Mitochondrial copper(I) transfer from Cox17 to Sco1 is coupled to electron transfer

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Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved February 13, 2008 (received for review January 2, 2008)

The human protein Cox17 contains three pairs of cysteines. In the mitochondrial intermembrane space (IMS) it exists in a partially oxidized form with two S–S bonds and two reduced cysteines ($HCox17_{2S\text{-}S}$). $HCox17_{2S\text{-}S}$ is involved in copper transfer to the human cochaperones Sco1 and Cox11, which are implicated in the assembly of cytochrome *c* oxidase. We show here that $Cu(I)HCox17_{2S\text{-}S}$, i.e., the copper-loaded form of the protein, can transfer simultaneously copper(I) and two electrons to the human cochaperone Sco1 (HSco1) in the oxidized state, i.e., with its metal-binding cysteines forming a disulfide bond. The result is Cu(I)HSco1 and the fully oxidized $apoHCox17_{3S\text{-}S}$, which can be then reduced by glutathione to $apoHCox17_{2S\text{-}S}$. The HSco1/$HCox17_{2S\text{-}S}$ redox reaction is thermodynamically driven by copper transfer. These reactions may occur *in vivo* because HSco1 can be found in the partially oxidized state within the IMS, consistent with the variable redox properties of the latter compartment. The electron transfer-coupled metallation of HSco1 can be a mechanism within the IMS for an efficient specific transfer of the metal to proteins, where metal-binding thiols are oxidized. The same reaction of copper–electron-coupled transfer does not occur with the human homolog of Sco1, HSco2, for kinetic reasons that may be ascribed to the lack of a specific metal-bridged protein–protein complex, which is instead observed in the $Cu(I)HCox17_{2S\text{-}S}$/HSco1 interaction.

cytochrome *c* oxidase assembly | copper chaperone | mitochondrial chemistry | NMR

Copper is an essential element for living organisms, in which it is required by a number of proteins responsible for a wide range of biological processes, including mitochondrial respiration (1). Specific mechanisms, involving a battery of proteins, are necessary for living organisms for safely and efficiently delivering copper ions from their entry in the cell to its final protein incorporation (2).

Cytochrome *c* oxidase (CcO), the terminal oxidase of the respiratory chain, needs three copper ions to perform its function (3). The CcO enzyme in mammals is located within the mitochondrial inner membrane and is constituted by 13 subunits, two of which contain two copper centers (4). One, called Cu_B, is a monocopper site in subunit I, and the other, called Cu_A, is a binuclear site in subunit II (4). A number of proteins are required for the metallation of these sites (5). For copper insertion into the Cu_A site, Cox17, Sco1, and Sco2 proteins have been proposed to have essential nonoverlapping roles within the mitochondrial intermembrane space (IMS), the first working as a metallochaperone to Sco1 and Sco2 (6, 7), whereas the other two have a cochaperone function during the copper insertion in the Cu_A site (7). Accordingly, lack of Cox17 or mutations on either Sco1 or Sco2 produce pathogenic conditions related to decreased CcO activity with fatal outcomes in mice and humans, respectively (8, 9). Also, yeast lacking Cox17 is respiratory deficient because of the lack of CcO activity, and this mutant phenotype is suppressed by the addition of 0.4% copper salts to the growth medium (10).

Human Cox17 (HCox17) is a 62-residue protein and contains six conserved Cys residues. A partially oxidized form of HCox17 with two disulfide bonds and two free cysteines ($HCox17_{2S\text{-}S}$) has been proposed to be the functionally competent state in the IMS capable of copper transfer to Sco1 protein partner (11–13). In the latter state, HCox17 is structurally organized in a coiled-coil–helix–coiled-coil–helix (CHCH) domain and binds one copper(I) ion with two consecutive Cys residues at positions C22 and C23 (12). The human Sco1 and Sco2 proteins (HSco1 and HSco2, respectively) contain two essential Cys residues in a fully conserved CXXXC metal-binding motif, arranged in a thioredoxin fold (14–16). These two Cys residues (C169 and C173 in HSco1, C133 and C137 in HSco2), together with a conserved His residue (H260 in HSco1, H224 in HSco2), constitute the copper-binding residues (14, 15). The specific role of these two proteins in the copper transfer to the Cu_A site is not yet understood and was recently the subject of an extensive scientific debate, in which several different functions, even completely novel and unrelated to the co-chaperone function, have been proposed (14, 17, 18).

Copper binding needs reduced Cys in both donor and accepting proteins. Even in the cytoplasm, although reduced glutathione (GSH) is preponderant with respect to the oxidized form (GSSG), thus providing a reducing environment, some copper-binding sites can require specific proteins to quantitatively reduce all Cys residues, such as glutaredoxin 1 in the case of the Menkes and Wilson proteins (19). No evidence exists for the presence in the IMS of glutathione reductase to maintain GSH/GSSG redox homeostasis (20, 21), thus suggesting that the environment in the IMS can be less reducing than in the cytoplasm, and might depend on its specific regions and cell conditions. Therefore, the question arises about how the accepting copper proteins located in the IMS, e.g., Sco1, Sco2, and CcO subunit II, which bind copper through two Cys residues, are kept reduced. Accordingly, the highly negative reduction potential values of the two metal-binding Cys residues in apoHSco1 and apoHSco2 (14, 15) suggest that a reductant capable of reducing their disulfide bonds is needed for them to be able to bind the copper(I) ion. This feature is even more compelling in Gram-positive and Gram-negative bacteria, where a similar pathway for copper transport and incorporation in CcO, involving Sco proteins (22, 23), occurs outside of the cell or in the periplasmic space, respectively, where Cys residues in Sco and Cu_A sites can presumably be oxidized by the aerobic environment.

