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Dynamic Profile of the Copper Chaperone CopP from *Helicobacter Pylori* Depending on the Bound Metals

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Abstract Copper is an elemental ion in living organisms. CopP from *Helicobacter Pylori* (HpCopP) is a copper(I)-binding protein and was suggested as regulator of copper metabolism *in vivo*. Previously, the metal binding property of HpCopP for Ag(I), Cu(I), and Cu(II) as well as the tertiary structure of HpCopP was shown. In this study, the dynamic profiles of HpCopP depending on metal binding were studied using $\{^{1}H\}$-^{15}N steady-state NOE analysis. The heteroNOE experiment was performed for apo-CopP or metal-bound CopP. The obtained NOE values were analyzed and compared to figure out the effect of metals on the structural flexibility of HpCopP. As a result, Ag(I) and Cu(I) ions improved the rigidity of the structure while Cu(II) ion increased the flexibility of the structure, suggesting the oxidation of the CXXC motif decreases the structural stability of HpCopP.

Keywords HpCopP, Copper chaperone, CXXC motif, Oxidation, *Helicobacter Pylori*, NMR

Introduction

Copper is an elemental ion in living organisms and it is well regulated since it can produce hydroxyl radicals (OH•) through conversion between oxidized Cu(II) and reduced Cu(I).[1] DNA, proteins, phospholipids, and other macromolecules can be damaged by reactive hydroxyl radicals so that tight regulation of copper homeostasis is vital for living organism. [2-3]

Copper chaperones are important in the intracellular copper trafficking. The copper chaperones consist of around 70 amino acids with the motif, C-X-X-C, which binds to the copper (I) and is mainly located in the loop between β1 and α1.[3] HpCopP is a homologue of CopZ that is a copper chaperone found in *Enterococcus hirae*[4] and *Bacillus subtilis*.[5]

The regulation mechanism of copper in *E. hirae* is well studied.[1] In *E. hirae*, four proteins are involved in the regulation. CopA and CopB that are transport Cu(I) into inside/outside of cells.[6] CopY, a gene repressor regulates the expression of CopA and CopB.[7] CopZ acts as a copper(I) delivery machine from CopA to CopY.[8] In *H. pylori*, the level of copper was suggested to be maintained by two proteins CopA and CopP. The composition of the cop operon is quite different from that of the *E. hirae* cop operon.[9] CopA is a copper ATPase family and acts as a copper exporter.[10] CopP is a homologue of CopZ, consisting of 66 amino acids. Interestingly, *H. pylori* copAP operon does not contain the physiological equivalents of CopY, suggesting that copper trafficking and regulation is quite different in *H.*

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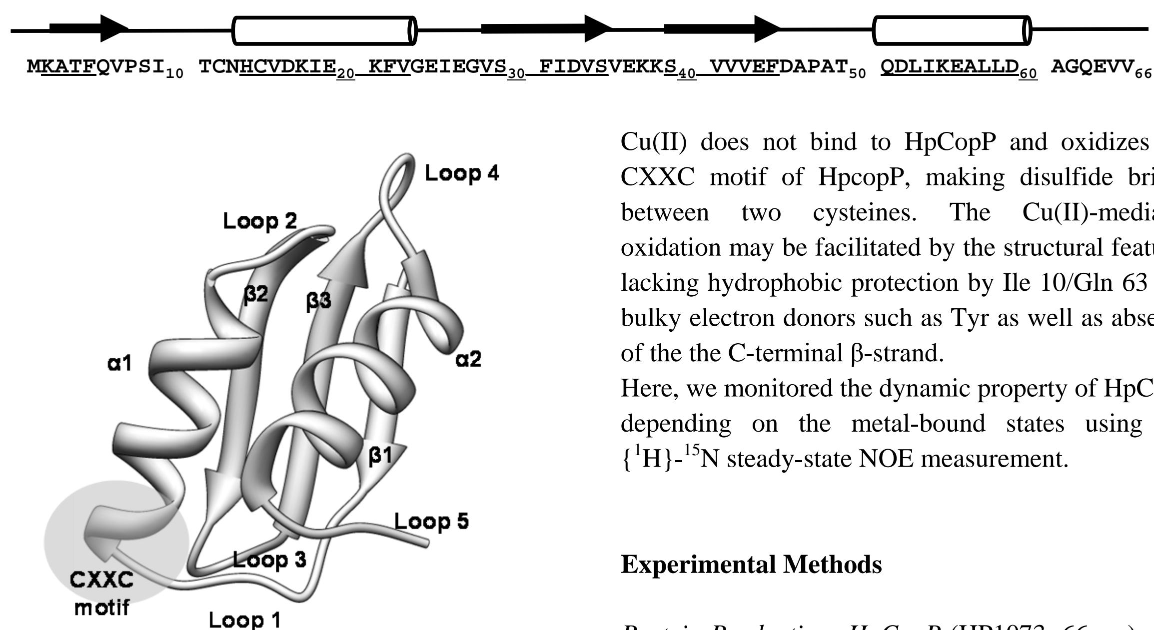

