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The green cupredoxin CopI is a multicopper protein able to oxidize Cu(I)

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Abstract: Anthropogenic activities in agriculture and health use the antimicrobial properties of copper. This has led to copper accumulation in the environment and contributed to the emergence of copper resistant microorganisms. Understanding bacterial copper homeostasis diversity is therefore highly relevant since it could provide valuable targets for novel antimicrobial treatments. The periplasmic CopI protein is a monodomain cupredoxin comprising several copper binding sites and is directly involved in copper resistance in bacteria. However, its structure and mechanism of action are yet to be determined. To study the different binding sites for cupric and cuprous ions and to understand their possible interactions, we have used mutants of the putative copper binding modules of CopI and spectroscopic methods to characterize their properties. We show that CopI is able to bind a cuprous ion in its central histidine/methionine-rich region and oxidize it thanks to its cupredoxin center. The resulting cupric ion can bind to a third site at the N-terminus of the protein. Nuclear magnetic resonance spectroscopy revealed that the central histidine/methionine-rich region exhibits a dynamic behavior and interacts with the cupredoxin binding region. CopI is therefore likely to participate in copper resistance by detoxifying the cuprous ions from the periplasm.

1. Introduction

Copper (Cu) is an essential micronutrient but is also a cytotoxic element [1-3]. Living organisms need to tightly control its homeostasis to prevent a deficiency that could perturb essential biological processes but also to avoid its toxicity due to an excessive accumulation. In particular, Gram-negative bacteria have evolved various Cu detoxification systems, some of them have yet to be characterized [4-7]. Such systems comprise [4-6, 8] (i) Cu sensors and regulators to adjust the response of bacteria to changes in Cu concentration in their environment or within the cells (ii) membrane Cu-exporters (the Cu-P_{1B}-type ATPase CopA [9] or the Cus efflux pump [10]) (iii) Cu binding proteins to sequester Cu or oxidize Cu(I) into the less toxic Cu(II) (multicopper oxidases). In addition, Cu resistance mechanisms

are important for the virulence of human pathogens [11, 12]. Bacterial Cu homeostasis has been well studied only in few species. In *Escherichia (E.) coli*, CopA extrudes Cu(I) from the cytoplasm to the periplasm and it is widely conserved among bacteria; the Cus efflux system and the periplasmic multi-copper oxidase CueO clear Cu(I) from the periplasm [4-6]. In *Salmonella enterica* serovar Typhimurium, the Cus system is absent and periplasmic Cu-binding proteins are important for Cu resistance [13, 14]. Most importantly, inactivation of CopA and some of these periplasmic proteins suppress virulence of the host invading pathogens in medically relevant cases [11, 13]. Yet, systems involved in Cu uptake and control in the periplasm are still poorly studied. A new type of protein involved in Cu resistance has started to be reported in the past two decades. These proteins described in different organisms (see next) are all homologs. In 2006, it was shown that the disruption of the *PA2807* gene in *Pseudomonas (P.) aeruginosa* led to a Cu sensitive phenotype [15]. The corresponding protein was not isolated nor studied. In 2007, a gene encoding an unusual methionine-rich (Met-rich) cupredoxin was found in the Cu-inducible *cin* operon in *P. putida* [16]. The deletion of *cinA* did not significantly influence the Cu sensitivity of the bacteria [16]. The corresponding protein was isolated and characterized by UV-visible spectroscopy and its redox potential measured [16], but there was no further study. Those results showed that it belongs to the cupredoxin protein family, well known for its characteristic Cu binding site and electron transfer role in biology [17, 18]. In 2012, in the pathogenic *Vibrio (V.) cholerae*, which is responsible for cholera outbreaks still occurring in developing countries, it was reported that the *cot* gene expression is activated by Cu and involved in Cu resistance under aerobic conditions [19]. In 2015, in the environmental *Rubrivivax (R.) gelatinosus*, the *copI* gene was reported to encode for a periplasmic protein CopI required for the Cu tolerance of the bacterium mainly under anaerobiosis or microaerobiosis [20]. As stated earlier, the proteins encoded by the *PA2807*, *cinA*, *cot* and *copI* genes are all homologs. Interestingly, in *R. gelatinosus* and *V. cholerae*, CopA is present and involved in Cu homeostasis, but the Cu-oxidase and the Cus system are absent, while they are

present in *P. aeruginosa* [21]. Thus the *R. gelatinosus* and *V. cholerae* Cu homeostasis systems are similar, and different from that of *E. coli* and *P. aeruginosa*. In *P. aeruginosa*, it is not yet clear which system is active under which conditions.

Recently, we characterized the CopI protein from *R. gelatinosus* [22] and showed that it was a green Cu type cupredoxin [17] able to bind up to three Cu(II) ions: one in the cupredoxin site, one likely at the N-terminus while the third site remained unidentified [22]. The amino acid sequence of CopI comprises the well conserved cupredoxin ligands (His70, Cys125, His130 and Met135), a His-rich and a His/Met-rich sequence at the N-terminus and in the central region of the protein (residues 83 to 95), respectively. The latter is highly conserved among CopI homologs and required for Cu resistance, while the former is not conserved and not required for Cu resistance [22]. Since the structure of the CopI protein is still unknown, we aimed at better characterizing the Cu binding sites of this protein for both the cuprous and cupric ions. To this aim, we introduced mutations in the three different regions (His-rich N-terminus, His/Met-rich sequence and cupredoxin site) and studied their effect on the Cu binding properties by different spectroscopic techniques. In addition, we purified the apoprotein and made specific addition of Cu(I) and/or Cu(II) to the protein to study the possibility of intramolecular electron transfer. The results show that CopI is able to oxidize Cu(I) into Cu(II) *in vitro*, unveiling the role it could play in the periplasm, conferring Cu resistance to the bacterium.

2. Material and methods

Molecular biology. WT and mutated *copI* genes (described later and labelled HMH and T1PC), including the signal peptide encoding sequence (MKNRILRPALLCVAALFATTAQA) [20], were produced by DNA synthesis services (GENEWIZ) and used for complementation assays in *R. gelatinosus*. To produce the soluble forms of these proteins missing the signal peptide sequence in *E. coli*, PCR DNA fragments were amplified using primers *copI*-NcoI (GAGCCGCCATGGAGAACCG) and *copI*-XhoI (ATCTAGATCTCGAGTCCATC) and cloned at the NcoI-XhoI site in pET28a plasmid which led to the deletion of the peptide signal and the first two amino-acids Asp and Ala in the three proteins CopI^{ADA}, CopI^{HMH} and CopI^{T1PC} (Fig. 1). To add the first two amino-acids back, we performed PCR amplification of the pET28a plasmid containing the *copI*^{ADA} gene using primers *copI*_{DA_fw} (AGGAGATATACCATGGACGCGAGCCATGACCA CGGCA) and *copI*_{DA_rev} (GGTATATCTCCTTCTTAAAGTTAA AC). *E. coli* BL21(DE3) strain was transformed with the corresponding plasmids and clones were selected on LB medium agar plates containing 30 µg/L of kanamycin

Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-ToF) analysis. 1 µL of each sample (about 50 picomoles) was spotted onto a target plate and 1 µL of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix was added. The global mass was measured on a MALDI-ToF Microflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) in linear positive mode (*m/z* range from 4000 to 20000).