We have already proposed that Cu(I)HSco1 may reduce the disulfide bond in the oxidized $apoCu_A$ site of CcO, giving rise to oxidized HSco1 ($HSco1_{1S\text{-}S}$ hereafter) and copper-bound Cu_A

Author contributions: L.B. and I.B. designed research; S.C.-B., T.H., M.M., and P.P. performed research; S.C.-B., T.H., M.M., and P.P. analyzed data; and L.B., I.B., S.C.-B., and P.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0800019105/DCSupplemental.

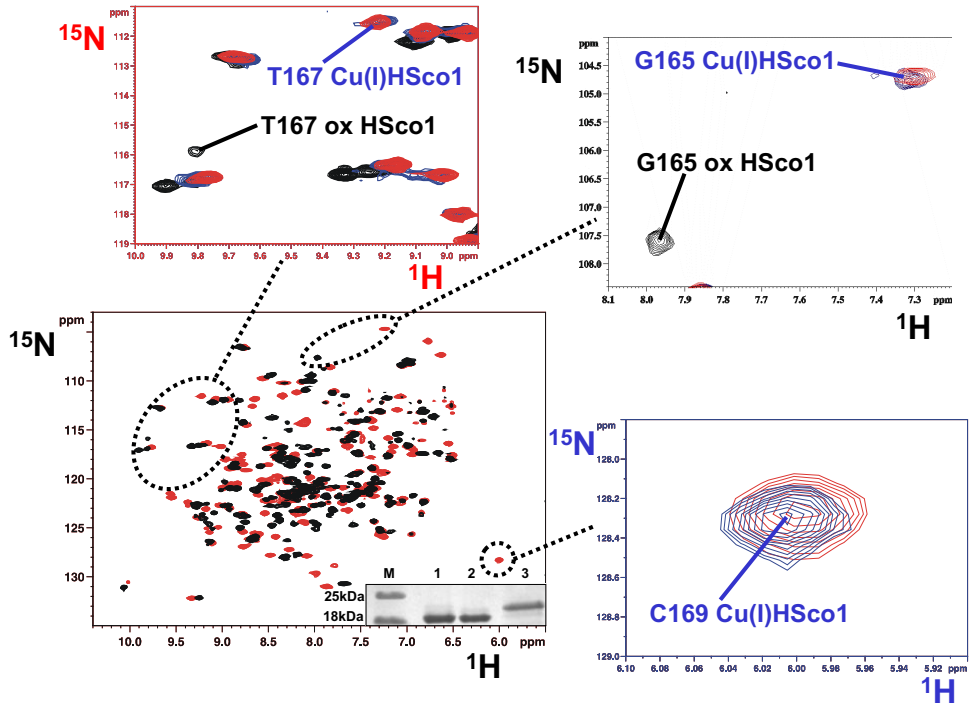

Fig. 1. Titration of ^{15}N-labeled HSco1$_{1S\text{-}S}$ with unlabeled Cu(I)HCox17$_{2S\text{-}S}$ followed through chemical shift changes in the ^{1}H–^{15}N HSQC spectra. The ^{1}H–^{15}N HSQC spectrum of ^{15}N-labeled HSco1$_{1S\text{-}S}$ (in black) is overlaid with the ^{1}H–^{15}N HSQC spectrum of a 1:1 ^{15}N-labeled HSco1$_{1S\text{-}S}$/unlabeled Cu(I)HCox17$_{2S\text{-}S}$ mixture (in red). In enlarged views selected regions of ^{1}H–^{15}N HSQC spectra are shown in which also the ^{1}H–^{15}N HSQC spectrum of ^{15}N-labeled Cu(I)HSco1 (in blue) is overlaid. The assignment of the NH resonances of G165 and T167 in Cu(I)HSco1 and HSco1$_{1S\text{-}S}$ forms is reported. NH resonance of C169 is detected only in the copper(I)-bound form. (*Inset*) Nonreducing SDS/PAGE gel. Lane M, protein marker; lane 1, HSco1; lane 2, HSco1$_{1S\text{-}S}$ modified with acetamido-4-maleimidylstilbene-2,2-disulfonic acid (AMS); lane 3, AMS-modified HSco1$_{2SH}$.

(14). Here, we show that Cu(I)HCox17$_{2S\text{-}S}$, i.e., human Cox17 protein containing two disulfide bridges and one Cu(I) ion bound to two remaining reduced cysteines, is capable of transferring *in vitro* copper(I) and electrons to HSco1$_{1S\text{-}S}$, giving rise to Cu(I)HSco1 and fully oxidized apoHCox17$_{3S\text{-}S}$ containing three disulfide bonds, thus performing an electron transfer-coupled metallation of HSco1$_{1S\text{-}S}$. The same is not true for oxidized HSco2 (HSco2$_{1S\text{-}S}$ hereafter), although the copper transfer from Cu(I)HCox17$_{2S\text{-}S}$ to reduced apoHSco2 (apoHSco2$_{2SH}$) occurs similarly to what has already been found for reduced apoHSco1 (apoHSco1$_{2SH}$) (11).

