Copper trafficking in biology: an NMR approach

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Copper ions are essential for living organisms because they are involved in several fundamental biological processes. Biomolecules interacting with copper ions have to be characterized as such, when bound to the metal ion, and when they interact with other biomolecules or substrates. The characterization is both structural and dynamic. In this context, NMR is a preferred tool of investigation because it allows shedding light on what happens in solution. Here, the NMR contribution to the copper trafficking is described, providing precious information on biochemical pathways, which are essential to understand the mechanisms of life at the molecular level. [DOI: 10.2976/1.3078306]

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INTRODUCTION

Living organisms interact with metal ions as part of the lithosphere from which they originate and where they eventually merge. The interaction with metal ions must be controlled in any moment of life: from the uptake to the trafficking within the living organisms for their utilization, until the excretion when appropriate (Huffman and O'Halloran, 2001; Pena *et al.*, 1999; Rae *et al.*, 1999; Valentine and Gralla, 1997). Essential transition metals such as zinc and copper and nonessential metals as cadmium, lead, and mercury can be indeed toxic at the cellular, tissue, and organ levels. In all cells there are mechanisms for metal ion homeostasis that frequently involve a balance between uptake and efflux systems (Finney and O'Halloran, 2003; Rosen, 2002; Tottey *et al.*, 2005). Specific pathways for resistance to the heavy transition metal ions have been also determined in a wide variety of organisms (Rensing *et al.*, 1999; Silver, 1996). The heavy metal ions form strong coordination bonds with groups such as the thiolates of cysteine residues and the imidazole nitrogens of histidine residues. When they react with those residues in proteins, they frequently inhibit catalytic or biological activity. Because

these heavy metals are widespread in nature, there had been pressure early in evolution to develop resistance mechanisms. Although there are a number of possible ways in which cells can become resistant, the most frequent ones are those involving transport, either the loss of an uptake system or the development of an efflux system (Gatti *et al.*, 2000). Another widespread resistance mechanism consists of trapping the metal in specific cell compartments, called vacuoles or of expressing metallothioneins for metal chelation (Vasak, 2005).

The variety of environments, which the metal ions encounter in the life cycle is impressive. They interact indeed with several molecules, such as proteins in a variety of ways (Banci and Rosato, 2003) and small ligands such as glutathione (Carroll *et al.*, 2004; Cobine *et al.*, 2004). The characterization of the metal-protein complexes requires a structural analysis, which goes beyond the x-ray analysis and the associated processes of crystallization, which may not be innocent. NMR is a precious tool of investigation in this case and largely contributed in the recent years to detail the molecular mechanisms of copper transfer between proteins and between small ligands and

proteins. In particular, we will here describe NMR contributions to copper trafficking processes occurring in three cellular compartments, i.e., cytoplasm, periplasm, and intermembrane space of mitochondria (IMS), which largely handle copper cellular requirements.

COPPER(I) IN THE CYTOPLASM: FROM CHAPERONE TO ATPASE

At variance with other metal ions, the proteins involved in copper trafficking are about 50% of the entire copper proteome, i.e., about half of all cellular copper proteins (Andreini *et al.*, 2008), and they traffic copper as copper(I) ions. In eukaryotes and in a few bacterial systems such as cyanobacteria, copper(I) ions pass through the membranes of the cells using permeases or ATPases and enter the cytoplasm (Cavet *et al.*, 2003; Kim *et al.*, 2008). The metal is necessarily imported in the cells as copper(I) since the copper(II) ion would be reduced in the cytoplasm to copper(I) without the necessary control. Copper(I) is highly dangerous because of its capability to generate reactive oxygen species and to interact with protein ligands (Valko *et al.*, 2005); therefore it has to be bound to a protein as soon as it enters the cell to be kept under control. Such type of proteins are called copper-chaperones because they accompany the metal to the next target (Field *et al.*, 2002; O'Halloran and Culotta, 2000). In the cytoplasm of eukaryotes, one of the main copper pathway involves copper(I) delivery to the subcellular Golgi organelle where the production of extracellular or membrane copper enzymes occurs (Yuan *et al.*, 1995). This pathway involves a small copper(I) chaperone and a copper(I)-transporting P-type ATPase (Fig. 1). The latter protein has a changeable number of copper-binding domains

in the N-terminal region, involved to directly accept copper ions from the cytoplasmic chaperone (Hamza *et al.*, 1999), to play a role in the ATPase localization between Golgi and plasma membranes (Cater *et al.*, 2007) and/or presumably to assist in delivering copper to the transmembrane channel of the ATPase.