External mass calibration was performed on the averaged [M + H]⁺ of the protein standard I (Bruker Daltonics). Measurement error was 200 ppm (manufacturer's specification)

N-terminal sequence determination. N-terminal sequence determination was performed by stepwise Edman degradation using an automatic sequencer model PPSQ 31B (Shimadzu, Kyoto, Japan)

Protein expression and purification. The native WT CopI protein was purified from *R. gelatinosus* as previously described.[22] *E. coli* BL21(DE3) strains harboring pET28a plasmid containing the different *copI* mutated genes were grown in minimum auto inducible medium C-750501[23] to obtain the apo proteins. We used the C-750501 medium with labelled ¹⁵NH₄Cl in order to obtain the uniformly ¹⁵N labelled protein for nuclear magnetic resonance (NMR) or electron paramagnetic resonance (EPR) experiments. An overnight pre-culture in LB medium with 60 µg/L of kanamycin was used to inoculate the appropriate medium. Cells were grown at 37 °C up to 0.6 absorbance at 600 nm, then the temperature was decreased to 20 °C. Cells were then harvested 48 h after inoculation by centrifugation at 5000g for 20 min, frozen in liquid nitrogen and stored at -80 °C until protein purification

Bacterial cell pellets were unfrozen, suspended in lysis buffer (Tris/HCl 50 mM pH 7.5, NaCl 500 mM, glycerol 10%, lysozyme 1 mg/mL) in the ratio 3 mL of buffer per gram of pellet, proteases inhibitors were added to the buffer. Cell lysis was performed using a cell disruptor (CellID), followed by a centrifugation at 15000g for 25 min at 4°C to pellet cell debris and unbroken cells. The supernatant (soluble fraction) was used to purify the CopI protein in two steps. First, an immobilized metal affinity chromatography was performed on a Nickel Sepharose High Performance (HP) affinity resin and eluted using 100 mM imidazole in Tris/HCl buffer 50 mM (pH 7.5) containing 500 mM NaCl and 10% glycerol. Then, size exclusion chromatography was performed on a 26 × 600 mm Superdex 200 resin with 50 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. Both chromatographic steps were performed using an Äkta Go FPLC (Cytiva). The relevant fractions were pooled and concentrated to around 1 mM on a Vivaspin 20 concentrator (Sartorius) with a 5 kDa cut-off. The protein aliquots were frozen in liquid nitrogen and stored at -80°C without addition of glycerol. Protein concentration was determined by UV-visible spectroscopy by using the theoretical extinction coefficient $\epsilon_{280\text{ nm}} = 6990\text{ cm}^{-1}\cdot\text{M}^{-1}$

Metalation of the apo-proteins. To metalate the purified apo-proteins with Cu(II), the desired equivalent of Cu(II) was added directly to the solution containing the protein from a CuSO₄ stock solution and incubated for 30 min at room temperature. No desalting step was performed

Optical absorption spectroscopy. Absorption ultraviolet (UV)-visible spectra were recorded on a Cary 60 spectrophotometer. The baseline was recorded on a buffer sample (sodium phosphate 50 mM, NaCl 0.15 M pH 7.5) and automatically subtracted from all spectra

EPR spectroscopy. EPR spectra were recorded on a Bruker Elexsys 500 spectrometer equipped with a nitrogen continuous flow Bruker variable temperature (BVT) apparatus (Bruker). 10% of glycerol was added to the samples before loading them in EPR tubes and freezing them in liquid nitrogen. Simulations were performed by using the Easyspin [24] toolbox and routines locally written

NMR spectroscopy. NMR spectra were recorded on a 600 MHz Bruker Advance III spectrometer equipped with a cryoprobe. Samples were prepared using the labelled ^{15}N or $^{15}\text{N}^{13}\text{C}$ apo protein in a 50 mM sodium phosphate buffer at pH 7.5 containing 150 mM NaCl and 10% (v/v) D_2O and traces (few μM) of sodium trimethylsilylpropanesulfonate used for referencing of the proton frequency, in 3 mm tubes. ^1H - ^{15}N HSQC spectra [25] were recorded using 8 scans, ^1H acquisition time of 243 ms and ^{15}N acquisition time of 35 ms. For the titrations, different equivalents of Cu(II) were added onto the apo proteins and the reduction of the Cu(II) ions was done by addition of 10 mM dithionite. The data were recorded at 300 K. The spectra were transformed in nmrPipe [26] and the cross-peak intensities measured by using NMRFAM-SPARKY [27]

Electron transfer experiments. The experiments were performed in a glovebox. Tetrakis(acetonitrile) copper(I) hexafluorophosphate was resuspended in ultrapure water with 2% acetonitrile (ACN). $[\text{Cu}(\text{I})(\text{ACN})_4]^+$ concentration of this stock solution was determined by measuring the absorbance at 485 nm of solutions with different dilution factors of the stock solution in a solution of bathocuproinedisulfonic acid (BCS) 30 mM in water. To prepare the samples, different equivalents of $[\text{Cu}(\text{I})(\text{ACN})_4]^+$ were added to the protein of interest

3. Results

3.1. Proteins expression and purification.

WT or mutated CopI proteins were purified either from *R. gelatinosus* (named hereafter native CopI protein) [22] or from heterologous expression in *E. coli*. The latter approach was performed to ease the production of mutated proteins, to obtain the desired proteins in large quantities and to perform ^{15}N and/or ^{13}C isotopic labeling in view of NMR and EPR studies. The mutated protein CopI^{ADA}, had the first two amino acids (Asp and Ala) deleted (Fig. 1). The other mutants were made from this construct. CopI^{HMH} had in addition the two His and the three Met residues of the central His/Met-rich region replaced by Ser residues to probe their role in copper binding. To try to mimic a blue T1 copper site [17], the CopI^{T1PC} protein was designed to have the same sequence as the plastocyanin (PetE) from the cyanobacterium *Synechocystis* 6803 around the ligand of the cupredoxin site. To probe the role of the first two amino acids (Asp and Ala) in CopI properties, they were added by site directed mutagenesis to produce the last mutant CopI^{MDA} (see further). The *E. coli* cultures were performed in minimum auto-inducible culture medium. [23] Typically, 1 L of culture yielded 15 g of

bacterial cells. All proteins were expressed to high levels in *E. coli* and a good yield of purification was obtained: we reproducibly obtained 1-1.5 μmol of protein per liter of culture. Only the CopI^{MDA} mutant was obtained in lower amount (about 0.7 to 0.8 μmol per liter of culture) compared to the other proteins. In a minimum culture medium [23], the protein was in the apo-form, in contrast with the native production of CopI in *R. gelatinosus* requiring a high concentration of Cu to induce the production of the protein which is then in the holo-form [22].

The purified proteins were analyzed by mass spectrometry (Fig. S1). The results show that the initiator methionine was deleted in *E. coli* in all cases except the one where the first two amino acids were introduced. Therefore, the protein with the exact native sequence could not be obtained by heterologous expression in *E. coli*. For clarity and to distinguish this protein from the native protein we will name it CopI^{MDA} hereafter. The presence of the initiator methionine was also checked by Edman sequencing of the purified protein. In our work, when dealing with the heterologously expressed proteins, we mainly used CopI^{ADA} rather than CopI^{MDA} as the model for CopI since the mutants were derived from this construct.