Results

When Cu(I)HCox17$_{2S\text{-}S}$ is mixed with apoHSco1$_{1S\text{-}S}$, i.e., with the two cysteines of the copper-binding site in the oxidized state forming an intramolecular disulfide bond, we observe the simultaneous transfer of copper(I) and of two electrons from Cu(I)HCox17$_{2S\text{-}S}$ to HSco1$_{1S\text{-}S}$, obtaining Cys-reduced Cu(I)HSco1 and apoHCox17$_{3S\text{-}S}$. This process was monitored through NMR. The NMR signals of ^{15}N-labeled apoHSco1$_{1S\text{-}S}$, titrated with unlabeled Cu(I)HCox17$_{2S\text{-}S}$ in the absence of reducing agents, indicated the formation of the Cu(I)HSco1 species, as monitored from the appearance of its typical NH resonances in the ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectra (Fig. 1). As an example, the NH cross-peaks of Gly 165 and Thr 167, which are close to the CXXXC metal binding motif and whose ^{1}H,^{15}N chemical shifts drastically change depending on the redox and/or metallation state of HSco1, disappear upon addition of Cu(I)HCox17$_{2S\text{-}S}$ from the position it has in apoHSco1$_{1S\text{-}S}$ with the simultaneous appearance of the corresponding cross-peaks, typical of the Cu(I)HSco1 species (Fig. 1). Accordingly, the NH of C169, not detected in apoHSco1$_{1S\text{-}S}$, appears in the ^{1}H–^{15}N HSQC spectra of the protein mixture, with the same chemical shift as the Cu(I)HSco1 species (Fig. 1). The changes on HCox17$_{2S\text{-}S}$ cannot be followed by ^{1}H–^{15}N HSQC spectra because the NH signals of both C22 and C23, involved in copper(I) binding and redox reaction with HSco1$_{1S\text{-}S}$, and of the only two other residues (A24 and C25) whose NH chemical shifts are significantly affected by metallation and/or redox state changes (12), broaden beyond detection when Cu(I)HCox17$_{2S\text{-}S}$ is mixed with apoHSco1$_{1S\text{-}S}$ [supporting information (SI) Fig. S1]. Broadening of signals, still being detectable, occurs upon addition of apoHSco1$_{1S\text{-}S}$ also for other NH resonances of HCox17$_{2S\text{-}S}$ mainly located in the α-helical hairpin (Fig. S1). This behavior originates from exchange processes through a HSco1/Cu(I)$_1$/HCox17$_{2S\text{-}S}$ transient low-populated intermediate (11). This broadening is detected only for HCox17$_{2S\text{-}S}$ because the difference in ^{1}H–^{15}N chemical shifts is smaller between Cu(I)HCox17$_{2S\text{-}S}$ and HCox17$_{3S\text{-}S}$ with respect to the shift observed between oxidized apoHSco1 and Cu(I)HSco1. The relative difference in ^{1}H–^{15}N chemical shifts makes the latter slow on the NMR time scale and makes the former intermediate. The same behavior was also observed when ^{15}N-labeled Cu(I)HCox17$_{2S\text{-}S}$ was titrated with unlabeled apoHSco1$_{2SH}$ in a reducing environment. Therefore, to analyze the redox state of the cysteines in the Cu(I)HCox17$_{2S\text{-}S}$/apoHSco1$_{1S\text{-}S}$ reaction, we produced a sample with ^{13}C-selectively-labeled cysteines and monitored their redox state in the protein mixture on the basis of the ^{13}C chemical shifts of their C$^{\beta}$ carbons. Indeed, the latter shifts have very specific and completely distinct values whether the sulfur atoms are reduced or oxidized to form a disulfide bond (24). The large ^{13}C chemical shift difference ensures that the equilibrium is slow on the NMR time scale. When the 1D ^{13}C NMR spectra of (^{13}C,^{2}H,^{15}N)Cys-selectively labeled HCox17$_{2S\text{-}S}$ and (^{13}C,^{2}H,^{15}N)Cys-selectively labeled HCox17$_{3S\text{-}S}$ are compared, the spectral signatures of the two redox states are clearly