NMR studies were able to characterize at the molecular level protein-protein recognition as well as the copper transfer mechanism between the copper(I) chaperone and the copper-binding domains of the ATPase. In particular, it has been found that the transfer of the copper(I) ion from the chaperone to the copper-binding domains of the ATPase can occur through a metal-mediated protein-protein complex, i.e., through a complex where the acceptor and the donor proteins provide the copper coordinating ligands. The best studied case of a metal-mediated complex is that involving the yeast copper chaperone Atx1 and the first soluble domain of a copper(I)-ATPase, called Ccc2a (Arnesano *et al.*, 2001a; Banci *et al.*, 2006c). A number of x-ray structures of these copper chaperones were reported (Rosenzweig *et al.*, 1999; Wernimont *et al.*, 2000), including that of a homodimer for the human homolog of Atx1, i.e., Hah1. The latter forms in the presence of copper(I), as a crystallization artifact, the complex Cu(I)Hah1₂, resulting as not so informative except for the fold knowledge. NMR is the most suitable tool for characterizing the structural features of the various states of these proteins in a functionally relevant condition, i.e., apo protein with reduced Cys and copper adduct with the correct coordination. NMR provided the structures of Atx1 (Arnesano *et al.*, 2001b) and of Ccc2a (Banci *et al.*, 2001), with and without copper (Fig. 2). The copper protein ratio is 1:1. The fold of these proteins is ferredoxinlike, also known as $\beta\alpha\beta\beta\alpha\beta$, with a β sheet and two helices above the β sheet. Copper(I) ion is bound by two Cys residues of a CXXC metal-binding motif, conserved among eukaryotic and bacterial homologs (Arnesano *et al.*, 2002a). The first Cys is at the N-terminus of the first helix, and the second Cys is in loop 1. This arrangement seems suitable for metal binding. The two Cys are disordered in the apoAtx1 structure while they become ordered when coordinated to the metal ion (Fig. 2). A Lys, located in the loop spatially close to the metal-binding site (loop 5), in Atx1 moves toward copper when it is bound to neutralize the negative charge of the Cu(I)Cys₂ moiety (Arnesano *et al.*, 2001b). In Ccc2a, this residue is substituted by a highly conserved phenylalanine (Arnesano *et al.*, 2002a), which contributes to the structural stability of the metal-binding loop in both apo and Cu(I) forms of Ccc2a (Banci *et al.*, 2001). The two apo proteins do not interact with each other without a metal ion (Banci *et al.*, 2006c). On the other hand, the delivery of copper from the Atx1 donor to the Ccc2a acceptor has to occur through a complex, which has to be weak, otherwise it would be a final state and the process would not further proceed. Copper

Figure 1. Copper trafficking pathways in a eukaryotic cell. Copper uptake is mediated by cell surface copper transporters (Ctr). Copper then is eventually transferred to cytosolic SOD1 (via a pathway involving CCS), or to a copper P-type ATPase (involving Atx1 in yeast or Hah1 in humans) in order to be incorporated into the Golgi organelle where the metal ion is used for the production of extracellular or membrane copper enzymes. CCS and SOD1 are also present in the IMS. Some mitochondrial copper proteins involved in the assembly of copper sites of CcO, i.e., the ones discussed in the text, are also shown.