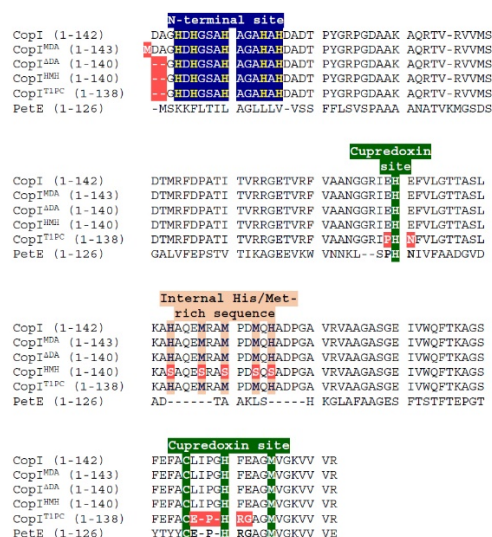


Figure 1. Sequence alignment of the native mature CopI protein from *R. gelatinosus* (CopI) and mutants studied in this work. The sequence of the plastocyanin PetE has been added to better visualize the mutations made for the T1PC construct. Except for the native CopI, isolated from *R. gelatinosus*, all proteins were obtained by heterologous expression in *E. coli*. Mutations are highlighted in red. The four residues ligand of the cupredoxin site are highlighted in dark green (His70 Cys125 His130 Met135 in *R. gelatinosus* CopI numbering). The His-rich N-terminus and the internal His/Met-rich regions are highlighted in dark blue and in light brown, respectively.

3.2. The structural properties of the recombinant proteins are similar to those of the native protein.

The global structural properties in solution of the native CopI and recombinant CopI^{ADA} and CopI^{MDA} purified proteins were compared using 1D ^1H NMR spectroscopy. The 1D ^1H NMR spectra (Fig. 2) obtained for both the oxidized and reduced states of the proteins were highly similar, indicating that there is no major

structural change between the native holoprotein and the recombinant apoprotein reconstituted with copper. Diffusion ordered NMR spectroscopy (DOSY) [28] on the native protein at 282 K led to the determination of the translational diffusion coefficient with a value of about $8.5 \cdot 10^{-11} \text{ m}^2 \cdot \text{s}^{-1}$, which corresponds to a hydrodynamic radius of $1.9 \cdot 10^{-9} \text{ m}$ according to the Stokes-Einstein relation. [29] These values are compatible with a monomeric globular protein of 15 kDa. In the reduced state additional resonances were observed, in particular at 10.2, 10.8 and 11.8 ppm at low field in the amide region. Their absence in the oxidized state is due their broadening beyond detection because of the paramagnetic relaxation enhancement effect (PRE) induced by the proximity between the proton giving rise to the resonance and the cupric ion. [30] The relaxation effect depends on the distance between the nuclei and the Cu(II), as well as the nature of its coordination. Of interest, the only Trp residue in the sequence would contribute to one of these low field shifted resonances that undergo PRE effect. This indicates that the Trp residue is in the vicinity of one of the Cu(II) sites in the folded protein. In some cases, at distance more than 4.6 Å, the PRE effect is not enough to broaden the NMR signals beyond detection [31]. This is the case here for about half of the resonances (Fig. S2). This indicates that the three Cu(II) binding sites are in close proximity.

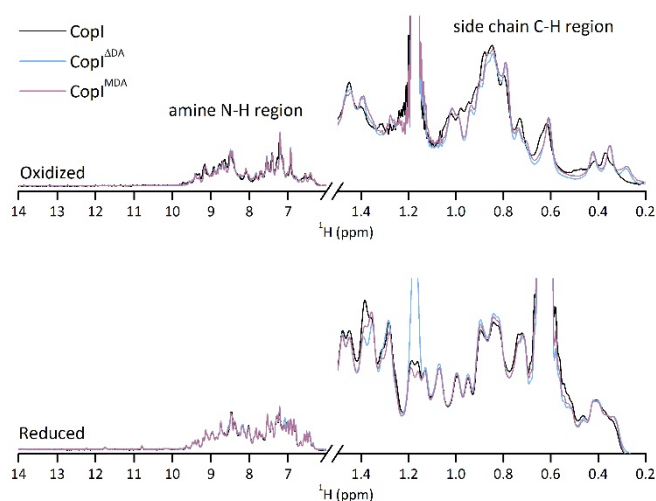


Figure 2. Comparison of the ^1H NMR spectra of the native (CopI) and the recombinant CopI^{ADA} and CopI^{MDA} proteins with 3 equivalents of Cu(II) ions added (Oxidized) and after supplementary addition of 10 mM dithionite (Reduced). Protein concentration 300 μM (oxidized) and 150 μM (reduced), KPI buffer 50 mM pH 7.5, 150 mM NaCl.

The ^{15}N - ^1H HSQC NMR spectrum of the CopI^{ADA} apoprotein is shown in Fig. 3 (blue spectrum). Several broad resonances can be seen overlapping as a massif in the 7.5-8.5 ppm region of the proton frequency, and the low field shifted resonances characteristic of the native folded protein were not observed. This is characteristic of a disordered or partially unfolded protein. By contrast, after addition of one equivalent of Cu(I) (black spectrum, Fig. 3) the massif disappeared and the resonances linewidth became narrower. The dispersion of the correlation peaks and the observation of the three low field shifted resonances mentioned

above indicated that the recombinant protein folds into its native structure upon binding of one Cu(I) ion. In *E. coli*, the apoprotein is produced in the cytoplasm, in contrast with *R. gelatinosus* where the protein is exported to the periplasm. The apoprotein purified from *E. coli* does not fold into its fully folded structure without the metal ion, but addition of only one copper per protein induced the folding.

Only 114 resonances were observed on the ^1H - ^{15}N HSQC spectrum of CopI^{ADA} in presence of one Cu(I), while the protein possesses 140 residues (among them six prolines and the terminal amine will not give rise to amide correlation). A region of the protein is thus not observed in the ^1H - ^{15}N HSQC, and indeed broad resonances are also observed when the signal threshold is decreased. This can be explained by the fact that not all the residues are detected by NMR due to dynamics of at least one region of the protein that broaden the NMR signal beyond detection.

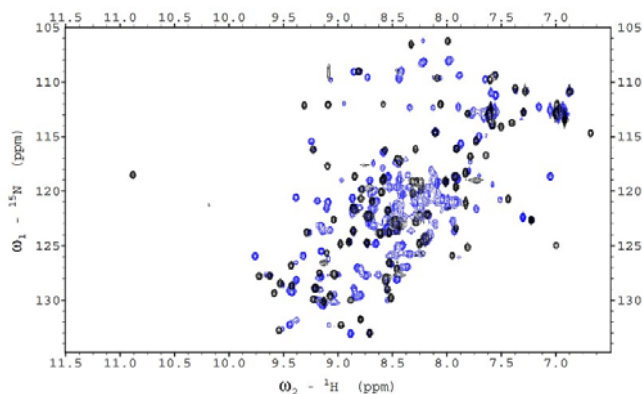


Figure 3. ^{15}N - ^1H HSQC of the recombinant apo protein CopI^{ADA} (650 μM) before (blue) and after (black) addition of 1 equivalent of Cu (I).

To gain further insights into the binding of Cu to the protein we performed several spectroscopic studies on the different mutants to characterize the copper sites.