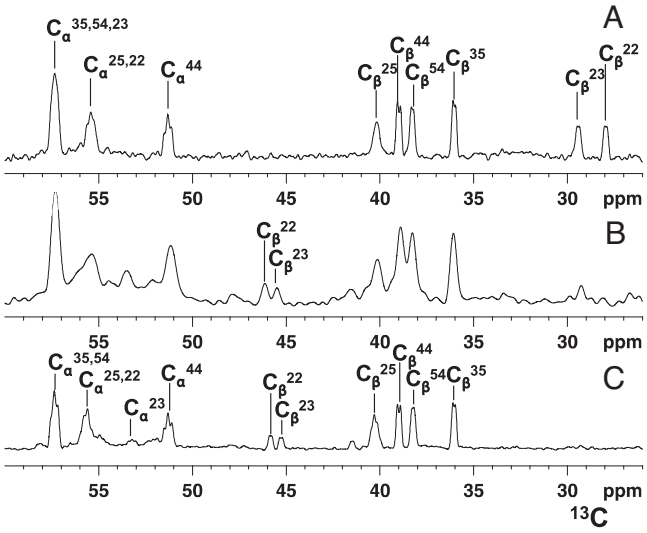

Fig. 2. Oxidation state of HCox17 cysteines monitored in the redox reaction between (^{13}C,^{2}H,^{15}N)Cys-selectively labeled Cu(I)HCox17$_{2S\text{-}S}$ and ^{15}N-labeled HSco1$_{1S\text{-}S}$. Chemical shift changes of C$^{\alpha}$ and C$^{\beta}$ carbons of Cys residues of Cu(I)HCox17$_{2S\text{-}S}$ were followed by 1D ^{13}C NMR spectra. (*A*) (^{13}C,^{2}H,^{15}N)Cys-selectively labeled Cu(I)HCox17$_{2S\text{-}S}$. (*B*) 1:1 (^{13}C,^{2}H,^{15}N)Cys-selectively labeled Cu(I)HCox17$_{2S\text{-}S}$/^{15}N-labeled HSco1$_{1S\text{-}S}$ mixture. (*C*) (^{13}C,^{2}H,^{15}N)Cys-selectively labeled HCox17$_{3S\text{-}S}$ obtained by air oxidation. The assignment of the C$^{\alpha}$ and C$^{\beta}$ resonances of the Cys residues of both Cu(I)HCox17$_{2S\text{-}S}$ and HCox17$_{3S\text{-}S}$ forms is reported. C$^{\beta}$ signals of the two Cys residues (C22 and C23) of Cu(I)HCox17$_{2S\text{-}S}$ that are involved in the disulfide exchange reaction drastically reduce their intensity in the protein mixture (*B*) with the concomitant formation of the corresponding C$^{\beta}$ signals with chemical shifts typical of HCox17$_{3S\text{-}S}$.

evident not only from the C$^{\beta}$ shifts but also from the C$^{\alpha}$ shifts (Fig. 2). So, when (^{13}C, ^{2}H, ^{15}N) Cys-selectively labeled Cu(I)HCox17$_{2S\text{-}S}$ is mixed with ^{15}N-labeled apoHSco1$_{1S\text{-}S}$ in the absence of reducing agents, it is observed that HSco1$_{1S\text{-}S}$ is reduced and metallated, similarly to what was observed in the previous titration, whereas C22 and C23 of HCox17$_{2S\text{-}S}$ are oxidized, as shown by their ^{13}C chemical shift values, thus forming HCox17$_{3S\text{-}S}$ (Fig. 2*B*).

When unlabeled apoHCox17$_{2S\text{-}S}$ is mixed with ^{15}N-labeled apoHSco1$_{1S\text{-}S}$ in the absence of reducing agents, apoHSco1$_{2SH}$ is not formed appreciably. The NH resonances typical of apoHSco1$_{2SH}$ (i.e., T167 and G165) are indeed not observed in the ^{1}H–^{15}N HSQC spectra of the mixture, but a new set of signals not belonging to either oxidized or reduced apoHSco1 appeared, originating from a species in slow exchange on the chemical shift time scale (Fig. S2). This observations suggests that apoHSco1$_{1S\text{-}S}$ is interacting with apoHCox17$_{2S\text{-}S}$ although the redox reaction does not occur appreciably. Addition of copper(I) to the latter apoHCox17$_{2S\text{-}S}$/apoHSco1$_{1S\text{-}S}$ mixture produces the same species obtained by mixing Cu(I)HCox17$_{2S\text{-}S}$ and apoHSco1$_{1S\text{-}S}$, proving that the presence of copper(I) is necessary to promote the disulfide exchange redox reaction (Fig. S2).

When Cu(I)HCox17$_{2S\text{-}S}$ is mixed with apoHSco2$_{1S\text{-}S}$ no reaction occurs, at variance with what is found for apoHSco1$_{1S\text{-}S}$, as monitored through ^{1}H–^{15}N HSQC spectra analysis (Fig. 3*D* and Fig. S1). However, when working in a reducing environment, through addition of DTT at millimolar concentrations, the behavior of the two proteins is similar. Indeed, similarly to what already found for apoHSco1$_{2SH}$ (11), Cu(I)HCox17$_{2S\text{-}S}$ quantitatively transfers the metal ion to apoHSco2$_{2SH}$ (Fig. S3). ESI MS experiments performed in the presence of reducing agent (0.5 mM DTT) did not detected any complex between Cu(I)HCox17$_{2S\text{-}S}$ and HSco2 (Fig. 3*C*). This finding is at variance with what was observed for the Cu(I)HCox17$_{2S\text{-}S}$–HSco1 interaction, in which protein–protein interaction occurs with formation of ≈5% of the complex at micromolar concentrations of the proteins (Fig. 3*A*). Accordingly, no broadening of NH resonances of HCox17$_{2S\text{-}S}$ was observed in the mixture of Cu(I)HCox17$_{2S\text{-}S}$ and apoHSco2$_{2SH}$ (Fig. S3), at variance with