Figure 1. The structure of HpCopP published previously (PDB ID: 1YG0). The secondary structure of HpCopP consists of three β-strands and two α-helices (Top).

pylori.

For regulation of copper in cells, it is requisite to discriminate between Cu(I) and Cu(II) and selectively eliminate the toxic copper (I). The copper regulating proteins such as human Hah1, yeast Atx1, and bacterial CopZ bind to Cu(I) ion. Thus, copper proteins are Cu(I) binding proteins in general. However, the two redox forms of copper can indiscriminately bind to *E. hirae* CopZ.[11] CopB from *Archaeoglobus fulgidus,* a Cu(II)-ATPase, binds to copper(II) although its copper binding motif is not the conserved C-X-X-C.[12] CopC from *Psuedomonas syringae* can bind Cu(I) and Cu(II) at two different sites.[13] Therefore, the selective binding CopP to copper is one of our concern. For this purpose, we studied copper ion binding properties of HpCopP by NMR spectroscopy.

We previously published the tertiary structure of HpCopP using NMR.[14] It has the βαββα fold without the C-terminal β-strand that exists in the homologous CopZ (Figure 1). We proposed that the Cu(I)-binding property was well conserved in HpCopP. However, Cu(II) does not bind to HpCopP and oxidizes the CXXC motif of HpcopP, making disulfide bridge between two cysteines. The Cu(II)-mediated oxidation may be facilitated by the structural features lacking hydrophobic protection by Ile 10/Gln 63 and bulky electron donors such as Tyr as well as absence of the the C-terminal β-strand.

Here, we monitored the dynamic property of HpCopP, depending on the metal-bound states using the $\{^{1}H\}$-^{15}N steady-state NOE measurement.

Experimental Methods

Protein Production- HpCopP (HP1073, 66 a.a) gene was amplified from the genomic DNA of *H. pylori* (ATCC 700392), and was incorporated into the commercial pGEX-4T-1 vector. The precise procedure is described elsewhere[14]. The uniform ^{15}N-labeled HpCopP was expressed in *E. coli* strain DH5α.[15] Cells were grown at 37 °C in minimal medium containing ^{15}N ammonium sulfate. The lysate of bacteria was centrifuged and the supernatant was purified using a Glutathione-Sepharose 4B column. The elution of GST- HpCopP was conducted with the elution buffer (30 mM glutathione in 50 mM Tris-Cl, pH 8.0). The fusion protein, GST was removed by a thrombin cleavage. Then, the reaction mixture was further purified using a DEAE-Sepharose column.[16] The final sample was anaerobically stored in 50 mM Na Phosphate (pH 7.0), 500 mM NaCl, 1 mM EDTA, and 10 mM DTT at 4°C.

NMR measurement and analysis - NMR spectra were obtained using a Bruker AVANCE 600 MHz spectrometer at 303 K. The heteronuclear NOEs were calculated from spectra recorded with NOE experiment and without NOE experiment a proton presaturation period of three seconds.[17] Saturation of amide protons was achieved using 120° pulses prior