3.3. The green colour of the protein is due to the cupredoxin site

The experimental and simulated EPR spectra of the WT native and mutant proteins loaded with 1 or 2 Cu(II) ions are shown in Figure 4. The simulation parameters are reported in Table 1. In all cases, the spectra were composed of two contributions: one from the cupredoxin site and the second from the N-terminal His-rich region square-planar site. [22] We will first focus on the former one. As expected, in all cases except for the CopI^{T1PC} mutant, the cupredoxin site had the same EPR properties as those of the native WT protein. The recombinant apo-proteins were titrated by addition of Cu(II). This was followed by EPR (Fig. 5) and UV-Visible (Fig. 6) spectroscopies. Of particular interest for the CopI^{MDA} protein, the EPR spectrum obtained by using a sub-stoichiometric quantity (up to 0.5 equivalent) of Cu(II) exhibited only the contribution from the cupredoxin site (see Fig. 5 and S3). In this case, the affinity of this site is therefore higher than that of the N-terminal site. By using the corresponding UV-Vis spectrum, it was therefore possible to determine the ϵ values for the different

transitions observed in the visible region and responsible for the colour of the protein (see Fig. S4). These values are 1365, 1916, 1779 and 1259 $\text{cm}^{-1}\cdot\text{M}^{-1}$ for the bands at 400, 450, 584 and 720 nm, respectively. By contrast, both sites filled up simultaneously with the addition of Cu(II) for the CopI^{ADA} and the CopI^{HMH} constructs, reflecting a similar affinity of both sites. The band at 400 nm is observed as a shoulder from the band at 450 nm. Finally, for the CopI^{T1PC} mutant, only the N-terminal site contributed to the EPR spectra (see Fig. 5 and S5) for sub-stoichiometric (0.25 equivalent) quantity of Cu(II). The cupredoxin site was therefore destabilized by the mutations around the cupredoxin ligands and its affinity for Cu(II) lowered in this case (see further). The UV-visible spectrum of the modified cupredoxin site in the CopI^{T1PC} mutant was also modified compared to the native one. In addition to the bands at 450, 584 and 720 nm, the band at 400 nm became better resolved compared to the other variant (Fig. 6). This reflects a distortion in the cupredoxin site geometry induced by the mutations around the Cu ligands and is responsible for the small change in colour observed for that mutant compared to the other proteins.

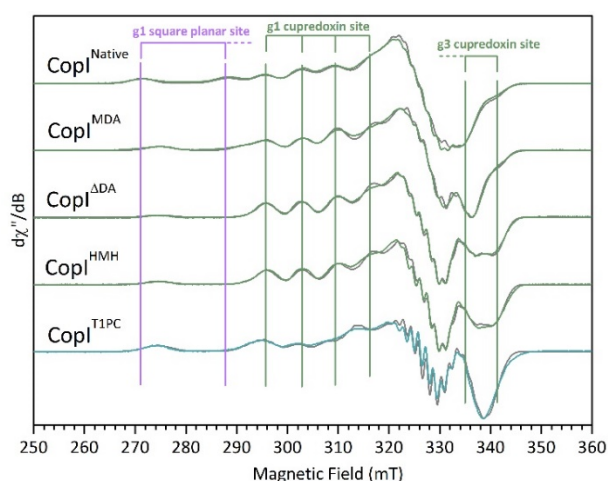


Figure 4. X-band cw EPR spectra (green and blue green lines) of the native protein CopI and of the proteins CopI^{MDA}, CopI^{ADA}, CopI^{HMH} and CopI^{T1PC} (300 μM) expressed in *E. coli*. Native CopI and CopI^{MDA} contained two equivalents of Cu(II) while CopI^{ADA}, CopI^{HMH} and CopI^{T1PC} contained only one equivalent of Cu(II) to ensure the absence of contribution from the third Cu(II) binding site. Simulated spectra are shown in grey superimposed on the experimental spectra. Simulation parameters are reported in Table 1. Experimental conditions: microwave frequency 9.43 GHz, microwave power 1 mW, modulation amplitude 0.5 mT, T 115 K. The vertical purple lines on the left mark the position of the first two hyperfine lines of the g_1 region of the N-terminal site of the native protein. The four green vertical lines in the center mark the position of the four hyperfine lines of the cupredoxin g_1 region, while the two green vertical lines on the right (around 340 mT) mark the position of the last two hyperfine lines of the cupredoxin g_3 region of the native protein.

3.4. The N-terminal amine binds Cu(II)

If we focus on the N-terminal Cu(II) binding site, for all the proteins produced in *E. coli*, the EPR parameters differed from those observed for the native protein (Fig. 4 and Table 1). In addition, CopI^{ADA}, CopI^{HMH} and CopI^{T1PC} exhibited the same parameters for

this site, while CopI^{MDA} had different ones. The coordination of this Cu(II) site is therefore very sensitive to the first residue of the protein and variation of only one amino acid induced differences of the spectroscopic properties. Thus the N-terminal amine is involved in the coordination of the Cu(II) ion in this binding site. The EPR parameters suggested a 3N1O binding mode for CopI^{MDA} as for the native protein, and a 4N coordination for CopI^{ADA}, CopI^{HMH} and CopI^{T1PC}.

In the case of the 3N1O binding mode, the binding of the terminal amine leaves thus two other N ligands that could be provided by side chains of histidine residues. For the native protein, the O ligand could be provided by a carbonyl from the backbone or the side chain of the initial Asp residue. If the carboxylate side chain of the Asp residue were involved, this would bring a negative charge into the coordination sphere which would affect the actual g -values (though they would of course still be compatible with 3N1O binding mode). As we noted previously, [22] the g -values are the same than those of the low-pH complex of the Cu(II)-amyloid beta complex which has a neutral coordination sphere $\{\text{NH}_{2\text{term}}, \text{C}=\text{O}_{\text{Asp}}, \text{N}_{\text{His}}, \text{N}_{\text{His}}\}$. [32] Therefore, the binding of the backbone carbonyl O is favored over the side chain of the Asp1 residue for the O ligand. It is also possible to have the carbonyl from the C=O from one of the two His ligands instead of the Asp1 as it leads to the same g -values but this happens in the amyloid beta complex only when the Asp1 is removed. [32] In the case of the MDA variant, the g_1 and A_1 values are different enough from the native protein and we cannot exclude that the modification removes the N-terminal amine as a ligand to provide a different equatorial coordination sphere but still neutral, for example $\{\text{N}_{\text{His}}, \text{C}=\text{O}_{\text{His}}, \text{N}_{\text{His}}, \text{N}_{\text{His}}\}$. In this case the variation in g -values may reflect the change of ligand from the terminal amine to a histidine.

The 4N coordination mode observed for the CopI^{ADA}, CopI^{HMH} and CopI^{T1PC} proteins was additionally supported by the superhyperfine structure in their EPR spectra in the g_2/g_3 region (around 325 mT, Fig. 4 and Fig S5). It is in agreement with a characteristic binding motif when a His residue is in second position of the sequence at the N-terminus.[33, 34] This also explains the increase in affinity of this N-terminal binding site compared to the CopI^{MDA} construct or the native protein.[22]

3.5. The internal His/Met-rich region binds primarily Cu(I)

As previously seen, the cupredoxin and the N-terminal were the first sites to bind to copper in a titration experiment, indicating that they both have high affinity for the cupric ion. When a third equivalent of Cu(II) was added, the evolution of the UV-visible and EPR spectra were similar to what had been observed for the native protein [22] for both the CopI^{MDA} and the CopI^{ADA} mutated proteins (Fig. 5 and 6). In UV-visible a band at 337 nm became well-resolved while the EPR spectra increased in intensity with a clear broadening of the cupredoxin site resonances. This broadening can be attributed to spin-spin interaction and is indicative of a third binding site in the vicinity of the cupredoxin center. When more Cu(II) was added on these two proteins, the EPR line shape changed drastically with a strong overall broadening. The total Cu(II) detected by EPR reached a plateau and the residual increase in EPR intensity can be attributed to the

