

## **[4Fe-4S] cluster assembly in mitochondria and its impairment by copper**

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## **ABSTRACT**

The cellular toxicity of copper is usually associated with its ability to generate reactive oxygen species. However, recent studies in bacterial organisms showed that copper toxicity is also strictly connected to iron-sulfur cluster proteins and to their assembly processes. Mitochondria of eukaryotic cells contain a labile copper(I) pool localized in the matrix where also the mitochondrial iron-sulfur (Fe/S) cluster assembly machinery resides to mature mitochondrial Fe/S cluster-containing proteins. Misregulation of copper homeostasis might therefore damage mitochondrial Fe/S protein maturation. To describe, from a molecular perspective, the effects of copper(I) toxicity on such maturation process, we have here investigated the still unknown mechanism of [4Fe-4S] cluster formation conducted by the mitochondrial ISCA1/ISCA2 and GLRX5 proteins, and defined how copper(I) can impair this process. The molecular model here proposed indicates that the copper(I) and Fe/S protein maturation cellular pathways needs to be strictly regulated to avoid copper(I) ion to block mitochondrial [4Fe-4S] protein maturation.

## INTRODUCTION

Early hypotheses on cellular copper toxicity were based on its ability to generate reactive oxygen species (ROS).<sup>1-3</sup> Later on it has been shown that copper accumulation and its toxicity increases in *Escherichia coli* and in *Saccharomyces cerevisiae* under anaerobic growth conditions, in which no oxygen is available for ROS generation,<sup>4-6</sup> and that copper toxicity is primarily due to the disruption of iron metabolism via non-ROS mechanisms.<sup>7,8</sup> Most of the ROS-independent copper toxicity likely stems from copper thiophilic nature, which favors its binding and/or the displacement of other metal ions or metal cofactors that are less tightly bound to thiolate or sulfide ligands,<sup>9</sup> as predicted by the Irving-Williams series.<sup>10</sup> Excess copper can therefore damage iron-sulfur (Fe/S) protein biogenesis<sup>11-14</sup> as well as disrupt matured Fe/S cluster-containing metalloenzymes.<sup>7,15</sup> Specifically, it has been shown that copper(I) ions: i) rapidly inactivate Fe/S cluster dehydratases in *E. coli* cells by displacing iron atoms from the solvent-exposed cluster of the dehydratases, leading to a non-functional enzyme;<sup>7</sup> ii) block the [4Fe-4S] cluster assembly process by targeting, in *E. coli*, the Iron-sulfur cluster Assembly protein (IscA), whose cluster binding site has a strong copper(I) binding affinity;<sup>11</sup> iii) target, in *Bacillus subtilis*, the sulfur mobilization protein S (SufU),<sup>16</sup> which loses its bound [4Fe-4S] cluster upon copper(I) addition.<sup>14</sup>

Humans contain two highly homologous IscA proteins, named ISCA1 and ISCA2. Both of them are located in the mitochondrial matrix, being part of the mitochondrial Iron-Sulfur Cluster (ISC) assembly machinery and they are fundamental for the maturation of mitochondrial [4Fe-4S] cluster-containing proteins,<sup>17-20</sup> via the formation of a heterocomplex.<sup>21</sup> Recently, we have characterized such system, showing that a ISCA1/ISCA2 heterodimeric complex is the functional unit that receive two [2Fe-2S]<sup>2+</sup> clusters from the physiological cluster-donor, the GLRX5 protein,<sup>22</sup> to assemble a [4Fe-4S] cluster prior to its transfer to the final target apo proteins.<sup>21</sup> The molecular mechanism through which the [4Fe-4S] cluster is formed remains, however, completely undefined. In particular, considering that the two ISCA proteins have three conserved cysteines which are all critical for their function in Fe/S protein formation,<sup>23,24</sup> it is not known how they drive the [4Fe-4S] cluster assembly process.<sup>25</sup>

Mitochondria of eukaryotic cells contain a large pool of copper(I) ions localized in the matrix, possibly bound to a soluble low molecular weight ligand (L), which accounts for around 70-85% of the total mitochondrial copper.<sup>26,27</sup> Although the identity of the copper(I)-ligand complex (CuL) remains unresolved,

several experimental evidences showed that the mitochondrial matrix CuL pool is accessible to copper-binding proteins.<sup>28</sup> Specifically, it has been shown that targeting copper-binding proteins to the matrix attenuates the level of the CuL complex within the matrix, without affecting the total mitochondrial copper levels.<sup>28</sup> The mitochondrial iron-sulfur cluster (ISC) assembly machinery and, specifically, the ISCA1/ISCA2 and GLRX5 proteins responsible of the [4Fe-4S] cluster assembly process,<sup>19,29,30</sup> therefore operate in an environment that contains an accessible CuL pool. This raises questions whether misregulation of copper homeostasis can damage the mechanisms of Fe/S protein assembly in mitochondria, and how the mitochondrial labile CuL pool can avoid possible molecular damages to Fe/S protein maturation also under the physiological cellular conditions.

To describe, from a molecular perspective, the effects of copper toxicity on the mitochondrial Fe/S protein assembly process, we have investigated the still unknown mechanism of [4Fe-4S] cluster formation accomplished by mitochondrial ISCA1/ISCA2 and GLRX5 proteins and studied how copper(I) can impair various steps of this process. Specifically, we were able to define the role of the three conserved cysteines of ISCA proteins in driving the [4Fe-4S] cluster formation upon receiving two [2Fe-2S]<sup>2+</sup> clusters from GLRX5. Our data allowed us to propose a molecular model of how the [4Fe-4S] cluster formation takes place on the ISCA1/ISCA2 heterodimeric complex and how copper can impair this process.

## **EXPERIMENTAL SECTION**

### *Protein production*

The C79S, C144S and C146 ISCA2 mutants were obtained through site-directed mutagenesis (Agilent QuikChange site-directed mutagenesis kit) according to the producer's manual, using the Gateway® pETG-30A vector, containing the sequence coding for the full-length protein, as a template. <sup>15</sup>N-labeled and unlabeled GLRX5, ISCA1, wild-type and mutated ISCA2 human proteins in their apo and holo forms were obtained following previously described protocols.<sup>21,31</sup> (<sup>13</sup>C, <sup>15</sup>N)Cys-selectively labeled ISCA2 was produced following the procedure reported in <sup>32</sup>.

### *Spectroscopic techniques*

Paramagnetic  $^1\text{H}$  NMR experiments were performed on a Bruker AVANCE600 spectrometer, equipped with a  $^1\text{H}$  selective probe.<sup>33</sup> Experiments were performed using 85 ms and 230 ms as acquisition and recycle delay, respectively. Water presaturation was applied during the recycle delay with a selective pulse. Spectral window was 192 kHz. From 100 000 to 500 000 scans were acquired. Experiment time was varying from 9 to 48 h.