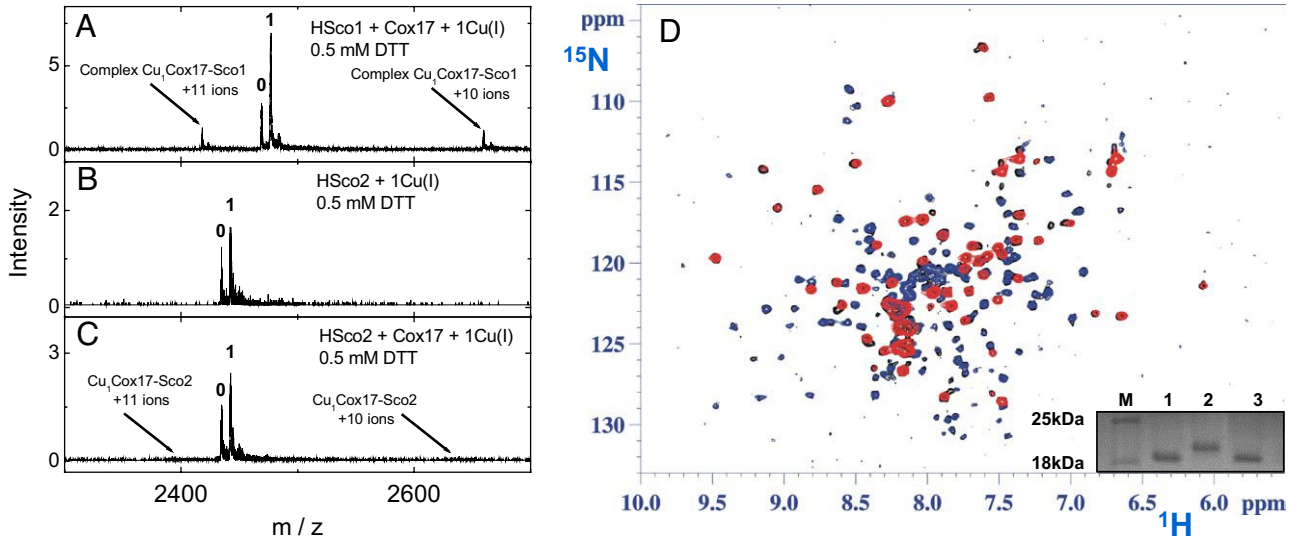

Fig. 3. Electrospray ionization (ESI) MS analysis of Cu(I)HCox17$_{2S\text{-}S}$/HSco protein–protein complex formation in the presence of 0.5 mM DTT (*Left*) and NMR data on Cu(I)HCox17$_{2S\text{-}S}$/HSco2$_{1S\text{-}S}$ mixture in the absence of reducing agents (*Right*). (*A*) Mixture of HSco1 (3 μM), HCox17$_{2S\text{-}S}$ (2 μM), and Cu(I)DTT (2 μM). (*B*) Mixture of HSco2 (3 μM) and Cu(I)DTT (2 μM). (*C*) Mixture of HSco2 (3 μM), HCox17$_{2S\text{-}S}$ (2 μM), and Cu(I)DTT (2 μM). Conditions: 20 mM ammonium acetate, pH 7.5, 0.5 mM DTT. ESI MS spectra were recorded as described in *Materials and Methods*. Charge state +8 ions are presented for HSco1 and HSco2, and numbers on the peaks denote the metal stoichiometry of the complex. Peaks corresponding to +10 and +11 ions of HCox17$_{2S\text{-}S}$/Cu(I)$_1$/HSco1 complexes (*A*) and positions of theoretical peaks for HCox17$_{2S\text{-}S}$/Cu(I)$_1$/HSco2 complexes (*C*) are indicated with arrows. (*D*) ^{1}H–^{15}N HSQC spectrum of a 1:1 ^{15}N-labeled HSco2$_{2S\text{-}S}$/^{15}N-labeled Cu(I)HCox17$_{2S\text{-}S}$ mixture (in black) is overlaid with ^{1}H–^{15}N HSQC spectra of ^{15}N-labeled HSco2$_{1S\text{-}S}$ (in blue) and of ^{15}N-labeled Cu(I)HCox17$_{2S\text{-}S}$ (in red). The NH resonances of the mixture and of the starting materials are well superimposed and no new NH resonances of Cu(I)HSco1 and apoHCox17$_{2S\text{-}S}$ species are detected, indicating that no reaction occurs. (Inset) Nonreducing SDS/PAGE gel. Lane M, protein marker; lane 1, HSco2; lane 2, AMS-modified HSco2$_{2SH}$; lane 3, AMS-modified HSco2$_{1S\text{-}S}$.

what was observed in Cu(I)HCox17$_{2S\text{-}S}$/HSco1 interactions (Fig. S1), indicating essentially the absence of an equilibrium involving a stable protein–protein complex. Therefore, we suggest that the Cu(I) ion transfer to HSco2 passes through a very low populated complex that is not detected.

Discussion

Recent studies overthrow the previously held view that the presence or absence of disulfide bonds in proteins is determined by the cell compartment and that the simple ratio between reduced and oxidized glutathione in all cases determines the status of intracellular thiol groups (25). Instead, the specific nature of the proteins and their interactions with other proteins determine the redox states of Cys residues. Indeed, glutaredoxin 1 has been proposed to be necessary for completely maintaining the Cys copper(I) ligands of Menkes and Wilson proteins in the reduced state (19), despite the fact that they are in the reducing environment of the cytoplasm. Moreover, a number of proteins have been also found to oxidize Cys in the IMS according to specific pathways (26). Overall, the redox status of thiol groups is in equilibrium with the redox potential of the specific cellular compartment, but apparently this thermodynamic equilibrium involving disulfide formation and electron flow is strongly modulated by protein–protein interactions so that, actually, only some pathways are allowed.