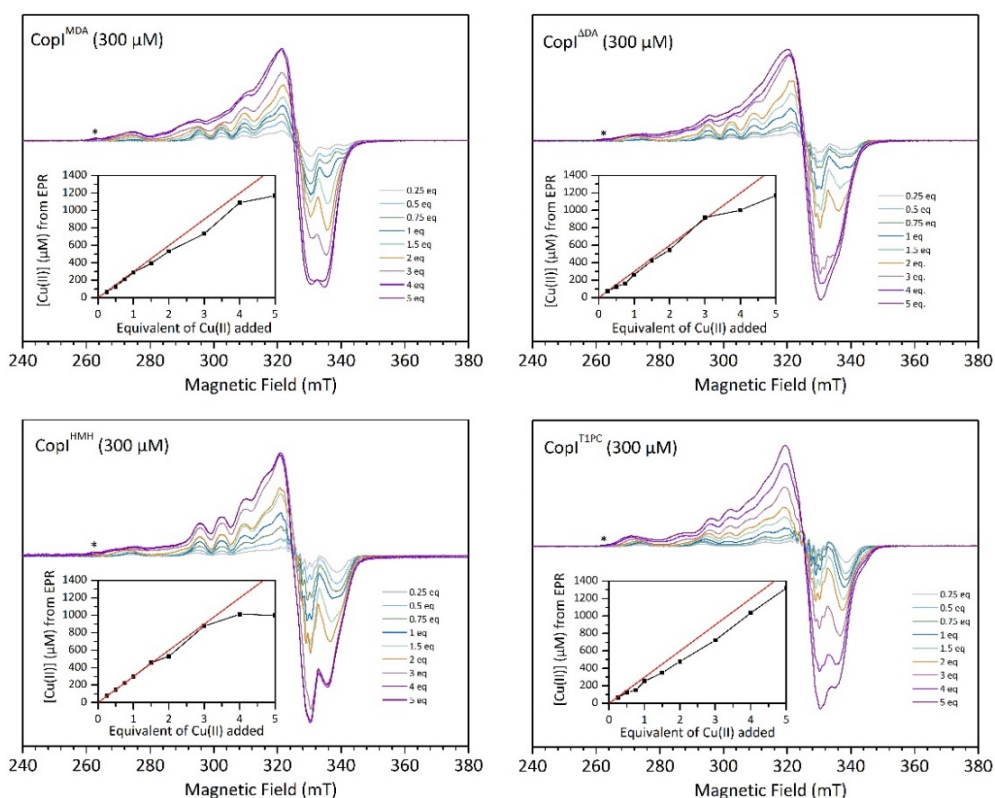


Figure 5. X-band cwEPR spectra of the CopI^{MDA}, RgCopI^{ΔDA}, RgCopI^{HMH} and RgCopI^{T1PC} apo-proteins (300 μM) titrated by addition of different Cu(II) equivalents. Experimental conditions: microwave frequency 9.43 GHz, microwave power 1mW, modulation amplitude 0.5 mT, T 115 K. Insets: Cu(II) concentrations observed experimentally and obtained by double integration of the EPR spectra, the red line represents the theoretical values that should be obtained if all the Cu(II) added were observed.

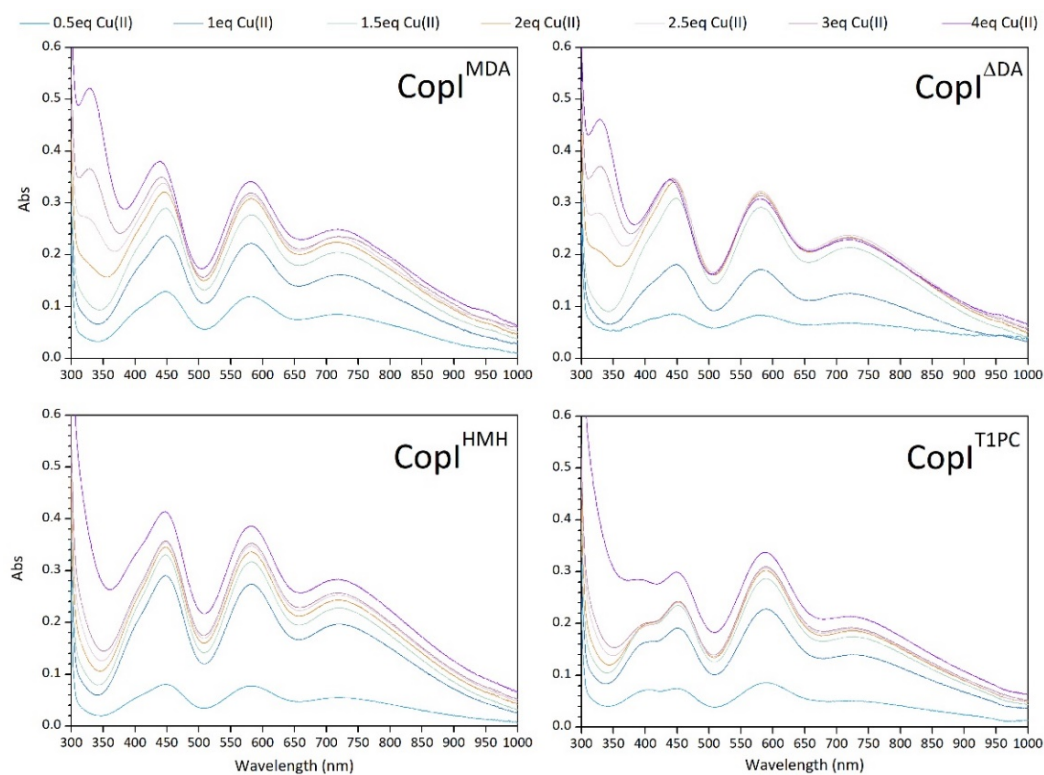


Figure 6. UV-visible spectra of the CopI^{MDA}, RgCopI^{ΔDA}, RgCopI^{HMH} and RgCopI^{T1PC} apo-proteins (150 μM) titrated by addition of different Cu(II) equivalents. Experimental conditions: KPi buffer pH 7.5, NaCl 150 mM, room temperature.

Table 1. EPR parameters for the first two Cu(II) sites in *RgCopl* WT and mutants

Protein	Cu(II) site	g_1	g_2	g_3	A_1 (MHz)	A_2 (MHz)	A_3 (MHz)	Ligands ^[a]
Native <i>Copl</i> / <i>Copl</i> ^{MDA} / <i>Copl</i> ^{ADA} / <i>Copl</i> ^{HMH}	T1.5	2.199	2.062	2.019	207	41	143	2N, 2S
<i>Copl</i> ^{T1PC}	T1.5	2.208	2.085	2.018	175	40	120	2N, 2S
Native <i>Copl</i>	T2	2.262	2.056	2.056	536	41	41	3N, 1O
<i>Copl</i> ^{MDA}	T2	2.242	2.049	2.049	528	45	45	3N, 1O
<i>Copl</i> ^{ADA} / <i>Copl</i> ^{HMH} / <i>Copl</i> ^{T1PC}	T2	2.217	2.051	2.051	592	45	45	4N

[a] First coordination sphere ligands. Isotropic nitrogen superhyperfine coupling was taken into account for the simulations: 2N $a_{iso}=22$ MHz for all T1.5; 3N $a_{iso}=38$ MHz for T2 native *Copl*; 3N $a_{iso}=38$ MHz for T2 *Copl*^{MDA}. 4N $a_{iso}=43$ MHz for all other T2 sites.