The diamagnetic NMR experiments were recorded on BRUKER AVANCE III 950, Bruker AVANCE 900 and Bruker AVANCE 700 spectrometers on  $^{15}\text{N}$ -labeled or ( $^{13}\text{C}$ ,  $^{15}\text{N}$ )Cys-selectively labeled samples in degassed 50 mM phosphate buffer, pH 7.0, containing 10% (v/v)  $\text{D}_2\text{O}$  and 5 mM DTT. 5 mM GSH was also present in the buffer when GLRX5 protein was used to perform the NMR experiments. All NMR spectra were collected at 298 K, processed using the standard BRUKER software (Topspin 2.1) and analyzed through the CARA program.<sup>34</sup>  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  resonance assignment for GLRX5 and ISCA2 in their apo and holo forms were already available.<sup>21,31</sup> To follow copper(I) and Fe/S cluster binding to protein, cluster transfer between proteins, cluster release from protein, and protein-protein interactions, chemical shift changes were followed in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra collected along the titration experiments. All titrations were performed anaerobically, i.e. in degassed buffers and by adding to the NMR tube the required components in a glove box.  $^{15}\text{N}$  heteronuclear relaxation experiments on  $^{15}\text{N}$ -labeled GLRX5 before and after the addition of copper(I) in the presence and in the absence of 5 mM GSH were collected to measure  $^{15}\text{N}$  backbone longitudinal and transverse relaxation rates and heteronuclear  $^{15}\text{N}\{^1\text{H}\}$  NOEs. These values allow us to estimate the correlation time value following standard procedures.<sup>35</sup>

To estimate the apparent Cu(I)-binding affinities of ISCA2 and GLRX5, each apo  $^{15}\text{N}$ -labelled protein was first titrated with stoichiometric amounts of Cu(I) (added as  $(\text{Cu(I)}(\text{CH}_3\text{CN})_4)\text{PF}_6$  directly to the NMR tube in anaerobic conditions) to follow the complete formation of Cu(I) form of GLRX5 or ISCA2, and then each Cu(I)-protein was anaerobically titrated with DTT as ligand competitor up to reach a DTT concentration able to completely remove copper(I) from the protein. Since the protein amide resonances affected by copper(I) binding are in a slow exchange regime, relative to NMR time scale, between their free and bound forms, we have estimated the apparent dissociation constant ( $K_{\text{Cu}}$ ) of Cu(I)-GLRX5 and Cu(I)-ISCA2 by integrating

apo vs. Cu(I) cross-peaks in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR experiments recorded along DTT additions and fitting the data following the procedure used by us for measuring apparent Cu(I)-binding affinities of mitochondrial copper chaperones.<sup>36</sup> In this way, we can consistently and significantly compare the  $K_{\text{Cu}}$  values of GLRX5 and ISCA2 with those of the mitochondrial copper(I) chaperones.

UV/visible spectra were anaerobically performed in degassed 50 mM phosphate buffer at pH 7.0, 5 mM DTT, 5 mM GSH on a Cary 50 Eclipse spectrophotometer. The iron and inorganic sulfur content and the protein concentration were estimated following standard chemical assays described in<sup>37</sup>. Copper and iron content was quantified by inductively coupled plasma mass spectrometry (ICP-MS).

## RESULTS

### *Fe/S cluster binding properties of ISCA2*

ISCA2 contains three completely conserved cysteine residues in a  $\text{CX}_n\text{CGC}$  sequence motif ( $n$  is usually 63–65, but it is increased by a 21-residue insert in some eukaryotic proteins such as in *Saccharomyces cerevisiae* Isa2), is a symmetric dimeric in solution (i.e. in the diamagnetic  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of ISCA2 only one set of backbone NH resonances are indeed observed for all the detected signals) and is able to bind either one [2Fe-2S] or one [4Fe-4S] cluster per dimer.<sup>21</sup> Each subunit of the dimer can thus potentially provide three cysteine residues as Fe/S cluster ligands, with a total of six possible sulfur-donor ligands. To discriminate which are the four iron ligands among the available conserved cysteines (i.e. Cys 79, Cys 144, Cys 146), we characterized, by NMR, the Fe/S binding properties of Cys-to-Ser single mutants for each conserved cysteine. While, upon Fe/S cluster chemical reconstitution, we already demonstrated<sup>21</sup> that the wild-type protein is converted in a mixture of three [4Fe-4S] cluster-bound dimeric species (accounting for the ~90% of the holo form) with a 1D paramagnetic NMR spectrum (**Figure 1a**) featuring signals typical of a reduced [4Fe-4S]<sup>+</sup> bound-cluster and of a minor dimeric species (~10%) with NMR signals typical of a [2Fe-2S]<sup>2+</sup> bound-cluster, 1D  $^1\text{H}$  paramagnetic NMR spectra of the chemically reconstituted ISCA2 mutants showed that the three mutants are unable to bind a [4Fe-4S] cluster (**Figure 1b-d**). Binding of Fe/S clusters of any type was essentially abolished in the C79S ISCA2 mutant as assessed by the analysis of iron and inorganic

sulfur content (**Table S1**). Consistent with this, no paramagnetic NMR signals were observed in the 1D  $^1\text{H}$  NMR spectrum optimized for the detection of paramagnetic signals<sup>38,39</sup> (**Figure 1b**) and the UV/vis spectrum showed a very weak signal in the visible region (inset in **Figure 1b**). On the contrary, 1D  $^1\text{H}$  paramagnetic NMR spectra of the cluster-reconstituted C144S and C146S ISCA2 mutants showed two broad signals at 35 and 30 ppm and a broad unresolved signal at 30 ppm, respectively, with anti-Curie temperature dependences (**Figure 1c-d**). The chemical shifts, the line-widths and the temperature dependence of these signals are consistent with the binding of a  $[\text{2Fe-2S}]^{2+}$  cluster.<sup>40,41</sup> UV/vis spectra confirm the NMR data interpretation as both C144S and C146S mutants show absorption peaks in the electronic spectra respectively at 320 (320), 418 (413), 460 (452, shoulder), 511 (510) and 590 (592) nm, typical of a protein-bound,  $[\text{2Fe-2S}]^{2+}$  cluster<sup>42</sup> (insets in **Figure 1c-d**). Iron, and acid-labile sulfur analysis of the cluster-reconstituted C144S and C146S ISCA2 mutants showed the presence of 1.0 and 0.75  $[\text{2Fe-2S}]$  cluster per homodimer, respectively (**Table S1**). In conclusion, these data indicate that: i) Cys 79 is essential to bind any type of Fe/S clusters to ISCA2; and ii) Cys 144 and Cys 146 are critical to bind a  $[\text{4Fe-4S}]$  cluster in ISCA2, since by mutating one of them only a  $[\text{2Fe-2S}]$  cluster is bound to the protein. They are therefore required to switch from a  $[\text{2Fe-2S}]$  cluster-bound species to a  $[\text{4Fe-4S}]$  cluster-bound species.