In this frame, we are here suggesting a possible dual functional role for HCox17, which involves the simultaneous reduction of the metal-binding Cys residues of HSco1 and the metal transfer to HSco1. The latter has a thioredoxin fold and was also proposed to have a function not only of copper transfer but also of reducing the Cys residues of the Cu_A site in CcO (14). Coupling of metal transfer with specific redox chemistry adds an additional level of control into the metal transfer pathway, warranting in particular a high metal specificity of this process for proteins capable of binding metals other than copper with high affinity, as occurs for the Sco1 protein family (14, 27). Such a specificity of HCox17$_{2S\text{-}S}$ action toward HSco1$_{1S\text{-}S}$ metallation is highlighted by the fact that redox reaction does not occur with HSco2$_{1S\text{-}S}$. HCox17 can therefore work to specifically metallate and reduce HSco1$_{1S\text{-}S}$ with respect to HSco2$_{1S\text{-}S}$, being able in this way to select between two proteins with similar copper(I)-binding affinity.[§] Because the redox potentials of apoHSco1$_{1S\text{-}S}$ and apoHSco2$_{1S\text{-}S}$ are similar (11, 15), the different behavior of the two proteins toward redox chemistry has to rely on kinetic grounds, which might arise from the occurrence or absence of specific protein–protein interactions. Previously we demonstrated by ESI MS that Cu(I)HCox17$_{2S\text{-}S}$ and HSco1$_{2SH}$ form a specific metal-bridged HSco1/Cu(I)$_1$/HCox17$_{2S\text{-}S}$ complex, which might serve also as transient metal transfer complex in *vivo* (11). Current ESI MS results confirm the formation of a specific low fraction (≈5%) metal-bridged HSco1/Cu(I)$_1$/HCox17$_{2S\text{-}S}$ complex at micromolar concentrations of proteins. However, in similar conditions, no adduct was detected between Cu(I)HCox17$_{2S\text{-}S}$ and HSco2$_{2SH}$. The specific protein–protein interactions between HSco1 and HCox17 might therefore be crucial for electron transfer from Cu(I)HCox17$_{2S\text{-}S}$ to apoHSco1$_{1S\text{-}S}$, thus establishing the molecular grounds of the selectivity of redox chemistry observed between HCox17$_{2S\text{-}S}$ and HSco1$_{1S\text{-}S}$ with respect to HCox17$_{2S\text{-}S}$ and HSco2$_{1S\text{-}S}$. The protein–protein interaction can be indeed crucial to position the disulfide bond of HSco1 in the correct orientation facilitating electron transfer reaction.

This view is in agreement with previous findings showing that the metallation of HSco1, but not of HSco2, when expressed in the yeast cytoplasm, depends on the coexpression of HCox17 (28), suggesting that HCox17 is the specific protein partner of HSco1. Therefore, the nonoverlapping functions of HSco1 and HSco2 proteins in mitochondrial copper delivery (7) can rely on their different redox behavior when interacting with Cu(I)HCox17$_{2S\text{-}S}$ but not on their copper-binding capabilities, which are indeed similar. This finding could be important to establish the molecular basis for the distinct clinical presentation of patients with mutations in *Sco2* with respect to that of *Sco1* patients (8). The failure of electron and copper transfer processes from HCox17$_{2S\text{-}S}$ to HSco2 also suggests that an additional partner of HSco2 could be necessary to reduce its disulfide bond to allow the copper(I) incorporation into the Cu_A site. The recently observed different backbone dynamic properties of apoHSco2 with respect to apoHSco1 (15) can indeed play an important role in selecting different protein partners in the molecular recognition process.

HCox17$_{3S\text{-}S}$ is quickly reduced *in vitro* to HCox17$_{2S\text{-}S}$ by millimolar concentrations of GSH (13). It is reasonable then to assume that, after each redox metal transfer cycle, the fully oxidized HCox17$_{3S\text{-}S}$ could be reduced by GSH in the IMS and the protein be recycled. Because the reduction potential of the redox couple apoHSco1$_{1S\text{-}S}$/apoHSco1$_{0S\text{-}S}$ is more negative (−280 mV) (11) than that of apoHCox17$_{3S\text{-}S}$/apoHCox17$_{2S\text{-}S}$ (−198 mV) (13), the thermodynamic equilibrium disfavors the transfer of electrons to apoHSco1$_{1S\text{-}S}$. Indeed, when apoHCox17$_{2S\text{-}S}$ was mixed with apoHSco1$_{1S\text{-}S}$, no disulfide exchange reaction was observed. Therefore, the presence of copper(I) is necessary to drive the electrons toward the reduction of apoHSco1$_{1S\text{-}S}$ and the driving force of electron flow from HCox17$_{2S\text{-}S}$ to HSco1$_{1S\text{-}S}$ is determined by the higher thermodynamic stability of Cu(I)HSco1 with respect to that of Cu(I)HCox17$_{2S\text{-}S}$ (11).