Figure 2. The mechanism for Cu(I) transfer between Atx1 and Ccc2a. Backbone atoms of the solution structures of Cu(I)-Atx1, apo-Atx1, Cu(I)-Ccc2a, and apo-Ccc2a are reported as a tube with variable radius, proportional to the backbone rms deviation value of each residue. The side chains of Cys ligands, copper(I) ions, and the secondary structure elements (α -helices in red and β -strands in cyano) are shown. The structure of the Atx1-Cu(I)-Ccc2a adduct is also shown.

bridges the two proteins, but the protein-protein interface has to be slightly repelling in nature. The equilibrium:

has a constant of 1.5 (Huffman and O'Halloran, 2000) and is fast on the NMR time scale ($> 10^3 \text{ s}^{-1}$). From the NMR titration we can estimate that the proteins are forming the intermediate complex for about 70%, while the remaining 30% are as isolated (Arnesano *et al.*, 2001a). By site directed mutagenesis we found that two intermediates differing in metal ligands occur (Fig. 2) (Banci *et al.*, 2006c). In this complex equilibria in solution, NMR was capable of providing a structural model of the adduct as reported in Fig. 2 (Banci *et al.*, 2006c).

The above mentioned cytoplasmic copper pathway occurs in several organisms, from humans to protozoa (Arnesano *et al.*, 2004), often maintaining metal-mediated protein-protein interactions. The human homologs of Ccc2 are Menkes and Wilson proteins. They have six soluble copper(I)-binding N-terminal domains (Lutsenko and Petris, 2003) (Fig. 1). The human chaperone Hah1 makes detectable metal-mediated complexes with some of these domains, i.e., domains 1 and 4 in the case of Menkes protein (Banci *et al.*, 2005a; Banci *et al.*, 2007a) and domains 1, 2, and 4 in the case of Wilson protein (Achila *et al.*, 2006; Banci *et al.*, 2008a; Larin *et al.*, 1999). However, the chaperone is able to load with copper(I) ions all domains (Banci *et al.*, 2005b; Banci *et al.*, 2006b; Banci *et al.*, 2007a; Wernimont *et al.*, 2004; Yatsunyk and Rosenzweig, 2007). From these find-

ings, two possible working mechanisms can be active: (a) some domains, once metallated by Hah1, are able to transfer the metal from one domain to the other, as has been observed by NMR for the domain 4 of Wilson protein, which was indeed able to donate copper to domains 5 and 6 (Achila *et al.*, 2006); (b) Hah1 delivers copper(I) ion to all domains. NMR therefore resulted to be the tool of excellence to monitor concomitant copper transfer processes when several copper sites are exchanging the metal ion, by observing the chemical shifts of the various hydrogen, nitrogen, and carbon atoms. Furthermore, NMR data, together with available *in vivo* studies (Cater *et al.*, 2007; Hamza *et al.*, 2003), were able to suggest that the localization of Menkes protein between the trans-Golgi network and the plasma membrane may be regulated by the accumulation of the metal-mediated adduct of Hah1 with one or both domains 1 and 4 of the Menkes protein, whereas the main role of domains 5 and 6 is to assist copper(I) translocation (Banci *et al.*, 2007a).

In the case of bacteria, a similar NMR approach allowed the detection of metal-mediated protein complexes between the bacterial Atx1 homolog and the soluble N-terminal domains of copper(I)-ATPases (Banci *et al.*, 2003; Banci *et al.*, 2006d). These interactions in bacterial proteins show specific differences at the molecular level with respect to the eukaryotic ones. For example, in cyanobacteria the metallochaperone binds copper(I) with an additional His residue (Banci *et al.*, 2004a; Borrelly *et al.*, 2004a), which has been suggested to play a role in determining the direction of copper transfer (Cavet *et al.*, 2003). In *B. subtilis*, it is found by NMR that copper is largely transferred in the metal-mediated

protein-protein complex from the chaperone to the soluble domain of the copper(I)-ATPase, suggesting the involvement of these proteins in a copper detoxification process (Banci *et al.*, 2003). This has been subsequently confirmed by *in vivo* data (Radford *et al.*, 2003).

In conclusion, metal-mediated protein-protein interactions have been conserved along evolution and can be exploited for different cellular functions, from copper incorporation into cellular organelle in eukaryotes and bacteria, to metal detoxification in bacteria and regulatory role in the protein trafficking of mammal ATPases. NMR turns out to be the preferential tool to characterize these kinds of weak metal-mediated interactions, in such a way as being able to elucidate at the molecular level the above mentioned functional processes.