Since in C144S and C146S ISCA2 mutants only four cysteines per dimer (Cys 79 and Cys 146 in C144S mutant, Cys 79 and Cys 144 in C146S mutant) are available to bind the  $[\text{2Fe-2S}]^{2+}$  cluster and considering that  $[\text{2Fe-2S}]$  clusters are typically tetra-coordinate,<sup>43,44</sup> the experimental data support a coordination mode for the two mutants where the  $[\text{2Fe-2S}]^{2+}$  cluster is bound by the two existing cysteines provided by each subunit of the dimer, as schematically drawn in the insets of **Figure 1c-d**. Wild-type ISCA2 purified from *E. coli* (i.e. not chemically reconstituted)<sup>21</sup> binds a  $[\text{2Fe-2S}]^{2+}$  cluster and shows a 1D  $^1\text{H}$  paramagnetic NMR spectrum with broad signals at 38, 35, 31 and 23 ppm (**Figure 1e**). Like in the case of the C144S and C146S mutants, the NMR features of the  $^1\text{H}$  paramagnetic NMR spectrum of the purified, wild-type protein are indicative of a  $[\text{2Fe-2S}]^{2+}$  bound cluster. However, its 1D  $^1\text{H}$  paramagnetic spectrum differs from those of the mutants in terms of chemical shifts and number of resolved signals (**Figure 1b-e**), indicating a coordination mode different from those of the mutants. Considering that Cys 79 is necessary for binding any type of cluster, these paramagnetic NMR data indicate that the oxidized  $[\text{2Fe-2S}]^{2+}$  cluster in the wild-type protein is coordinated by two Cys 79, provided by each subunit of the wild-type ISCA2 dimer, and by Cys 146 from

one subunit of the homodimer and by Cys 144 from the other subunits of the homodimer, as schematically drawn in the inset of **Figure 1e**.

$^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of a ( $^{13}\text{C}$ ,  $^{15}\text{N}$ )Cys selectively-labeled wild-type ISCA2 sample, acquired on the apo and on the chemically reconstituted [4Fe-4S] forms, showed that the NH signals of Cys 79, Cys 144 and Cys 146 are all observable in the apo form, but they are all broadened beyond detection in the chemically reconstituted [4Fe-4S] ISCA2 as a consequence of paramagnetic relaxation effects typically affecting signals of nuclei near the paramagnetic center (**Figure 1f-g**). This indicates that Cys 79, Cys 144 and Cys 146 residues from both subunits of the ISCA2 dimer are close to the cluster. Since we have here shown that Cys 79 is essential to bind a [4Fe-4S] cluster, we can assume that Cys 79 from each subunit of the dimer is required to coordinate the [4Fe-4S] cluster, as proposed for the [2Fe-2S] cluster coordination. Moreover, since no backbone NHs of Cys 144 and Cys 146 are detected in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the [4Fe-4S] cluster bound form of the ( $^{13}\text{C}$ ,  $^{15}\text{N}$ )Cys selectively-labeled ISCA2, both CXC motifs of the C-terminal region of the two molecules of the homodimer are necessarily involved in cluster coordination. Indeed, as Cys 144 and Cys 146 are located in a flexible and unstructured C-terminal segment, the participation in [4Fe-4S] cluster coordination of one cysteine from each C-terminal segment of the ISCA2 dimer is consistent with the fact that their backbone NH are undetected in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum. Otherwise, the C-terminal segment of one molecule of the homodimer would be freely moving in solution and thus Cys 144 and Cys 146 would be far from the paramagnetic center and their backbone NHs should be detected as it occurs in apo ISCA2. Since we found that the binding of a [4Fe-4S] cluster requires all the three conserved cysteines (Cys 79, Cys 144 and Cys 146), the overall data suggest that the cluster coordination proposed in [2Fe-2S] $^{2+}$  ISCA2 might also be preferential in [4Fe-4S] ISCA2, as pictured in the inset of **Figure 1a**.

In conclusion, the spectroscopic data on wild-type and mutants of ISCA2 suggest that Cys 79, by acting as a ligand from both subunits of the ISCA2 dimer, is essential for binding a [2Fe-2S] or a [4Fe-4S] cluster per dimer, and that Cys 144 and Cys 146 from one and the other subunit of the homodimer preferentially provide the other two binding residues. Moreover, our data showed that the binding of a [2Fe-2S] cluster can be also possible with the availability of Cys 79, and of either Cys 144 or Cys 146 only, without the need of all three conserved Cys of ISCA proteins.



### *Characterization of copper(I) binding to ISCA proteins*

Copper(I) binding to apo ISCA2 and to the apo ISCA1/ISCA2 1:1 heterodimeric complex was characterized by NMR, titrating  $^{15}\text{N}$ -labelled ISCA2, either as homo or heterodimer, with copper(I). The final mixtures were then analyzed by ICP-MS, once passed through a PD-10 desalting column to remove the unbound copper(I) ions. From the ICP-MS data, it resulted that both the homodimeric apo ISCA2 and the heterodimeric apo ISCA1/ISCA2 complex bind one copper(I) ion ( $0.9 \pm 0.1$  and  $1.1 \pm 0.1$  moles of Cu per mole of ISCA2 dimer, respectively). The NMR data showed chemical shift changes, in a slow chemical shift regime on the NMR time scale, between the apo and the Cu(I)-bound forms, which are complete once the 1:1 Cu(I)/protein ratio was reached (**Figure 2a-b**). The Cu(I)-binding affinity of ISCA2 was then measured performing a titration of  $^{15}\text{N}$ -labelled Cu(I)-ISCA2 with DTT used as competitor ligand and following the copper(I) removal by  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR experiments. We obtained that ISCA2 binds copper(I) with an apparent dissociation constant ( $K_{\text{Cu}} = 4.5(\pm 0.1) \times 10^{-14}$  M, **Figure S1**) that is comparable to those of mitochondrial copper chaperones ( $K_{\text{Cu}}$  (COX17) =  $1.7 \times 10^{-14}$  M;  $K_{\text{Cu}}$  (SCO1) =  $3.1 \times 10^{-15}$  M;  $K_{\text{Cu}}$  (SCO2) =  $3.7 \times 10^{-15}$  M;  $K_{\text{Cu}}$  (CCS) =  $2.4 \times 10^{-15}$  M).<sup>36</sup> In order to identify the cysteines involved in copper(I) binding, the copper binding properties of the C79S, C144S and C146S ISCA2 mutants were analyzed. From these data it resulted that copper(I) binding was essentially abolished in the C146S ISCA2 mutant as, upon step-wise additions of copper(I) to the apo protein up to an excess of copper(I), the formation of the copper(I) form was essentially not observed in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra (**Figure 2c**), and the copper content of the C146S ISCA2 mutant at the end of the titration resulted to be  $0.1 \pm 0.1$  moles of Cu per mole of ISCA2 dimer as estimated by ICP-MS, after passing it through a PD-10 desalting column. The C144S ISCA2 mutant was partially metallated as both the NMR titration of the apo protein with an excess of copper(I) and the ICP-MS analysis on the final PD-10 treated sample showed ~50% of the protein in the copper(I) bound form (**Figure 2d**). Finally, NMR and ICP-MAS data showed that the C79S ISCA2 mutant retains the same copper(I) binding properties as the wild-type protein with one copper(I) ion bound per dimeric ISCA2 (Cu:protein dimer ratio  $1.0 \pm 0.1$ ) (**Figure 2e**). Overall, the data showed that Cys 146 is essential to bind copper(I), Cys 144 stabilizes copper(I) binding, and Cys 79 is not involved in Cu(I) binding.

