high level of conservation and sequence homology associated with cluster assembly machinery across domains [60]. In spite of this, we have demonstrated that these systems are not completely interchangeable and that significant differences can arise. An example of this would be frataxin and its homologues displaying contradictory data across model systems. In the mammalian system frataxin was shown to increase cysteine desulfurase activity by binding to the ISCU-Nfs-Isd11 complex [61]. This is in contrast to the yeast system, where the frataxin homolog increases desulfurase activity, but this is independent of ISCU binding [62], and the bacterial system which actually showed a decrease in cluster synthesis with the addition of frataxin homolog [63]. In the case of GLRX5, the HSPA9/HSC20 chaperone pair is dispensable regarding cluster transfer from holo ISCU, but they may still serve a regulatory role according to the needs of the cell. HSPA9 has been found to be associated with multiple processes for the cell and interacts with p53, so the modulation of cluster assembly and transfer may be in response to other metabolic pathways HSPA9 is involved in [23, 24, 26, 64]. ISCU activity is also regulated by the protease Pim1 [65], so HSPA9 modulating ISCU transfer activity most likely reflects another means to better regulate cluster assembly and iron levels in response to cellular conditions.

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Highlights

- Cluster exchange chemistry for human glutaredoxin 5 is distinct from bacterial homologs.
- Heat-shock type chaperone proteins HSPA9 and HSC20 control transfer directionality.
- Heat-shock type chaperone proteins inhibit cluster transfer from scaffold proteins.
- Iron-sulfur cluster carrier protein NFU1 is the most likely donor to glutaredoxin 5.
- The work extends the map of physiologically relevant cellular Fe-S cluster transfers.

Figure 1. Crystal structure of human GLRX5 (PDB: 2WUL). Although the structure proper shows a tetrameric organization this has been considered an artifact of the crystal, and that the functional unit is a bridged homodimer. A [2Fe-2S] cluster is ligated by two monomers of GLRX5 (green) with two more ligands coming from exogenous glutathione molecules (blue).

Figure 2.

(top) Cluster transfer from 40 μM holo ISU to 40 μM apo GLRX5 in the absence of chaperones was monitored by CD spectroscopy. (bottom) The time-dependent change in CD signal at 412 nm was fit to yield an apparent second-order rate constant of $10,300 M^{-1}min^{-1}$. Reactions were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5.

Figure 3.

(top) Cluster transfer from 40 μM holo GLRX5 to 40 μM apo ISU in the presence of chaperones (20 μM each), 40 mM $MgCl_2$ and 2 mM ATP, was monitored by CD spectroscopy over the course of an hour. (bottom) The time-dependent change in CD signal at 328 nm yielded an apparent second-order rate constant of $7,500 M^{-1}min^{-1}$. Reactions were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5.

Figure 4.

(top) Cluster transfer from 40 μM holo GLRX5 to 40 μM apo ISU D37A in the absence of chaperones was monitored by CD spectroscopy over the course of an hour. (bottom) Monitoring the time-dependent change at 375 nm yielded an apparent second-order rate constant of 3,500 $M^{-1}min^{-1}$. Reactions were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5

Figure 5.

Cluster transfer from 40 μM holo GLRX5 to 40 μM apo NFU1. (top) Successful transfer was seen within 2 h, with characteristic features of NFU1 at 325 nm and 365 nm appearing. (bottom) An apparent rate constant of $950 M^{-1}min^{-1}$ was acquired by monitoring change in CD signal at 365 nm over the course of the reaction. Reactions were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5

Figure 6.

Cluster transfer from 40 μ M holo GLRX5 to 40 μ M apo ferredoxin 1. (top) Unidirectional cluster transfer was seen, with key spectral features associated with holo FDX1 appearing at 325, 375, and 425 nm over a period of 2 h. (bottom). The change in absorbance at 380 nm was monitored over the course of the reaction to yield an apparent rate constant of 2,000 $M^{-1}min^{-1}$. Reactions were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5.

Figure 7.

Cluster transfer from 40 μM holo GLRX5 to 40 μM apo ferredoxin 2. (top) Unidirectional cluster transfer was seen, with key features of holo Ferredoxin 2 appearing over the reaction duration of two hours. (bottom) The change in CD signal was monitored at 380 nm over the course of the reaction to yield an apparent rate constant of $650 M^{-1}min^{-1}$. Reactions were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5.

Table 1

List of successful cluster transfers involving GLRX5 and their rates, as well as the presence or absence of any chaperones.

Donor Protein	Acceptor Protein	Chaperones	Transfer Rate ($M^{-1}min^{-1}$)
ISCU	GLRX5	Yes	none
		No	$10,300 \pm 1800$
GLRX5	ISCU	Yes	$7,500 \pm 2,300$
		No	none
GLRX5	D37A ISCU	No	$3,500 \pm 500$
GLRX5	NFU1	Yes	none
		No	950 ± 450
NFU1	GLRX5	Yes	$28,500 \pm 7,500^a$
		No	$35,100 \pm 2,000^a$
GLRX5	FDX1	No	$2,000 \pm 700$
GLRX5	FDX2	No	650 ± 250

^aData taken from [66].