COPPER(I) IN THE CYTOPLASM: FROM CHAPERONE TO SUPEROXIDE DISMUTASE

Another cytoplasmic copper pathway (Fig. 1) involves copper insertion into the superoxide dismutase 1 (SOD1), the antioxidant enzyme that guards against superoxide toxicity by catalyzing the disproportionation of superoxide anion to oxygen and hydrogen peroxide (Valentine *et al.*, 2005). SOD1 is a homodimeric protein containing in each subunit a copper and zinc ion (Banci *et al.*, 2002b). Even if it has long been known that the apo-form of SOD1 can be generated *in*

vitro and then reconstituted with the native copper and zinc ions or with other metals (Bertini *et al.*, 1998), most cells do not leave the insertion of copper to diffusion reactions and employ an accessory protein known as the copper chaperone for SOD1 (CCS) to facilitate this process. CCS docks with and transfers the metal ion to the disulfide-reduced apo SOD1 (Culotta *et al.*, 1997; Rae *et al.*, 2001). A mechanism shown in Fig. 3 has been proposed where CCS activates its target SOD1 by a very different mechanism than that used by the copper chaperone Atx1 (Furukawa *et al.*, 2004). It has been suggested indeed that CCS copper chaperone employs a more complex oxygen- and disulfide-dependent mechanism (Brown *et al.*, 2004; Furukawa *et al.*, 2004). This mechanism can be present not only in the cytoplasm but also in the intermembrane space of mitochondria (Fig. 1), being in the latter case responsible, not only for SOD1 activation, but also for the SOD1 accumulation into this compartment (Field *et al.*, 2003; Kawamata and Manfredi, 2008). This mechanism has not yet been verified at the molecular level, and NMR can be the more appropriate tool to define the multiple molecular events occurring during the SOD1 formation. Still, indeed, it is not clear how a copper ion is donated by Cu(I)-CCS to apo or Zn-SOD1 and how and when a Zn ion is incorporated in SOD1 during the activation process.

Figure 3. Proposed mechanism of copper insertion into SOD1 by its metallochaperone, CCS. The copper chaperone acquires copper through unknown routes and then docks with a disulfide reduced form of monomeric Zn-SOD1. This complex is inert to further reaction unless exposed to oxygen or superoxide, at which point a disulfide-linked heterodimeric intermediate forms. An analogous complex containing mutant SOD1 has been trapped and structurally characterized (Lamb *et al.*, 2001). In the case of the WT protein, this complex undergoes disulfide isomerization to an intramolecular disulfide in SOD1. Copper is transferred at some point after introduction of oxygen, and the mature monomer is proposed to be released from CCS.

COPPER (AND OTHER METALS) DETOXIFICATION

NMR largely contributes to the investigation of the molecular mechanisms of copper detoxification in some bacterial organisms. The more deeply characterized system involves the copper resistance mediated by periplasmic and outer membrane proteins.

The bacterium *Escherichia coli* carries a number of chromosomal operons, which mediate copper tolerance (Munson *et al.*, 2001; Petersen and Moller, 2000). However, resistance in significantly higher levels of environmental copper requires plasmid-based resistance genes such as the *E. coli* *pco* gene cluster featuring the seven genes *pcoABCDRE* (Brown *et al.*, 1995). On the other hand, copper resistance in *Pseudomonas syringae* pathovar tomato carries six plasmid-borne genes *copABCDRS*, which are homologous to the equivalent *pco* genes (Cha and Cooksey, 1991). CopA and CopC are soluble proteins present in the oxidizing environment of the periplasm, CopB and CopD appear to be membrane-bound copper pumps while CopS and CopR form part of copper sensing and gene induction systems (Cooksey, 1994). The CopC protein is proposed to mediate sequestration of copper in the periplasm but in contrast to known cytoplasmic copper chaperones, it contains no cysteine residues (Cha and Cooksey, 1991). Solution structures of apo (Arnesano *et al.*, 2002b), Cu(I) (Arnesano *et al.*, 2003b), and Cu(II) (Arnesano *et al.*, 2003a) states of the CopC have been solved using a synergic approach between the NMR and x-ray absorption spectroscopy techniques (Banci *et al.*, 2005d), being able to elucidate the coordination environments of the two different Cu(I) and Cu(II) binding sites and to show that CopC functions do not sequester copper, but instead may play a role in copper trafficking. Moreover, NMR data show that the copper ion migrates from copper(I) site to copper(II) site and vice versa on change in its redox state, and that the two metal-binding sites have very high specificity for Cu(II) and Cu(I), respectively, being their affinity constants strongly dependent on the copper oxidation state (Arnesano *et al.*, 2003b). The NMR data on CopC were therefore able to suggest a general mechanism of copper trafficking triggered by redox-dependent coordination properties of the metal (Fig. 4).