To characterize copper(I) coordination in wild-type ISCA2, an apo ( $^{13}\text{C}$ ,  $^{15}\text{N}$ )Cys-selectively labeled ISCA2 sample was titrated with increasing amounts of copper(I), and copper(I) binding followed by NMR. In the

$^1\text{H}$ - $^{15}\text{N}$  HSQC spectra, no effects were observed on the HN signal of Cys 79 upon copper(I) additions (**Figure 1h**), consistently with the data on the C79S mutant showing that Cys 79 is not required for copper(I) binding. In the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra, also the NH chemical shifts of the not conserved Cys 51 and Cys 56 are not affected by the addition of increasing amounts of Cu(I), indicating that they are not involved in copper(I) binding. On the contrary, the NH signals of Cys 144 and Cys 146, already at substoichiometric amounts of copper(I), decreased in intensity in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra, and became unobservable at the 1:1 Cu(I)/ISCA2 ratio (**Figure 1h**), suggesting that Cys 144 and Cys 146 are affected by copper(I) binding to ISCA2. However, no new NH signals due to Cys 144 and Cys 146 in the copper(I) bound species appeared in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra (**Figure 1h**), suggesting conformational flexibility in the metal binding site region upon Cu(I) binding to Cys 144 and Cys 146. This behavior is also consistent with  $^1\text{H}$ - $^{13}\text{C}$  HSQC experiments, which showed that both  $\text{H}\alpha$ - $\text{C}\alpha$  and  $\text{H}\beta$ - $\text{C}\beta$  couplings for Cys 144 and Cys 146 disappeared upon copper(I) binding while no new signals appeared.

In conclusion, at variance with what observed for Fe/S cluster binding, Cys 79 is not required to bind copper(I), and copper(I) is bound to the protein through Cys 144 and Cys 146. Considering that the latter cysteine is essential for copper(I) binding, while Cys 144 only contributes in increasing copper(I) binding, and that 2- or 3-coordination numbers are commonly found in copper(I) transporting proteins with S-donor ligands,<sup>45</sup> copper(I) ion is likely coordinated by two Cys 146 residues from each subunit of the homodimer, and by a Cys 144 residue provided by one or by the other subunit of the homodimer, with the occurrence of a conformational equilibrium between the two ligands, each from one subunit of the dimer (inset of **Figure 1h**).

*Addressing the mechanism of cluster transfer from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> to ISCA proteins and how copper(I) can impair it*

The so far described copper(I) and Fe/S cluster binding properties of ISCA proteins showed that, while only the conserved cysteines at the C-terminus (Cys 144 and Cys 146 in ISCA2) are required to bind copper(I), all the three conserved cysteines (Cys 79, Cys 144 and Cys 146 in ISCA2) are required for  $[4\text{Fe-4S}]$  cluster binding, being the presence of both C-terminal cysteines determinant for switching from  $[2\text{Fe-2S}]$  cluster to  $[4\text{Fe-4S}]$  cluster binding. We then addressed: i) the specific role of each of the three conserved cysteines of

ISCA proteins in the  $[2\text{Fe-2S}]^{2+}$  cluster transfer process from the physiological cluster donor, i.e. the homodimeric  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>, to apo ISCA2 and to the apo ISCA1/ISCA2 heterodimeric complex, and ii) whether and how copper(I) can impair the  $[4\text{Fe-4S}]$  cluster assembly in ISCA proteins upon receiving the cluster from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>.

To address the first issue, <sup>15</sup>N-labelled C79S, C144S and C146S ISCA2 mutants in their apo forms were titrated with <sup>15</sup>N-labelled  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> up to a 1:1 protein ratio (obtained considering that ISCA2 is a dimer in both apo and holo forms) and the cluster transfer process was followed by diamagnetic 2D <sup>1</sup>H-<sup>15</sup>N HSQC and paramagnetic 1D <sup>1</sup>H NMR experiments, and by UV/vis spectra. It resulted that the C144S and C146S ISCA2 mutants were not able to receive the  $[2\text{Fe-2S}]^{2+}$  cluster from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>, which remains indeed stably bound to GLRX5 (**Figure 3a-b** and **Figure S2**), at variance with what occurs for wild-type ISCA2 and for the heterodimeric ISCA1/ISCA2 complex, which both stoichiometrically receive  $[2\text{Fe-2S}]^{2+}$  clusters from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> (**Figure 3d** and **Figure S3**, respectively).<sup>21,31</sup> On the contrary, the C79S ISCA2 mutant was able to receive the cluster from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>, although at lower extent than wild-type ISCA2 (**Figure 3c** and **Figure S2**). At a 1:1 protein ratio, only 65% of the cluster was transferred to C79S ISCA2 mutant. NMR data on this mixture showed indeed the presence in solution of both apo GLRX5 and  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> species in equilibria with apo and  $[2\text{Fe-2S}]^{2+}$  species of C79S ISCA2. No detectable amount of a protein-protein adduct was observed, indicating that the cluster transfer occurs via a transient, low-populated complex, as it is the case of cluster transfer between wild-type  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> and dimeric ISCA2 or the ISCA1/ISCA2 complex.<sup>21,31</sup> Indeed, the  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>-C79S ISCA2 1:1 mixture is stable upon passing it on a PD-10 desalting column, i.e. no cluster release is observed in solution. The peak intensity ratio of the apo vs. the  $[2\text{Fe-2S}]^{2+}$  species in the <sup>1</sup>H-<sup>15</sup>N HSQC spectra recorded before and after the PD-10 desalting column remain in fact unchanged. Overall, the data showed that Cys 144 and Cys 146, but not Cys 79, are fundamental for transferring the cluster from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> to ISCA2. Since Cys 144 and Cys 146 are located in a completely unstructured and flexible protein region, we suggest that, upon the formation of the transient protein-protein complex observed in solution (described in <sup>31</sup>), Cys 144 and Cys 146 have the proper flexibility/ability to invade the coordination sphere of the  $[2\text{Fe-2S}]^{2+}$  cluster bound to dimeric GLRX5 so to drive the cluster transfer to dimeric ISCA2. On these basis, we can therefore propose that the transient protein-protein complex between GLRX5 and ISCA2 contains a  $[2\text{Fe-2S}]^{2+}$  cluster

coordinated by Cys 144 of a ISCA2 subunit and Cys 146 from the other ISCA2 subunit and by the two conserved cysteines of GLRX5, being the two GLRX5-bound GSH molecules released in solution. However, the transfer is quantitative only once all the three Cys 79, Cys 144 and Cys 146 are present, indicating that Cys 79 has a role in efficiently driving cluster acquisition by ISCA2. In conclusion, Cys 144 and Cys 146 are required to trigger the  $[2\text{Fe-2S}]^{2+}$  cluster transfer from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> to apo ISCA2. On the contrary, Cys 79 is required to thermodynamically drive the  $[2\text{Fe-2S}]^{2+}$  cluster transfer from GLRX5 to ISCA2. According to that, we have here demonstrated that Cys 79 is essential for the stable binding of any type of Fe/S cluster to ISCA2.