The *in vitro* observation of the simultaneous electron and copper transfer reaction between Cu(I)HCox17$_{2S\text{-}S}$ and apoHSco1$_{1S\text{-}S}$ suggests that the same reaction can occur also *in vivo* when needed: copper(I) eventually transferred to the Cu_A site, with HSco1 acting as a thioredoxin (14). Living organisms could have, therefore, developed an efficient and rapid mechanism of metal incorporation capable of handling the complex redox scenario of IMS, enabling transfer of copper(I) to the Cu_A site both when Sco1 protein is in an oxidized and when it is in a reduced state. Our intriguing hypothesis involves HCox17$_{2S\text{-}S}$ as the origin of an electron cascade for the reduction of the Cys residues in the Cu_A site, through HSco1 (Fig. 4).

In conclusion, from this work we propose that copper trafficking in the IMS is a complex process requiring multiple steps that need to deal with various redox states of the involved proteins, in a system-dependent manner.

Materials and Methods

Protein Expression and Purification Procedures. Unlabeled and uniformly ^{15}N-labeled HSco1, HSco2, and HCox17$_{2S\text{-}S}$ proteins were expressed and purified from *Escherichia coli* by following already reported protocols (12, 14, 15). Aerobic oxidation of HSco1 and HSco2 were followed through nonreducing SDS/PAGE gels after AMS sample modification. Metallation of HCox17$_{2S\text{-}S}$ was performed by following a previously reported protocol (12). To produce (^{13}C,^{2}H,^{15}N)Cys-selectively labeled HCox17$_{2S\text{-}S}$, the Cox17-pETG-30A expression vector was transformed in a suitable cysteine-auxotrophic strain, BL21(DE3)cysE (29, 30), and the cells were grown in a minimal medium supplemented with 50 mg/liter of L-(U-^{13}C$_3$,U-^{2}H$_3$,^{15}N)cysteine (Cambridge Isotope Laboratories; each isotope at 98% replacement), 100 mg/liter each other unlabeled amino acid, 3 g/liter glucose, 1 g/liter $(NH_4)_2SO_4$. The growth of BL21(DE3)cysE/Cox17 cells and overexpression of (^{13}C,^{2}H,^{15}N)Cys-selectively labeled HCox17$_{2S\text{-}S}$ were carried out in a 2.5-liter Tunair shaking flask (IBI-Shelton Scientific) with a working solution of 0.5 liter. The culture was grown at 37°C up to OD_{600} = 0.8 and then the protein expression was induced by

[§]When, to a 1:1 mixture of ^{15}N-labeled apoHSco1 and ^{15}N-labeled apoHSco2, 1 eq of a copper(I) acetonitrile complex was added, formation of the copper form for the two proteins was observed in similar ratios, thus indicating similar cooper(I)-binding affinity (Fig. S4); the copper forms are in a slow exchange on the chemical shift time scale with the apo forms.

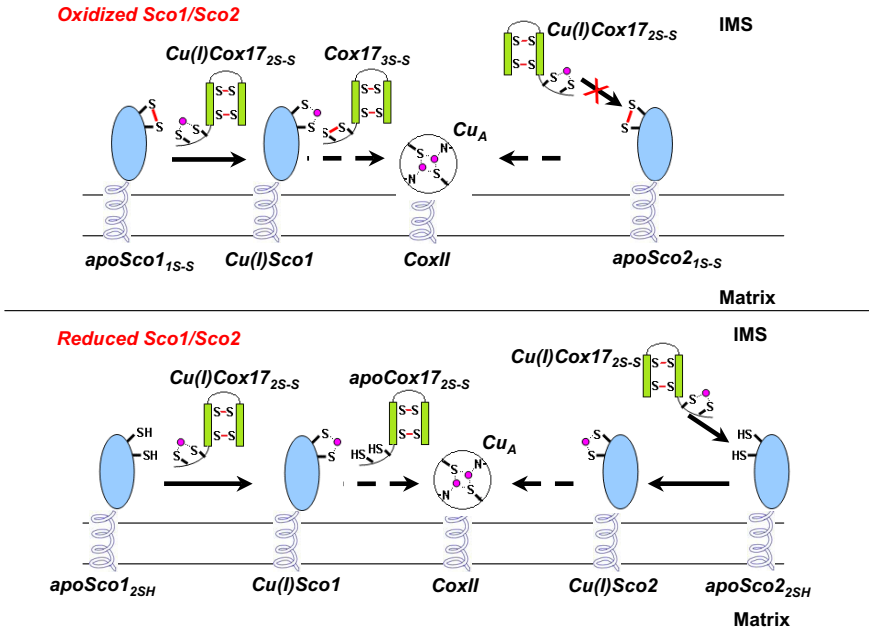

Fig. 4. Proposed mechanism of copper transfer from HCox17$_{2S-S}$ to the subunit II of CcO, through the assistance of HSco1 and HSco2. This model implies that HSco1, independently of the redox state of its Cys ligands in the IMS, may accept copper(I) from the mitochondrial chaperone Cu(I)HCox17$_{2S-S}$ to form Cu(I)HSco1 whereas HSco2 can accept copper(I) from Cu(I)HCox17$_{2S-S}$ only once its Cys ligands in the IMS are in a reduced state. HCox17$_{3S-S}$, produced by the redox HSco1/HCox17$_{2S-S}$ reaction, can be quickly reduced to HCox17$_{2S-S}$ by GSH, thus the latter protein being recycled for the following metal transfer.

addition 0.7 mM IPTG at 25°C for 16 h. Purification and metallation of (^{13}C,^{2}H,^{15}N)Cys-selectively labeled HCox17$_{2S-S}$ were performed by following the protocol in ref. 12.