Recently, NMR has also been applied to investigate several other periplasmic soluble copper resistance proteins, such as CusF (Kittleson *et al.*, 2006; Loftin *et al.*, 2005), CopH (Sendra *et al.*, 2006) and CopK (Bersch *et al.*, 2008), belonging to copper-transporting efflux systems. NMR has been exploited to structurally and dynamically characterize some of these proteins as well as to investigate their metal-binding properties, in particular being able to define that Cu(I) and Ag(I) ions are the physiological substrates of CusF (Kittleson *et al.*, 2006).

NMR contribution in metal detoxification mechanisms also concerns the study of the cytoplasmic efflux system involving P-type ATPase pumps. Two distinct subgroups

Figure 4. Molecular redox switch of CopC. The protein can exchange copper between Cu(I) and Cu(II) sites activated by a redox switch. The copper(II) ion and the side chains of copper(II) ligands are shown in red, while the copper(I) ion and the side chains of copper(I) ligands are in blue.

of the P-type ATPase family can be distinguished based on physiological data: those that transport monovalent metal ions such as Cu(I) and Ag(I), and those that predominantly handle divalent metals such as Zn(II), Cd(II), and Pb(II) (Rensing *et al.*, 1999). Similar to the copper(I) P-type ATPases, zinc/cadmium bacterial ATPases have one or two metal-binding domains at the N-terminus bearing a conserved —(M,L)XCXXC— metal-binding motif (Arnesano *et al.*, 2002a). Again, similar to the copper(I) P-type ATPases (Fan and Rosen, 2002; Mandal and Argüello, 2003; Voskoboinik *et al.*, 1999), these domains have been shown to increase the overall catalytic rate of metal transfer (Bal *et al.*, 2001; Mitra and Sharma, 2001). Therefore, an essential topic for fully understanding the mechanism of metal detoxification concerns the factors determining metal specificity of P-type ATPases and, in particular, if the metal specificity resides in the N-terminal cytoplasmic domain of P-type ATPase pumps.

In order to address this topic, an NMR-based approach started from a detailed sequence alignment study of the N-terminal cytoplasmic domains of Zn(II), Cd(II), and Cu(I) P-type ATPases (Arnesano *et al.*, 2002a). The results suggested that the presence of negatively charged residues in the vicinity of the metal-binding site may modulate the affinity of the protein for divalent over monovalent metal ions. In particular, an aspartate residue, located in the —GMD-CXXC— metal-binding motif of a divalent cation transporting P-type ATPase from *Escherichia coli* (ZntA) may be implicated in this role. The NMR structural data on the N-terminal domain of ZntA showed indeed that this Asp residue, fully conserved in ZntA-like P-type ATPases (Fig. 5), facilitate the binding of a divalent cation such as Zn(II) (Banci *et al.*, 2002a). The NMR structural characterization of the N-terminal domain of the P-type Cd(II)-ATPase from *Listeria monocytogenes* (CadA) also indicates that a conserved negatively charged residue, a glutamic acid, is involved in Cd(II) binding (Fig. 5) (Banci *et al.*, 2006e). In contrast to the *E. coli* ZntA N-terminus, in Cd(II)-ATPase, this residue is in loop 5 (between $\alpha 2$ and $\beta 4$, Fig. 5),