To address the second issue, the effects of copper(I) addition were evaluated by NMR, ICP-MS and UV/vis, at different steps of the  $[4\text{Fe-4S}]$  cluster assembly process on ISCA proteins upon receiving  $[2\text{Fe-2S}]^{2+}$  clusters from GLRX5. Three different experiments were performed: 1. copper(I) was stepwise added to the cluster donor, i.e. GLRX5 in both apo and  $[2\text{Fe-2S}]^{2+}$  forms; 2. copper(I) was stepwise added to apo ISCA proteins and then the  $[2\text{Fe-2S}]^{2+}$  cluster transfer from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> was investigated; 3. copper(I) was stepwise added to the  $[4\text{Fe-4S}]$  ISCA1/ISCA2 heterocomplex, formed by receiving two  $[2\text{Fe-2S}]^{2+}$  clusters from GLRX5.<sup>21</sup>

In the first experiment, <sup>15</sup>N-labelled apo GLRX5 was titrated with copper(I) up to 1.5 equivalents. In the <sup>1</sup>H-<sup>15</sup>N HSQC experiments, we observed the stoichiometric formation of Cu(I)-GLRX5, being the protein amide resonances affected by copper(I) additions in a slow exchange regime, relative to NMR time scale, between their free and bound forms (**Figure 4a**). The residues affected by copper(I) binding are located in the region close to the GLRX5 cluster ligand Cys 67; specifically the backbone NH chemical shifts of Gln 66, Cys 67, Phe 69 and Ser 70 are affected by copper(I) binding. At variance with what it occurs upon  $[2\text{Fe-2S}]^{2+}$  cluster binding,<sup>31</sup> residues of the GSH binding site in apo GLRX5 are not affected by copper(I) binding, suggesting that GSH is not a copper(I) ligand as it occurs for the iron ions in  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>.<sup>31,46</sup> Cu(I)-GLRX5 is dimeric in solution, as shown by the correlation time value ( $\tau_c$ ) obtained on the final 1:1 mixture by measuring <sup>15</sup>N R<sub>1</sub> and R<sub>2</sub> NMR relaxation rates. Overall, the data indicate that a copper(I) ion is bound to dimeric GLRX5 through Cys 67 provided by two GLRX5 subunits. The Cu(I)-affinity of GLRX5 was measured following the same approach described above to obtain the K<sub>Cu</sub> value of Cu(I)-ISCA2. We obtained that GLRX5 binds copper(I) with an weaker affinity (K<sub>Cu</sub> (GLRX5)= 8.2(±0.2) x 10<sup>-13</sup> M, **Figure**

**S1**) than ISCA2. Moreover, at variance to Cu(I)-ISCA2, the copper(I) binding is quite labile, since, passing the final mixture on a PD-10 desalting column, copper(I) is released to produce apo GLRX5 (**Figure 4a**), accordingly to what it also results from ICP-MS data ( $0.1 \pm 0.1$  moles of Cu per mole of GLRX5).  $^{15}\text{N}$ -labelled  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub> was also titrated with copper(I) and, by following the interaction through  $^1\text{H}$ - $^{15}\text{N}$  HSQC experiments and UV/vis spectra, it resulted that the stepwise additions of copper(I) progressively remove the  $[\text{2Fe-2S}]^{2+}$  cluster from GLRX5, which turns out to be in the copper(I)-bound state once 1.5 equivalents of copper(I) were added (**Figure 4b**). Again, passing the final mixture on a small gel filtration column, it is observed that copper(I) is largely released to produce apo GLRX5 (**Figure 4b**).

In the second experiment, either apo  $^{15}\text{N}$ -labelled ISCA2 or apo ISCA1/ $^{15}\text{N}$ -labelled ISCA2 heterocomplex were first titrated with copper(I) and the obtained solutions were passed through PD-10 desalting column to remove unbound copper(I) ions. Then, the obtained copper(I)-bound forms were titrated with  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub> and the interaction followed by  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR experiments and UV/vis spectra. From these data it resulted that the copper(I) ion remains tightly bound to dimeric ISCA2 and to the ISCA1/ISCA2 heterodimeric complex upon  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub> additions up to a 1:1 protein ratio, and that the  $[\text{2Fe-2S}]^{2+}$  cluster is released from GLRX5 without being acquired by either ISCA2 or the ISCA1/ISCA2 complex (**Figure 5a** and **Figure S4**), as a consequence of copper(I) occupancy of the cluster binding site in ISCA2 and in the ISCA1/ISCA2 complex. To provide a possible explanation of the copper(I)-driven destabilization mechanism of  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub>, we have repeated the titration shown in **Figure 5a** with  $^{15}\text{N}$ -labelled Cu(I)-C79S ISCA2 to document whether Cys 79 is responsible of cluster release from GLRX5. We observed that, the  $[\text{2Fe-2S}]^{2+}$  cluster on GLRX5 is still released by the mutant, as it occurs with the wild-type protein, thus being Cys 79 not involved in GLRX5 cluster destabilization. Overall, these results suggest that copper(I) binding by Cys 144 and Cys 146 is weakened upon protein-protein interaction, allowing attack of the  $[\text{2Fe-2S}]^{2+}$  cluster bound to GLRX5 by Cys 144 or Cys 146 of ISCA2, even in the presence of Cu(I), so that the consequent cluster transfer to ISCA2 can occur. Released copper(I) can then damage the  $[\text{2Fe-2S}]^{2+}$  cluster bound to either GLRX5 or ISCA2. Once the equilibrium conditions are reached, copper(I) is preferentially bound to ISCA2 than GLRX5, since ISCA2 has higher affinity for Cu(I) than GLRX5.

In the third experiment, the apo ISCA1/ $^{15}\text{N}$ -labelled ISCA2 complex was first treated with  $^{15}\text{N}$ -labelled  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub> up to a 1:2.5 protein ratio to form the  $[\text{4Fe-4S}]$  cluster bound form as already reported.<sup>21</sup>

Then, this mixture was titrated with copper(I). Both reactions were followed by  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR experiments and UV/vis spectra. In the first step, it resulted that the  $[\text{2Fe-2S}]^{2+}$  clusters are quantitatively transferred from GLRX5 to the ISCA1/ISCA2 complex (**Figure 5b**), to form a  $[\text{4Fe-4S}]$  cluster on the ISCA1/ISCA2 heterodimer. Then, copper(I) additions completely remove the cluster from the heterocomplex, generating the Cu(I)-ISCA1/ISCA2 complex (**Figure 5b**). Indeed, in the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the final ISCA1/ISCA2/GLRX5 mixture treated with Cu(I) up to 1.5 equivalents and then passed through a PD-10 desalting column to remove unbound Cu(I), those backbone NH peaks of the ISCA1/ISCA2 heterodimer experiencing different shifts in the Cu(I) vs. the apo form, thus making possible to determine whether Cu(I) is bound or not to the ISCA1/ISCA2 heterodimer, are univocally overlapped with those of the Cu(I)-ISCA1/ISCA2 heterocomplex (black and blue peaks, respectively, in **Figure 5b**). According to this, once the final mixture was passed through a PD-10 desalting column, copper and iron content, estimated by ICP-MS, resulted to be  $1.0 \pm 0.1$  moles of Cu per mole of ISCA1/ISCA2 complex and residual amounts of iron.

In conclusion, copper(I) can impair  $[\text{4Fe-4S}]$  cluster assembly at various levels, i.e. by occupying the cluster binding site on ISCA proteins or by removing the Fe/S cluster bound either to GLRX5 or to ISCA proteins. This is due to its high thiophilic nature, which is much higher than that of iron as predicted by the Irving-Williams series. Therefore, the cellular concentration of free copper(I) needs to be tightly regulated in order to prevent the impairment of the mitochondrial ISC machinery.