The numbering of HCox17 sequence follows the isolated functional mammalian Cox17 sequences (31, 32), in which the first Met is processed away posttranslationally. Therefore, the HCox17 sequence numbering starts with Pro-1.

NMR Titration of the Two Proteins. Titrations of Cu(I)HCox17$_{2S-S}$ with apoHSco1$_{1S-S}$ were performed with NMR spectroscopy, following the ^{1}H–^{15}N and ^{13}C chemical shift changes upon the addition of increasing amounts of the titrating protein. Two-dimensional ^{1}H–^{15}N HSQC and/or 1D ^{2}H-decoupled ^{13}C experiments were collected at 298 K during the titrations on Avance 900, 800, and 500 Bruker spectrometers operating at a proton nominal frequency of 900.13 MHz, 800.13 MHz, and 500.13 MHz, respectively. Aliquots were added in a Coy chamber under nitrogen atmosphere at 298 K to deliver unlabeled or labeled proteins to the labeled samples in NMR tubes. The titrations were performed in the absence of reducing agents, adding unlabeled Cu(I)HCox17$_{2S-S}$ or ^{15}N-labeled apoHSco1$_{1S-S}$ to ^{15}N-labeled apoHSco1$_{1S-S}$ or (^{13}C,^{2}H,^{15}N)Cys-selectively labeled Cu(I)HCox17$_{2S-S}$, respectively, and unlabeled apoHSco1$_{1S-S}$ to ^{15}N-labeled Cu(I)HCox17$_{2S-S}$. Protein mixture concentrations range between 0.1 and 0.4 mM. Similarly, HSco2/HCox17 interaction has been followed by NMR both in the presence (1 mM DTT) and in the absence of reducing agents. In the first case, ^{15}N-labeled apoHSco2$_{2SH}$ or ^{15}N-labeled Cu(I)HCox17$_{2S-S}$ was titrated with unlabeled Cu(I)HCox17$_{2S-S}$ or unlabeled apoHSco2$_{2SH}$, respectively. In the second case, ^{15}N-labeled apoHSco2$_{1S-S}$ was titrated with ^{15}N-labeled Cu(I)HCox17$_{2S-S}$ and ^{15}N-labeled Cu(I)HCox17$_{2S-S}$, with unlabled HSco2$_{1S-S}$. For all titrations, protein/protein ratio ranges from 0 to 2 equivalents.

ESI MS Studies. Cu(I)HCox17$_{2S-S}$ was prepared by addition of 1 eq of Cu(I)DTT complex to HCox17$_{2S-S}$ in 20 mM ammonium acetate, pH 7.5, containing 0.5 mM DTT. Cu(I)·DTT complex was prepared by dissolving of 150 μM Cu(II)acetate in argon-saturated 20 mM ammonium acetate, pH 7.5, containing 1 mM DTT. The Cu(I)HCox17$_{2S-S}$ sample obtained was subsequently mixed with the sample of HSco1 or HSco2 protein in 20 mM ammonium acetate, pH 7.5, containing 0.5 mM DTT. The obtained mixture containing 2 μM Cu(I)HCox17$_{2S-S}$ and 3 μM HSco1 or HSco2 was incubated for an additional 1 min at 298 K and injected by a syringe pump at 5 μl/min into the electrospray ion source of a QSTAR Elite ESI-Q-TOF MS instrument (Applied Biosystems). ESI MS spectra were recorded in the region from 500 to 3,000 Da at the following instrument parameters: ion spray voltage 5,500 V; source gas 30 liters/min; curtain gas 20 liters/min; declustering potential 60 V; focusing potential 320 V; detector voltage 2,300 V. ESI MS spectra were analyzed by the program Analyst (Applied Biosystems). Fractional content of the HCox17$_{2S-S}$/Cu(I)$_1$/HSco1 complex was calculated from peak intensities, taking into account all charge states of HSco1 (+9 and +8) and of the complex (+10 and +11) in the ESI MS spectra, assuming that ionization efficiency for all forms of HSco1 as well as of the HCox17$_{2S-S}$/Cu(I)$_1$/HSco1 complex is similar.

ACKNOWLEDGMENTS. We thank Marie-Paule Strub for providing us the cysteine-auxotrophic strain BL21(DE3)cysE. This work was supported by the European Commission (''European Network of research Infrastructures for providing Access and Technological Advancements in bio-NMR,'' Contract 026145; ''From Receptor to Gene: structures of complexes from signaling pathways linking immunology, neurobiology and cancer,'' SPINE2-COMPLEXES Contract LSHG-CT-2006-031220). The Italian Ministero dell'Universitá e della Ricerca Scientifica e Technologica COFIN03 and MIUR–FIRB (Ministero dell'Università e della Ricerca–Fondo per gli Investimenti della Ricerca di Base, Protocollo MIUR-RBLA032ZM7), and the Estonian Science Foundation (Project 7191) are also acknowledged for financing.

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