Figure 5. Structural basis for metal-binding specificity in the N-terminal domain of heavy metal ATPases. (A) Backbone atoms of the solution structure of the N-terminal domains of Zn(II)-ZntA and apo-CadA as a tube with variable radius, proportional to the backbone rms deviation value of each residue. The side chains of metal ligands, zinc(II) ion and the secondary structure elements (α -helices in red and β -strands in cyano) are shown. (B) Structure-based sequence alignments of the N-termini of various P-type ATPases. The N-terminal domain of CadA from *L. monocytogenes* and that of ZntA from *E. coli* were aligned with other N-terminal domains. Secondary structure elements of CadA N-terminal domain and of ZntA N-terminal domain are also shown above the CadA-like sequences and above the ZntA-like sequences, respectively, together with their amino acid numbering. Residues that are known or predicted to bind a metal ion are highlighted in yellow. Positions where hydrophobic residues are conserved are highlighted in magenta.

i.e., far from the CXXC motif in the sequence, thus determining a different arrangement of the metal-binding residues for ZntA-like and CadA-like proteins. The different relative location of the carboxylate moiety with respect to the CXXC binding metal motif could play a role in finely tuning the specificity for either Zn(II) or Cd(II).

From the structural analysis of the cytoplasmic N-terminal domains it emerges that the same ferredoxinlike fold, comprising the secondary structure elements $\beta\alpha\beta\beta\alpha\beta$, is shared by of Cu(I)-, Zn(II)-, and Cd(II)-ATPases and that a single amino acid change can be a key feature, which drives

the metal specificity (Figs. 2 and 5). On the other hand, it is found that the P-type ATPases metal specificity relies on the membrane transport sites (Hou *et al.*, 2001). Alignments of the eighth helix of P-type ATPases show indeed that a Met conserved among the Cu(I)-ATPases is changed to an Asp in the Cd(II)- or Zn(II)-ATPases (Argüello, 2003; Dutta *et al.*, 2006; Liu *et al.*, 2006), suggesting that the membrane site selectivity may also be based on the presence of an acidic residue. Up to now, the data suggest a model where the metal ion specificity does not reside only in the membrane region but also in the N-terminal domain. According to this

view, from *in vivo* data obtained with the cyanobacterium *Synechocystis* PCC 6803, it has been found that a chimera of a Co(II)-ATPase, bearing the N-terminus of a Zn(II)-ATPase (ZiaA), confers resistance to Zn(II) (Borrelly *et al.*, 2004b). From a functional point of view, the same metal-binding specificity in both the soluble N-terminal site and the membrane site of the ATPase could have a key role in optimizing metal transport activity. The metal specificities in both metal sites of the ATPase pump can therefore play an essential role in order to detoxify more efficiently a cell from deleterious heavy metal ion with respect to another. In support of this model, it has been recently found that the presence of two metal-binding domains at the N-terminus of a cyanobacterial Zn-ATPase enhances the Zn(II) selectivity (Liu *et al.*, 2007).

COPPER(I) IN THE MITOCHONDRIA: CcO ASSEMBLY

Mitochondria need copper to allow cells to respire, exploiting the copper enzyme cytochrome *c* oxidase (CcO). Performing this cellular process, mitochondria are the greatest producers of peroxides as a consequence of side reactions occurring along the respiratory chain (Cadenas and Davies, 2000). To delimitate peroxide mitochondrial accumulation, which are deleterious for cell survival, cells use superoxide dismutases (SOD1 and the manganese-containing SOD2) located in the IMS and in the mitochondrial matrix, respectively. Since the copper incorporation mechanism into mitochondrial SOD1 works similarly to what was already described in the previous paragraph for cytosolic SOD1 (Field *et al.*, 2003), in this section we will detail assembly of copper sites of CcO. Cytochrome *c* oxidase is the terminal enzyme of the energy transducing respiratory chain in mitochondria of eukaryotes and many prokaryotes. The catalytic cofactors in CcO include copper and heme. CcO contains two heme *a* moieties. One of the two heme *a* groups has a six coordinate iron, thus lacking the ability to bind ligands and functioning in the electron transfer process (Tsukihara *et al.*, 1995). The second heme *a*, designated heme *a*₃, is a five coordinate heme with an open coordination site where oxygen binds (Tsukihara *et al.*, 1995). Heme *a*₃ interacts with a mononuclear copper site where copper is coordinated by three His residues, forming a heterobimetallic site in subunit 1 (Cox1). This metal center, designated heme *a*₃-Cu_B, is buried 13 Å below the membrane surface (Tsukihara *et al.*, 1996). In addition to the copper ion present in the Cu_B site, two additional copper ions exist in another CcO subunit (Cox2) forming a cysteine-bridged, binuclear, mixed valent center, designated Cu_A, which functions as the electron acceptor from reduced cytochrome *c*. The Cu_A site in Cox2 is located within a ten-stranded β-barrel and two cysteine residues within a Cxxx motif bridge the two Cu ions (Tsukihara *et al.*, 1996). The Cox2 domain protrudes into the IMS with the Cu_A site 8 Å above the membrane surface (Tsukihara *et al.*, 1996).