## DISCUSSION

A few recent studies on bacterial organisms have shown that copper(I) can dramatically interfere with iron-sulfur cluster assembly pathways by acting as competitor, with iron/iron-sulfur clusters, for the same protein metal binding site.<sup>11,12,14</sup> Specifically, the  $[\text{4Fe-4S}]$  cluster assembly pathway involving iron-sulfur scaffold proteins, IscA and SufU, has been found to be copper-damaged.<sup>11,14</sup> In eukaryotes, the ISCA-driven  $[\text{4Fe-4S}]$  cluster assembly process occurs in mitochondria,<sup>47</sup> which contain the largest pool of cellular copper ions in an accessible form, i.e. as a soluble low molecular weight ligand Cu(I)-complex (CuL).<sup>26,27</sup> The arising query is therefore whether copper(I) can also damage the mitochondrial  $[\text{4Fe-4S}]$  assembly machinery. To address

this issue, we first elucidated the molecular aspects of the mechanism of [4Fe-4S] cluster assembly on ISCA proteins upon [2Fe-2S]<sup>2+</sup> cluster acquisition by GLRX5, and then addressed how copper(I) can impair each step of this process. The resulting findings indicate that a tight regulation of [4Fe-4S] cluster assembly and copper trafficking pathways is required in mitochondria to avoid deleterious cellular effects.

Recently, we showed that the [2Fe-2S]<sup>2+</sup> cluster-bound homodimeric form of GLRX5 efficiently transfer its clusters to homodimeric ISCA1 and ISCA2 via a specific protein-protein recognition mechanism based on a low-populated, cluster-mediated GLRX5-ISCA intermediate.<sup>21,31</sup> We also showed that the formation of a heterodimeric 1:1 ISCA1/ISCA2 complex is thermodynamically favored compared to their homodimeric forms, and that this complex is able to assemble a [4Fe-4S]<sup>2+</sup> cluster in a DTT-reducing environment, once it is mixed in its apo form with [2Fe-2S]<sup>2+</sup> GLRX5.<sup>21</sup> ISCA1 and ISCA2 are two homologous proteins having three fully conserved Cys, one located in a flexible loop (Cys 57 in ISCA1 and Cys 79 in ISCA2) and the other two (Cys 121 and Cys 123 in ISCA1, and Cys 144 and Cys 146 in ISCA2) in a completely unstructured and flexible C-terminal tail.<sup>21</sup> Although far apart in the solution structure of the dimeric apo ISCA2,<sup>21</sup> the two Cys-rich regions are located in structurally flexible regions and can therefore easily undergo to structural rearrangements.<sup>21,48-50</sup> Therefore, both regions could be involved in Fe/S cluster binding, thus potentially providing six Cys iron ligands in the homo/heterodimer. Furthermore, the large backbone conformational flexibility observed at the interface in dimeric apo ISCA2<sup>21</sup> is likely a driving factor allowing the two Cys-rich regions to come closer and to bind a Fe/S cluster. Spectroscopic data on ISCA2 mutants, here reported, showed that Cys 79 is essential for the binding of any type of Fe/S cluster. Moreover, Cys 79 together with either Cys 144 or Cys 146 is able to bind a [2Fe-2S]<sup>2+</sup> cluster, but not a [4Fe-4S] cluster, whose binding requires indeed all three cysteines. These results suggest that Cys 79, Cys 144, and Cys 146 play specific roles for the assembly of a [4Fe-4S] cluster on the ISCA1/ISCA2 heterodimeric complex. The investigation of the cluster transfer from [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub> to the single Cys to Ser ISCA2 mutants allowed us to define the different roles of the cysteines. Indeed, we found that the C-terminal Cys 144 and Cys 146 of ISCA2 are both required to remove the [2Fe-2S]<sup>2+</sup> cluster from GLRX5. Therefore, our data support a model in which the two C-terminal cysteines, located in the unstructured and flexible C-terminal tail of ISCA proteins,<sup>21,49,50</sup> extract the [2Fe-2S]<sup>2+</sup> cluster from GLRX5 by forming a transient, low-populated, cluster-mediated GLRX5-ISCA intermediate<sup>21,31</sup> that presumably share GLRX5 and ISCA cluster ligands (species *A'* in **Scheme 1**),

similarly to what it was already observed in protein-mediated, copper transfer processes.<sup>51,52</sup> This cluster-extraction mechanism from GLRX5 produces the formation of an ISCA2 species that binds the cluster via Cys 144 and Cys 146 (species *A* in **Scheme 1**). This *A* species is, however, transient and does not accumulate at enough levels to be observed, as, by chemically reconstituting the C79S ISCA2 mutant, no Fe/S cluster binding was observed. On the contrary, Cys 79 is not involved in the cluster transfer step, as the C79S ISCA2 mutant is still able to extract the  $[2\text{Fe-2S}]^{2+}$  cluster from GLRX5, again via the formation of the transient, low-populated intermediate *A'* (**Scheme 1**) with no cluster release in solution. However, the transfer is not as efficient as observed for the wild-type protein, suggesting a cluster-binding stability of species *A* lower than that of the  $[2\text{Fe-2S}]^{2+}$  cluster-bound forms of the C144S and C146S ISCA2 mutants. The latter two, indeed, at variance with the C79S ISCA2 mutant, can be isolated upon chemical reconstitution. Our data, therefore suggest that species *A* can evolve to the more thermodynamically favored species *B* (**Scheme 1**), which binds the  $[2\text{Fe-2S}]^{2+}$  cluster by a Cys 79 from each subunit of the ISCA2 homodimer (or Cys 79 from ISCA2 subunit and Cys 57 from ISCA1 subunit in the ISCA1/ISCA2 heterodimer), by Cys 144 from one ISCA2 subunit, and Cys 146 from the other ISCA2 subunit (or by Cys 144 from ISCA2 subunit and Cys 123 from ISCA1 subunit in the ISCA1/ISCA2 heterodimer). Spectroscopic NMR data suggest that this is the preferential coordination mode in wild-type ISCA2 upon binding of either  $[2\text{Fe-2S}]$  or  $[4\text{Fe-4S}]$  clusters, and presumably also in the heterodimeric ISCA1/ISCA2 complex. The formation of this species *B* would make also two cysteines in the C-terminal tail free and still available for coordination. In this way they are available to extract another  $[2\text{Fe-2S}]^{2+}$  cluster from GLRX5, upon the formation of another GLRX5-ISCA intermediate (intermediate *B'* in **Scheme 1**). This transient intermediate which contains two  $[2\text{Fe-2S}]^{2+}$  clusters might be therefore the species that, by accepting two electrons from a still unknown physiological electron donor, evolves to the final  $[4\text{Fe-4S}]^{2+}$  cluster-bound ISCA1/ISCA2 complex (*C* in **Scheme 1**). A reductive coupling of two  $[2\text{Fe-2S}]^{2+}$  clusters, which is a general pathway of  $[4\text{Fe-4S}]^{2+}$  cluster biosynthesis,<sup>53-55</sup> would therefore occur on species *C* to form a single  $[4\text{Fe-4S}]^{2+}$  cluster bound to the complex via Cys 79 from both subunits of the homodimer, Cys 144 from one subunit and Cys 146 from the other subunit, according to the spectroscopic NMR data consistent with this coordination mode of the  $[4\text{Fe-4S}]$  cluster. The overall molecular model agrees with *in vivo* data on yeast, which showed that the three conserved cysteines of Isa1 and Isa2 are essential for the maturation of  $[4\text{Fe-4S}]$  proteins.<sup>23,24</sup>