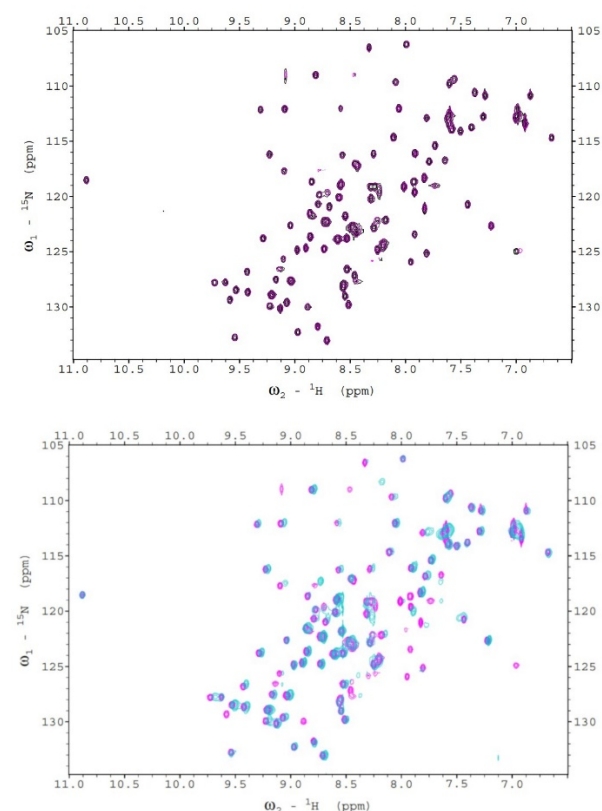


Figure 7. Comparison of the ¹⁵N-¹H HSQC NMR spectra of the recombinant *Copl*^{ADA} protein (650 μM) with 1 (black) and 2 (pink) equivalents of Cu(I) (top panel), and with 2 (pink) and 3 (cyan) equivalents of Cu(I) (bottom panel).

formation of a complex with the buffer as evidenced by the appearance of a new feature above 260 mT (indicated by an asterisk in Fig. 5, see also Fig. S6) before it precipitates in the buffer making it EPR silent. This is also supported by the UV-visible data which shows light scattering detected at four equivalents of Cu(II) added (Fig. 6). By contrast, the spectra evolved quite differently in the case of the *Copl*^{HMH} and *Copl*^{T1PC} mutants.

For *Copl*^{HMH}, the band at 337 nm in the UV-visible spectra was no longer visible in comparison with *Copl*^{MDA} and *Copl*^{ADA} (Fig. 6). The third binding site is therefore strongly affected in this mutant. This was confirmed by the EPR spectra where the broadening of the cupredoxin EPR signal was not observed anymore (Fig. 5)

indicating the binding of the third equivalent of Cu(II) is affected in *Copl*^{HMH}. The interaction of added Cu(II) with the buffer is already detected for 3 equivalents (Fig. 6). This is in favor of the removal of the third Cu(II) binding site in the *Copl*^{HMH} mutated protein, and coherent with the hypothesis that it is located in the central His/Met-rich region.

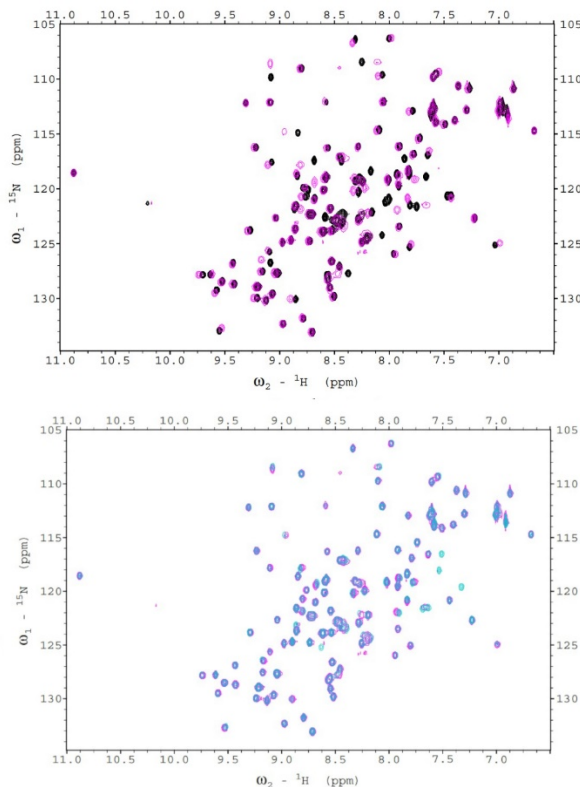


Figure 8. Comparison of the ¹⁵N-¹H HSQC NMR spectra of the recombinant *Copl*^{HMH} protein (400 μM) with 1 (black) and 2 (pink) equivalents of Cu(I) (top panel), and with 2 (pink) and 3 (cyan) equivalents of Cu(I) (bottom panel).

Surprisingly, the third Cu(II) binding site was also affected in the *Copl*^{T1PC} mutant. Similar to the *Copl*^{HMH} case, the band at 337 nm in the UV-visible spectra was no longer visible in comparison with the native *Copl* and *Copl*^{ADA}. In addition, the EPR spectra kept increasing in intensity with addition of Cu(II) without reaching a plateau and the interaction with the buffer was not obvious in this case. The changes in the EPR spectra were different compared to the other proteins. The amount of Cu(II) detected by EPR

remained always below the quantity added to the protein (Fig. 5, inset), indicating that the affinity of this mutant for Cu(II) was lower compared to the other proteins. This could be attributed to structural modifications induced by the mutations for this variant.

We also investigated the binding of the cuprous ion to the protein. For this, we used the CopI^{ADA} and CopI^{HMH} constructs in order to study more specifically the effect of the internal His/Met-rich region. The titration of these proteins by Cu(I) was followed by ¹⁵N-¹H HSQC NMR spectra (Fig. 7 and 8). Surprisingly, no changes in the NMR spectra was observed when a second equivalent of Cu(I) ion was added to the CopI^{ADA} mutated protein (Fig. 7). It was mentioned above that a region of the protein possesses resonances that are broadened beyond detection, and we hypothesized that the second Cu(I) binds in this NMR-invisible region. By contrast, differences are seen when a third equivalent of Cu(I) is added to the protein. The behavior was different with the CopI^{HMH} mutated protein. In this case, the addition of the second equivalent of Cu(I) induced significant shifts of the HSQC resonances while the third equivalent did not have any effect (Fig. 8). The comparison of both results suggested that Cu(I) binds to the His/Met-rich region that is NMR invisible. Altogether, these data indicated that once the cupredoxin site is filled, Cu(II) binds preferentially to the N-terminal site while Cu(I) binds to the His/Met-rich region.

3.6. Intra-protein electron transfer

Having insights into the binding of Cu(I) and Cu(II) to the different sites of the protein, it was interesting to investigate possible intramolecular electron transfer between these sites. This was performed by choosing adequate quantities of Cu(II) and Cu(I) to add. The additions were followed by EPR which shows the signatures of the Cu(II) sites. Cu(I) was added directly as [Cu(I)(ACN)₄]⁺ and no chemical reductants such as ascorbate or dithionite were used to avoid interference with Cu(II).