Copper insertion is required in the maturation of both Cox1 and Cox2, and a number of accessory proteins are known to be important in the copper metallation of CcO (Carr and Winge, 2003). These include Cox11, Cox17, Cox19, Cox23 proteins and Sco1/Sco2 protein homologs, all localized into the IMS (Fig. 1). In recent years, NMR largely contributed to characterize the proteins involved in copper CcO assembly.

NMR provided the first structure of a member of the Sco family showing that it is similar to some redox active proteins, including thioredoxins (Trx) and peroxiredoxins, with putative copper-binding Cys ligands located at the same positions as the conserved catalytic residues in Trx (Balatri *et al.*, 2003). Eukaryotic Sco proteins bind a copper(I) ion through the two above mentioned cysteine residues, located in a CPXXCP motif, which, in the absence of copper, can easily form intramolecular disulfide bonds in oxidative conditions and by a conserved His residue (Banci *et al.*, 2006a; Banci *et al.*, 2007d; Horng *et al.*, 2005) (Fig. 6). After these findings, several *in vivo* and *in vitro* investigations followed with the ultimate goal of specifically defining the functional role of this large protein family, which spreads indeed from bacteria to humans (Banci *et al.*, 2007b). In particular, the question was: are Sco proteins working like copper chaperones, thioredoxins, or both? From some studies (Horng *et al.*, 2004; Leary *et al.*, 2004), both Sco1 and Sco2 in mammals have been proposed to be part of the assembly of the Cu_A site working as cochaperones with a nonoverlapping but cooperative function and assisting the mitochondrial copper chaperone Cox17 in the metallation process. Redox disulfide processes have been recently found to be involved in the for-

Figure 6. Backbone atoms of the solution structure of human Cu(I)-Cox17, Cu(I)-Sco1, Cu(I)-Sco2, and *S. melliloti* apo-Cox11 as a tube with variable radius, proportional to the backbone rms deviation value of each residue. The side chains of two structural disulfides in Cox17, of metal ligands, the copper(I) ions, and the secondary structure elements (α -helices in red and β -strands in cyan) are shown.

mation of the Cu_A site. *In vitro* NMR data showed indeed that the metallation of oxidized human apoSco1 by Cu(I)Cox17 chaperone can simultaneously involve both reduction of the metal-binding Cys residues of Sco1 and metal transfer to Sco1 (Banci *et al.*, 2008b), as well as that, in the assembly of the Cu_A site of a bacterial organism, the Sco protein works like a thioredoxin, by reducing oxidized apoCox2 domain (Abriata *et al.*, 2008). The latter role is also substantiated by the crystal structure of a nickel derivative of oxidized human Sco1, where the metal is found to be bound to the conserved His residue and the two Cys residues of the CPXXCP motif in the oxidized state (Banci *et al.*, 2006a). The latter state can possibly represent a transient copper-delivery state existing just before the copper is transferred to the Cu_A site of the Cox2 subunit. In conclusion, NMR significantly contributed to the functional aspect of the Sco protein family revealing a general redox role of them in the Cu_A assembly mechanism, besides the copper chaperone role.