We can then establish at the molecular level how free copper(I) ions interfere with the described [4Fe-4S] cluster assembly process. We have observed that the thiolphilic nature of copper(I) makes it able to occupy the Fe/S cluster binding sites of both ISCA proteins (as homodimeric ISCA2 and heterodimeric ISCA1/ISCA2) and GLRX5, being copper(I) more stably bound to ISCA proteins than GLRX5 (**Scheme 1**). Specifically, copper(I) is coordinated by Cys 144 and Cys 146 in a tri-coordinated environment in ISCA2, and by Cys 67 in a di-coordinated environment, in GLRX5. Being both C-terminal cysteines of ISCA proteins stably binding copper(I), they are not able to bind the [2Fe-2S]<sup>2+</sup> cluster received by GLRX5. A release of the [2Fe-2S]<sup>2+</sup> cluster from GLRX5 is observed upon the interaction of [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub> with Cu(I)-ISCA proteins, but the cluster is not acquired by ISCA proteins, i.e. species *A* is not formed, and therefore the [4Fe-4S] cluster assembly is compromised (**Scheme 1**). Free copper(I) can also interfere even once a Fe/S cluster is already bound to ISCA proteins and GLRX5 proteins. This is due to the fact that the cluster binding sites in GLRX5 and ISCA proteins are solvent exposed, as a result of their required function to exchange the Fe/S clusters with other proteins in the Fe/S protein biogenesis pathways. Therefore, free copper(I) can easily displace the Fe/S clusters bound to ISCA proteins and GLRX5, forming the copper(I)-bound species, again compromising the [4Fe-4S] cluster assembly process. Moreover, the similarities of the  $K_{Cu}$  values between Cu(I)-GLRX5/Cu(I)-ISCA2 and the mitochondrial copper proteins support the physiological relevance of Cu(I) inhibition of mitochondrial Fe/S cluster assembly process. Overall, these *in vitro* findings show that a mitochondrial copper(I) misregulation, determining sizable levels of free copper(I), can dramatically damage the mitochondrial [4Fe-4S] assembly machinery. This result also opens several questions on how the mitochondrial labile copper(I) pool formed by CuL can avoid molecular damages on Fe/S protein maturation under the physiological cellular conditions. Indeed, although L binds Cu(I) stably, the L-bound Cu(I) ions are exchange-labile because either human Sod1 or Crs5 can compete for the L-bound Cu(I).<sup>26</sup> A kinetically labile copper pool within the mitochondria was also confirmed by x-ray fluorescence microscopy.<sup>56</sup> Finally, considering that it has been proposed that CuL can be mobilized from the mitochondrial matrix to inner membrane space, to be incorporated into COX17 and CCS,<sup>27</sup> we can predict that the copper(I) affinity of this ligand would be less than  $\sim 10^{-14}$  M ( $K_{Cu}$  value of COX17), thus being GLRX5 and ISCA2 effectively physiological competitors of CuL, considering that they have indeed  $K_{Cu}$  values in the  $10^{-14}$ - $10^{-13}$  M range. Therefore, the CuL pool needs to be tightly controlled to avoid any possible

deleterious crosstalk with the mitochondrial Fe/S cluster assembly machinery. Only by maintaining the copper(I) and Fe/S protein maturation pathways strictly regulated, copper(I) toxicity effects on mitochondrial [4Fe-4S] protein maturation can be indeed minimized. Since GLRX5 is essential, not only for iron-sulfur cluster biosynthesis, but also for haem biosynthesis<sup>57</sup> and for the maintenance of normal mitochondrial and cytosolic iron homeostasis,<sup>58</sup> CuL pool-GLRX5 interactions need also to be avoided to prevent deleterious effects on several other cellular processes fundamental for cell survival.

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## SUPPORTING INFORMATION

Supporting information contains four Figures reporting the determination of apparent dissociation constants of Cu(I)-GLRX5 and Cu(I)-ISCA2, UV/vis spectra of ISCA2 and its Cys to Ser mutants, <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectra of GLRX5 to follow [2Fe-2S]<sup>2+</sup> cluster transfer to apo ISCA1/ISCA2 heterocomplex, and UV/vis spectra to follow [2Fe-2S]<sup>2+</sup> cluster release from GLRX5 upon the interaction with Cu(I)-ISCA1/ISCA2 heterocomplex. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## FIGURE LEGENDS

**Figure 1. Fe/S cluster binding properties of ISCA2.** Paramagnetic 1D  $^1\text{H}$  NMR spectra of chemically reconstituted wild-type  $[\text{4Fe-4S}]^+$  ISCA2 (**a**), of the ISCA2 mutants C79S (**b**), C144S (**c**), C146S (**d**) subjected to Fe/S chemical reconstitution, and of wild-type  $[\text{2Fe-2S}]^{2+}$  ISCA2 purified from *E. coli* (**e**) in 50 mM phosphate buffer pH 7.0 at 600 MHz and 283 K. In the insets, Fe/S cluster coordination for the wild-type and the mutants are tentatively proposed on the basis of the spectroscopic analysis and the UV/vis spectra of wild-type and ISCA2 mutants are shown.  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra of ( $^{13}\text{C}$ ,  $^{15}\text{N}$ )Cys selectively-labeled ISCA2 in its apo (**f**) and chemically reconstituted  $[\text{4Fe-4S}]$  (**g**) form, and of Cu(I)-ISCA2 (**h**), in 50 mM phosphate buffer pH 7.0 and 298 K. The inset of the **h** panel shows the coordination mode of copper(I) involving ligands exchange process, proposed to occur on the basis of the NMR data.

**Figure 2. Copper(I) binding properties of ISCA2 and of the ISCA1/ISCA2 heterodimeric complex.**  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra of  $^{15}\text{N}$ -labelled ISCA2 (**a**) and ISCA1/ $^{15}\text{N}$ -labelled ISCA2 (**b**) in their apo (red) and copper(I) bound (black) forms. The insets show an overlay of  $^1\text{H}$ - $^{15}\text{N}$  HSQC experiments showing the backbone NH resonances of Asn136 observed upon stepwise additions of copper(I) (red, apo ISCA2; blue, 0.5 eq. of copper(I); black, 1 eq. of copper(I)), showing that a slow chemical shift regime on the NMR time scale between the apo and the Cu(I)-bound forms is operative upon copper(I) binding, which is complete once the 1:1 Cu(I)/protein ratio was reached. A region of  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra selected to monitor copper(I) binding to the C146S (**c**), C144S (**d**) and C79S (**e**)  $^{15}\text{N}$ -labelled ISCA2 mutants were shown. The spectra of the apo forms before (red) and after (black) their treatment with an excess of copper(I) followed by a PD-10 desalting column, are shown.

**Figure 3. The specific role of the three conserved cysteines of ISCA proteins in the cluster transfer process from  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub> to apo ISCA2.** Selected regions of  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra performed on 1:1 mixtures between  $^{15}\text{N}$ -labelled  $[\text{2Fe-2S}]^{2+}$  GLRX5<sub>2</sub> (30% of apo GLRX5 is present in the sample as previously described<sup>31</sup>) and C146S (**a**), C144S (**b**), C79S (**c**), wild-type (**d**)  $^{15}\text{N}$ -labelled apo

ISCA2 are overlaid with those of  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>. Specifically, backbone NH resonances of Gly120, Lys101, and Cys122 of GLRX5 were shown in red for  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> and in black for the mixtures. A slow chemical shift regime on the NMR time scale between the apo and the holo forms is observed upon  $[2\text{Fe-2S}]^{2+}$  cluster transfer, when it occurs. In the last column, the cross-peaks of the selected residues were integrated along the stepwise additions of  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> to ISCA proteins to calculate % molar fractions of apo GLRX5 (■) and of  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> (●), which is then plotted vs. the  $[\text{GLRX5}]/[\text{ISCA2}]$  molar ratio calculated considering that both GRLX5 and ISCA proteins are in a dimeric form.