The CopI^{ADA} protein was loaded with one molar equivalent of Cu(II), in order to only occupy partially the cupredoxin and the N-terminal sites (Fig. 9) without Cu(II) binding to the His/Met-rich site. As equivalents of Cu(I) were then added, the intensity of the Cu(II) cupredoxin EPR signal decreased indicating a reduction of this site. Concomitantly, a new Cu(II) EPR signal appeared superimposed to the other signatures. It was best detected in the g₁ region with hyperfine lines located at fields slightly lower compared to the N-terminal site hyperfine lines. The position of this signal agreed with the titration of the protein by copper when a third equivalent of Cu(II) was added (Fig. 5) and can therefore be attributed to Cu(II) bound to the His/Met-rich site. We can therefore propose that Cu(I) binds to the His/Met-rich region and is oxidized into Cu(II) by the cupredoxin center which is therefore reduced into Cu(I). By contrast, the N-terminal Cu(II) seemed unaffected during the course of the experiment. In particular, the well-resolved superhyperfine pattern typical of this binding site [33, 34] in this protein remained unchanged. We can conclude that the cupredoxin site of CopI is able to oxidize Cu(I) into Cu(II). When the N-terminal site was already filled with a cupric ion, the oxidized Cu(II) formed remained in the His/Met-rich region as indicated by the EPR signature.

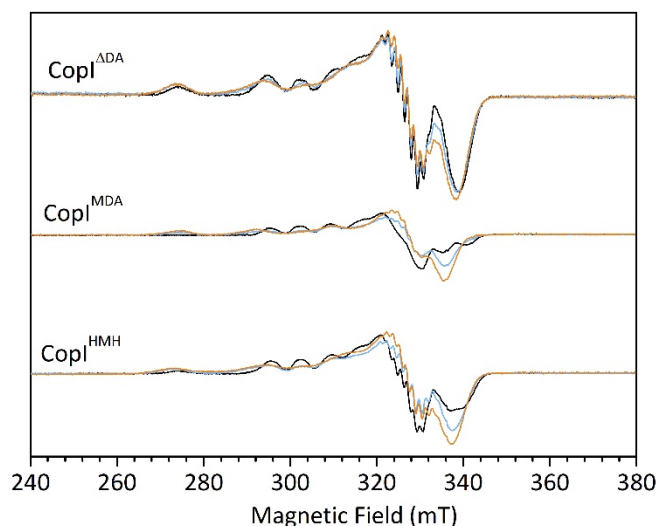


Figure 9. EPR spectra of CopI^{ADA}, CopI^{MDA} and CopI^{HMH} (300 μ M) loaded with 1 equivalent (CopI^{ADA}, CopI^{HMH}) or 0.5 equivalent (CopI^{MDA}) of Cu(II) (black lines) and with addition of different equivalents of Cu(I) (blue line: 1 eq; orange line: 2 eq). Experimental conditions: microwave frequency 9.4 GHz, microwave power 1 mW, modulation amplitude 0.5 mT, T 115 K.

We also investigated electron transfer when only the cupredoxin site was filled. As explained before, the CopI^{MDA} protein, loaded with half an equivalent of Cu(II), had only its cupredoxin site occupied. Addition of Cu(I) induced the decrease of the intensity of the EPR signal of the cupredoxin site indicating its reduction (Fig. 9). Another Cu(II) EPR signal appeared that had the same characteristics as the N-terminal site for this protein. In this case, it can be proposed that Cu(I) bound to the His/Met-rich region was oxidized by the cupredoxin center and the resulting Cu(II) ion moved to the N-terminal site. This is in agreement with the previous observations allowing to qualitatively determine the relative affinities for the different copper ions binding sites.

In order to check whether the His/Met-rich region, which is required for Cu resistance, [22] was required for the oxidation of Cu(I) as suggested above, we performed the same experiment with the CopI^{HMH} mutated protein (Fig. 9). We started with the protein loaded with one equivalent of Cu(II) so that only the cupredoxin and N-terminal sites contributed to the EPR spectrum. Upon addition of Cu(I), reduction of the cupredoxin center was observed. Furthermore, the evolution of the Cu(II) EPR signal was similar to what was observed with the titration of this protein by Cu(II) upon addition of two equivalents of Cu(II) (Fig. 5). Therefore, the His/Met-rich central region was not required for the oxidation of Cu(I) to Cu(II) by the cupredoxin center, at least *in vitro*.

4. Discussion

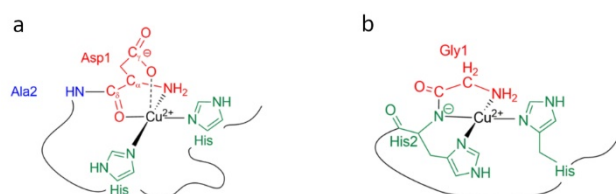
The CopI protein from *R. gelatinosus* is directly involved in copper resistance. This protein is able to bind up to three Cu(II) ions. From the sequence of the protein, three putative Cu binding sites can be identified: ligands characteristic of a cupredoxin center (His70, Cys125, His130 and Met135), a His-rich region at the N-terminus and a central His/Met-rich region. By targeting these

different regions with mutations, we were able to gain insight into the Cu coordination to the protein, to show that CopI is a multicopper protein that may be able to oxidize Cu(I), and to suggest an electron transfer route within the protein.

4.1. N-terminal binding site

Any mutation (even of a single amino acid) that affects the starting residue at the N-terminus modifies the EPR spectrum of the Cu(II) ion bound in this site. This implies that the N-terminal amine is involved in its coordination sphere. We noted previously [22] that the EPR parameters for this site are virtually identical to those measured for the low-pH form of the Cu(II)-amyloid β complex. [35] In addition, there is a good sequence similarity between the N-terminal regions (first sixteen residues) of CopI (DAGHDHGS AHAGAH AH) and the amyloid β peptide (DAEFRHDSGYEVHHQK). Five amino acids are strictly conserved (31% homology). The conserved amino acids include those providing the ligands to the Cu(II) ion for the amyloid β complex. Therefore, we propose that a likely structural model for the Cu(II) binding to the N-terminal site of CopI is the following (see Scheme 1a): the ion is coordinated by 3 nitrogen atoms (terminal amine and two histidine side chains) and one oxygen atom (carbonyl group of the first peptide bond). Regarding the histidine ligands, CopI has two additional His compared to the amyloid β and one in a different position so the two His ligands may not be those in position 6 and 14. These differences could also induce a different binding mode although it would have to be compatible with the g-values observed. An alternative binding mode could be that the carbonyl ligand is provided by one of the two histidine ligands instead of Asp1. This has been observed for the amyloid β peptide, although only for a truncated form at the N-terminus. [32]

For the variants where the first two amino acids are deleted (CopI^{ADA}, CopI^{HMH} and CopI^{T1PC}), a histidine is in the second position of the sequence leading to the possibility of a well-known and well-characterized motif in this case [33] (see Scheme 1b) with four nitrogen atoms as ligand in the equatorial plane (the terminal amine, the deprotonated amide of the first peptide bond and two histidine side-chains). When a methionine is added to the native sequence at the N-terminus (CopI^{MDA}), the positions of the aspartic acid and of the histidines in the sequence are shifted. This could lead to sufficient constraints to modify the coordination sphere and disengage the N-terminal amine that could be replaced by an additional histidine for example and reflect the modified EPR parameters but with still a neutral square planar coordination.