NMR also provided the first structures of human and yeast Cox17 (Abajian *et al.*, 2004; Arnesano *et al.*, 2005; Banci *et al.*, 2008c) (Fig. 6) as well as direct evidence how metal transfer processes from Cu(I)Cox17 toward both human Sco1 and Sco2 work at the molecular level (Banci *et al.*, 2007e; Banci *et al.*, 2008b), suggesting that the partially oxidized Cu(I)Cox17 is the functional metallochaperone species in the IMS. The solution structure of human Cox17 consists of a coiled coil-helix-coiled coil-helix domain stabilized by two disulfide bonds where one copper(I) ion is coordinated by the consecutive Cys residues, this representing the first example of a Cys-Cys binding motif in copper proteins (Fig. 6) (Banci *et al.*, 2008c).

The molecular mechanism of Cu_B site assembly is much less characterized than that of Cu_A site as Cu_B is inserted in the transmembrane Cox1 subunit and therefore difficultly available in the proper apo state as a stable sample in *in vitro* conditions. The accessory protein Cox11 was suggested

to play a role as cometallochaperone in the Cu_B site formation, as its absence appears to preclude Cu_B site formation without affecting binding of other cofactors (Hiser *et al.*, 2000; Tzagoloff *et al.*, 1990). NMR provided the only available structure of a bacterial homolog of Cox11, interestingly showing that Cox11 has an immunoglobulinlike fold with a novel type of β -strand organization where the copper binding motif is composed of two highly conserved cysteines located on one side of the β -barrel structure (Fig. 6) (Banci *et al.*, 2004b). At variance with Sco and Cox17 proteins, copper(I) binding determines the formation of a dimeric state with a binuclear copper(I) cluster at the protein-protein interface. Cox11 dimerization has been suggested to help in delivering copper(I) to the Cu_B site (Banci *et al.*, 2007c). Bioinformatic data indicate indeed that Cox11 dimeric state contains a membrane interaction site, which can help in copper delivery toward the transmembrane Cu_B site (Banci *et al.*, 2007c).

Finally, through an NMR-based approach, the first direct evidence for a periplasmic copper chaperone in bacteria was provided, allowing to functionally annotate a bacterial copper(I)-binding protein family, often located in the gene neighborhood of Sco proteins in some operons, as periplasmic copper chaperone of Cu_A site of bacterial oxidases (Abriata *et al.*, 2008; Banci *et al.*, 2005c). This bacterial copper(I)-binding protein (called here PCu_AC) can bind copper(I) through Met and His residues arranged in a highly conserved H(M)₁₀MX₂₁HXM motif, being specifically able to insert copper(I) ions into the active site of Cox2 of bacterial oxidases, giving rise to a native Cu_A site in its reduced form (Abriata *et al.*, 2008). This finding allows one to provide a model for the bacterial assembly of the Cu_A center, which involves also Sco protein (Fig. 7) (Abriata *et al.*, 2008).

Figure 7. Model for the assembly of the Cu_A center in *Thermus thermophilus* oxidase. PCu_AC protein acts as a metallochaperone by sequentially and specifically inserting copper(I) ions into the active site of subunit II of *Thermus thermophilus* oxidase, giving rise to a native Cu_A site in its reduced form. Instead, Sco1 is unable to deliver copper ions to this center, while it behaves as a thiol:disulfide reductase to maintain the adequate oxidation state of the Cys ligands of Cu_A site. Cys residues involved in redox disulfide chemistry are shown in green. Metal ligands are in magenta (PCu_AC) and in blue (Cu_A). The inset highlights the metal-binding site of Cu(I)PCu_AC chaperone.

CONCLUDING REMARKS

Biomolecules interacting with copper ions have to be characterized as such, when bound to the copper ion, and when they interact with other biomolecules or substrates. The characterization is both structural and dynamic. In this context, NMR is a preferred tool of investigation because it allows shedding light on what happens in solution. As metal ions are essential components of living organisms, it can be predicted that the investigation of their complexes with biomolecules will profit from more and more of NMR methodologies. Overall, the NMR approach has provided and will provide precious information on biochemical pathways, which are essential to understand the mechanisms of life at the molecular level.

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