**Figure 4. Copper(I) binding to apo and  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> forms.** Overlays of regions of  $^1\text{H-}^{15}\text{N}$  HSQC NMR experiments selected to monitor copper(I) binding to apo (a) and  $[2\text{Fe-2S}]^{2+}$  (b)  $^{15}\text{N}$ -labelled GLRX5<sub>2</sub> were shown. The spectra of the apo and holo forms before (red) and after (black) their treatment with a 1.5 equivalents of copper(I) were shown on the left column. Cross-peaks of the copper(I) form in a slow chemical shift regime on the NMR time scale observed upon copper(I) additions are indicated. On the right column,  $^1\text{H-}^{15}\text{N}$  HSQC spectra performed on the final Cu(I)-GLRX5 mixtures once passed through a PD-10 desalting column (blue) are compared with the starting materials (in red), i.e. apo and  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub>, respectively, showing that the apo form is mostly present. On the top of the NMR spectra, the occurring reactions are schematically reported.

**Figure 5. Monitoring copper(I) deleterious effects along the  $[4\text{Fe-4S}]$  cluster assembly process occurring on ISCA proteins upon receiving the  $[2\text{Fe-2S}]^{2+}$  cluster from GLRX5.** (a) Overlays of the  $^1\text{H-}^{15}\text{N}$  HSQC NMR spectrum of the 1:1  $^{15}\text{N}$ -labelled Cu(I)-ISCA2/ $^{15}\text{N}$ -labelled  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> mixture with those of Cu(I)-ISCA2 (black, left) and apo GLRX5 (blue, right). In the insets, amide resonances of ISCA2 (Asn136, left) and GLRX5 (Lys101, right) affected by  $[2\text{Fe-2S}]^{2+}$  cluster and copper(I) binding, respectively, in a slow exchange regime between the free and bound forms relative to NMR time scale are shown. The related spectral regions of the 1:1  $^{15}\text{N}$ -labelled Cu(I)-ISCA2/ $^{15}\text{N}$ -labelled  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> mixture are overlaid with those of Cu(I) (black, left) and apo (pink, left) ISCA2, and with those of  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> (brown, right;  $[2\text{Fe-2S}]^{2+}$  GLRX5 exists in solution as a mixture of two forms indicated here as

[2Fe-2S] GLRX5<sub>2a</sub> and [2Fe-2S] GLRX5<sub>2b</sub><sup>31</sup>) and apo GLRX5 (blue, right). **(b)** At the top, overlay of the <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectrum of <sup>15</sup>N-labelled apo ISCA2 (black) with that of the 1:1 <sup>15</sup>N-labelled apo ISCA2/unlabelled apo ISCA1 mixture (red). In the inset, a selected region of the latter superimposition is shown and overlaid with the spectrum of the 1:0.5 <sup>15</sup>N-labelled apo ISCA2/unlabelled apo ISCA1 mixture (blue), to show that a complete homodimer to heterodimer interconversion, occurring in a slow exchange regime on the NMR time scale, between the free and complexed ISCA2 forms is reached at the 1:1 protein ratio. 2.5 equivalents of <sup>15</sup>N-labelled [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub> were then added to the 'red' mixture. In the middle, two selected regions of the <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectrum acquired on the 2.5:1:1 <sup>15</sup>N-labelled [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub>/<sup>15</sup>N-labelled apo ISCA2/unlabelled apo ISCA1 mixture (in pink) are shown, specifically displaying GLRX5 backbone NH peaks. The two selected <sup>1</sup>H-<sup>15</sup>N HSQC regions are overlaid with the corresponding <sup>1</sup>H-<sup>15</sup>N HSQC regions of apo GLRX5 (in green) and of [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub> (in black). Stepwise additions of copper(I) to the previous ISCA1/ISCA2/GLRX5 mixture were performed till 1.5 equivalents of copper(I) were reached and, on this latter mixture, a PD-10 desalting column was performed. At the bottom, a selected region of the <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectrum, displaying ISCA2 backbone NH peaks, of the 2.5:1:1 <sup>15</sup>N-labelled [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub>/<sup>15</sup>N-labelled apo ISCA2/unlabelled apo ISCA1 mixture is shown before (in pink) and after Cu(I) additions and PD-10 column passage (in black). . This selected <sup>1</sup>H-<sup>15</sup>N HSQC region is superimposed with the corresponding <sup>1</sup>H-<sup>15</sup>N HSQC region of the Cu(I)-ISCA1/ISCA2 heterocomplex (in blue).

**Scheme 1. Model of [4Fe-4S] cluster assembly mechanism by [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub>, apo ISCA1/ISCA2 interactions and how copper(I) can impair it.** [2Fe-2S]<sup>2+</sup> GLRX5<sub>2</sub> interacts with the apo ISCA homo or heterodimer forming an intermediate *A'*, in which C-terminal cysteines of the ISCA proteins (Cys 144 and Cys 146 by following ISCA2 numbering, as shown in the figure, or Cys 121 and Cys 123 by following ISCA1 numbering) coordinate the [2Fe-2S]<sup>2+</sup> cluster upon the release of two GSH molecules. By releasing apo GLRX5, this intermediate evolves to species *A*, which coordinates the [2Fe-2S]<sup>2+</sup> with the C-terminal cysteines of ISCA proteins. Species *A* evolves to a more thermodynamically favored species *B*, which binds the [2Fe-2S]<sup>2+</sup> cluster via Cys 79 from each subunit of the ISCA2 homodimer (or Cys 79 from ISCA2

subunit and Cys 57 from ISCA1 subunit in the ISCA1/ISCA2 heterodimer), Cys 144 from one ISCA2 subunit, and Cys 146 from the other ISCA2 subunit (or by Cys 144 from ISCA2 subunit and Cys 123 from ISA1 subunit in the ISCA1/ISCA2 heterodimer). Species *B* can now receive another  $[2\text{Fe-2S}]^{2+}$  cluster from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> thanks to the availability of two free C-terminal cysteines. The latter coordinate the  $[2\text{Fe-2S}]^{2+}$  cluster in an intermediate *B'* formed upon the release of two GSH molecules. This intermediate might be the species receiving two electrons from a still unknown physiological partner (in our *in vitro* conditions DTT is the electron donor<sup>21,55</sup>) to form the final species *C*, which bind a  $[4\text{Fe-4S}]^{2+}$  cluster via the same ligands of species *B*. Copper(I) can impair such mechanism by ligating to both apo GLRX5 and apo heterocomplex, by displacing the  $[2\text{Fe-2S}]^{2+}$  or the assembled  $[4\text{Fe-4S}]^{2+}$  clusters in  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> and species *C*, respectively. The copper(I) ISCA1/ISCA2 heterocomplex is not able to receive the  $[2\text{Fe-2S}]^{2+}$  cluster from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> and triggers cluster release from  $[2\text{Fe-2S}]^{2+}$  GLRX5<sub>2</sub> to form apo GLRX5.

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