Scheme 1. Proposed models based on our data for the N-terminal binding site of Cu(II) in the native *Rg*CopI protein (a) and in the variants CopI^{ADA}, CopI^{HMH} and CopI^{T1PC} (b).

4.2. Cupredoxin and His/Met-rich region sites

The cupredoxin site is known to accommodate cuprous or cupric ions. [17, 18, 36] The titration of the different proteins by addition of Cu(II) and the results obtained on the purified native protein shows that this site fills up first and has therefore the highest affinity for Cu(II). Cu(II) binds next to the N-terminal site and then to the His/Met-rich central region. When titration is made with Cu(I), NMR points rather toward the binding to the cupredoxin site first, then the His/Met-rich region and eventually to a third site, possibly in the N-terminal region. The latter is expected from the number of His residues available that could bind Cu(I) in a linear geometry but we cannot exclude that the third Cu(I) binds elsewhere to the protein. These bindings of Cu(I) and Cu(II) to the different sites of CopI is consistent with the numerous results of coordination chemistry, the hard and soft acids and bases theory and what is known on copper proteins to date. Overall, CopI possesses a cupredoxin site accommodating both redox state, a Cu(II) binding site at the N-terminal region and a Cu(I) binding site in the His/Met-rich region. Both Cu(II) and Cu(I) binding sites are able to bind the other redox state but with lower affinity.

Our experimental evidences also point toward an interaction between the cupredoxin center and the His/Met-rich region. The broadening observed on the EPR spectrum of the cupredoxin center when Cu(II) binds to the His/Met-rich site indicates a dipolar coupling between the two centers and therefore a structural proximity. In addition, the UV band at 337 nm observed in relation to the His/Met-rich site is lost in the CopI^{HMH} mutant as expected but also when the cupredoxin site is modified in the CopI^{T1PC} mutant. This also underlines the interaction between both sites and that modifications on one of them affects the other one. In fact, the modifications generated by the mutations made for the CopI^{T1PC} protein seem to affect the overall structure of the protein leading to a somewhat destabilized form that has a lower affinity for Cu(II), including at the cupredoxin site. In addition to the consequence of the cupredoxin site mutations on the His/Met-rich site, it is possible that the N-terminal region is also affected with a more open conformation able to bind more than one Cu(II) ion which could explain the different evolution of the EPR spectra upon Cu titration in this case.

4.3. Electron transfer and role of CopI

The cupredoxin protein family is known for its role in electron transfer. [17, 18, 36] The results obtained on the CopI^{ADA} protein loaded with two equivalents of Cu(II) show that during Cu(I) additions, the cupredoxin is able to oxidize Cu(I) into Cu(II). Furthermore, when Cu(II) is only bound initially to the cupredoxin site, oxidation of Cu(I) to Cu(II) leads to the Cu(II) located in the N-terminal binding site while the Cu(II) in the cupredoxin is completely reduced. In the literature, it has been proposed that the His/Met-rich region of *E. coli* CueO enhances electron transfer. [37] The model we propose is that the His/Met-rich site would bind Cu(I) from the periplasm, this Cu(I) would then be oxidized by the cupredoxin and the N-terminal site would take up the newly formed Cu(II). Since this N-terminal site can only bind one ion, it is unlikely to be used as storage and the Cu(II) is potentially taken over by a partner protein or an export system. However, this His-

rich N-terminal region is not well conserved in CopI homologs and is not required for copper resistance so its necessity as an intermediate Cu(II) binder is questionable.

By contrast, the His/Met-rich region is required for Cu resistance of *R. gelatinosus*, thus a fair assumption is that CopI can oxidize Cu(I) only if it is bound to this site. However, the results obtained *in vitro* with the CopI^{HMH} mutant invalidate this hypothesis. Indeed, in the absence of the His/Met ligands, the cupredoxin is still able to play its role as an oxidant, *in vitro*. The requirement and role of the His/Met-rich region remains therefore to be confirmed. Some multicopper oxidases, such as *E. coli* CueO, also have a His/Met-rich region that is thought to play a role in Cu(I) storage and it can bind several Cu(I) ions, unlike CopI. [38] It is therefore unlikely that its role is similar in CopI. Another feature that we have identified here is the dynamical structural properties of the His/Met-rich region which residues are broadened in the NMR spectra. [39] Disordered regions are often involved in protein-protein interaction, and this dynamical region could play a role in the interaction with a partner protein.

For the Cu(I) oxidation mechanism to be catalytically viable, it is necessary that the cupredoxin site be reoxidized. This requires the participation of a redox partner that remains to be identified. Indeed, in Cu oxidases this is done by a tri-nuclear Cu center with dioxygen being the terminal electron acceptor [40]. CopI does not possess the equivalent of the tri-nuclear site and therefore requires a redox partner. Without this partner, the cupredoxin site will remain reduced and not be able to oxidize the next Cu(I) recovered from the periplasm. The proteins CopH and CopJ are encoded in the same operon as CopI and would be primary candidates to study as potential partners for CopI for this role or another. The hypothesis of the role of the His/Met region in a possible interaction with such a redox partner protein could explain the loss of copper resistance *in vivo* when one of these two sites is deleted. Also, as CopI is overexpressed under anaerobic and microaerobic conditions [20], oxygen seems unlikely to be the terminal electron acceptor in this system.

5. Conclusion

The periplasmic CopI protein is involved in bacterial copper resistance in several environmental and pathogenic bacteria, some of them being devoid of the classical multi-copper oxidase/Cus export systems. This is the case for our model of study, the CopI protein from the purple photosynthetic bacterium *R. gelatinosus* [20]. It is a green cupredoxin monodomain protein able to bind two additional Cu ions [22]. By using mutants of the different putative Cu binding modules identified in the primary sequence, we have shown that the His/Met-rich central area primarily binds Cu(I) while the His-rich N-terminus binds primarily Cu(II). The His/Met-rich region exhibits dynamic behavior and interacts with the cupredoxin site. In addition, CopI is able to oxidize Cu(I) into Cu(II) via its cupredoxin center. *In vitro*, the His/Met-rich region was not necessary for this oxidation to occur, leaving its exact role still open since it is required for Cu-resistance *in vivo*. [22] CopI could therefore act similarly to the multicopper oxidase system by detoxifying Cu(I). However, it lacks a moiety to reoxidize the cupredoxin to turn over and is

unlikely to use dioxygen as terminal electron acceptor. It could potentially interact with partner proteins that remain to be identified. Two other proteins CopJ and CopH are encoded by the same operon [20] and are potential candidates. More work is needed to identify the full chain of electron transfer in this detoxification system and to determine whether Cu(I) oxidation is the only role of CopI. The description of this new protein family will provide a significant step forward in bacterial Cu homeostasis and could provide new means to interfere with pathways involved in bacterial virulence.

CRedit author statement

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

All relevant data are presented within the paper and its supporting information. The files corresponding to the experimental data presented in the Figures are available on the repository entrepot.recherche.data.gouv.fr (doi: [10.57745/1UXNR8](https://doi.org/10.57745/1UXNR8)).

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Appendix A. Supplementary data

Experimental results: MALDI-ToF spectra, NMR HSQC spectra of CopI^{ADA} with different equivalents of Cu(I), cwEPR and UV-visible spectra of CopI cupredoxin site, cwEPR spectrum of CopI^{T1PC} N-

terminal Cu site, cwEPR spectra of CopIMDA titrated by Cu(II) and of Cu(II) in phosphate buffer.

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