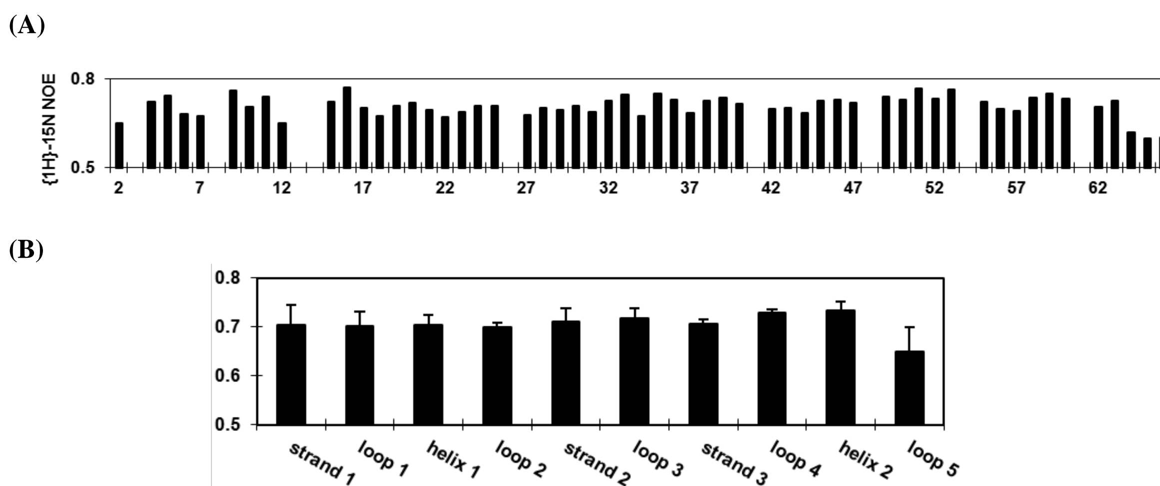

Figure 2. Hetero NOE values of apo-HpCopP. (A) Full NOE values are depicted along the amino acids sequence. X-axis is the residue number and Y-axis is the NOE value. (B) The average NOE value of each secondary structural elements is shown. Standard deviations are depicted.

to the measurement. 2048 data points and 256 increments were recorded in the direct and indirect dimensions, respectively. The assignment of residues was described elsewhere.[14] The protein samples were dissolved in 90% H_2O/10% $2H_2O$, containing 500 mM NaCl, 50 mM Na Phosphate, 10 mM DTT, 1 mM EDTA with pH 7.0. The concentration of the samples was ranged between 200 and 400 μM. Chemical shift calibrations for all nuclei were performed relative to the proton resonance of DSS. Spectra were processed with NMRPipe and analyzed with NMRView 5.[18]

To measure the dynamic differences between apo-HpCopP and metal-bound HpCopP, heteronuclear NOEs for each sample were obtained. The NOE value of each residue was grouped into the secondary structural elements in which they are involved. The grouped NOE values were averaged.

The binding of Cu(I) and Ag(I) to HpCopP was performed in 50 mM Na Phosphate buffer (pH 7.0) supplemented with 500 mM NaCl, 1 mM PMSF, and 5 mM DTT. Ag(I) was used as a Cu(I) mimetic metal since it is stable in air without oxidation. CuCl was dissolved in 0.1 M HCl/1M NaCl under anaerobic conditions and used as the stock solution.[19] The stock solution of Ag(I) was prepared by dissolving $AgNO_3$ in acetonitrile. For the Cu(II)-bound sample, HpCopP was dialyzed against 50 mM Na Phosphate (pH 7.0), 500 mM NaCl, and 1 mM PMSF and mixed with $CuCl_2$. The stock solution of Cu(II) was prepared by dissolving $CuCl_2$ in H_2O.

The minimized average structure of HpCopP (PDB ID: 1YG0) was used for visualization.

Results and Discussion

Flexibility of Apo-HpCopP- The heteronuclear NOE information can provide a qualitative measure of the mobility of the protein backbone on a sub -nanosecond time scale at local sites throughout amino acids sequence. The flexibility of apo-HpCop was estimated at first. Figure 2 shows the NOEs for the backbone amides throughout the whole sequence. The average value of all NOEs was about 0.7 for the apo-form, indicating the structure is rigid and ordered. For convenience of comparison, the average NOE value of each secondary structural element was also depicted. The C-terminal loop 5 (residues 62–66) appeared to be relatively flexible since the average value was 0.64.

Flexibility of metal-bound HpCopP- The NOEs of metal-bound HpCopP were also monitored and

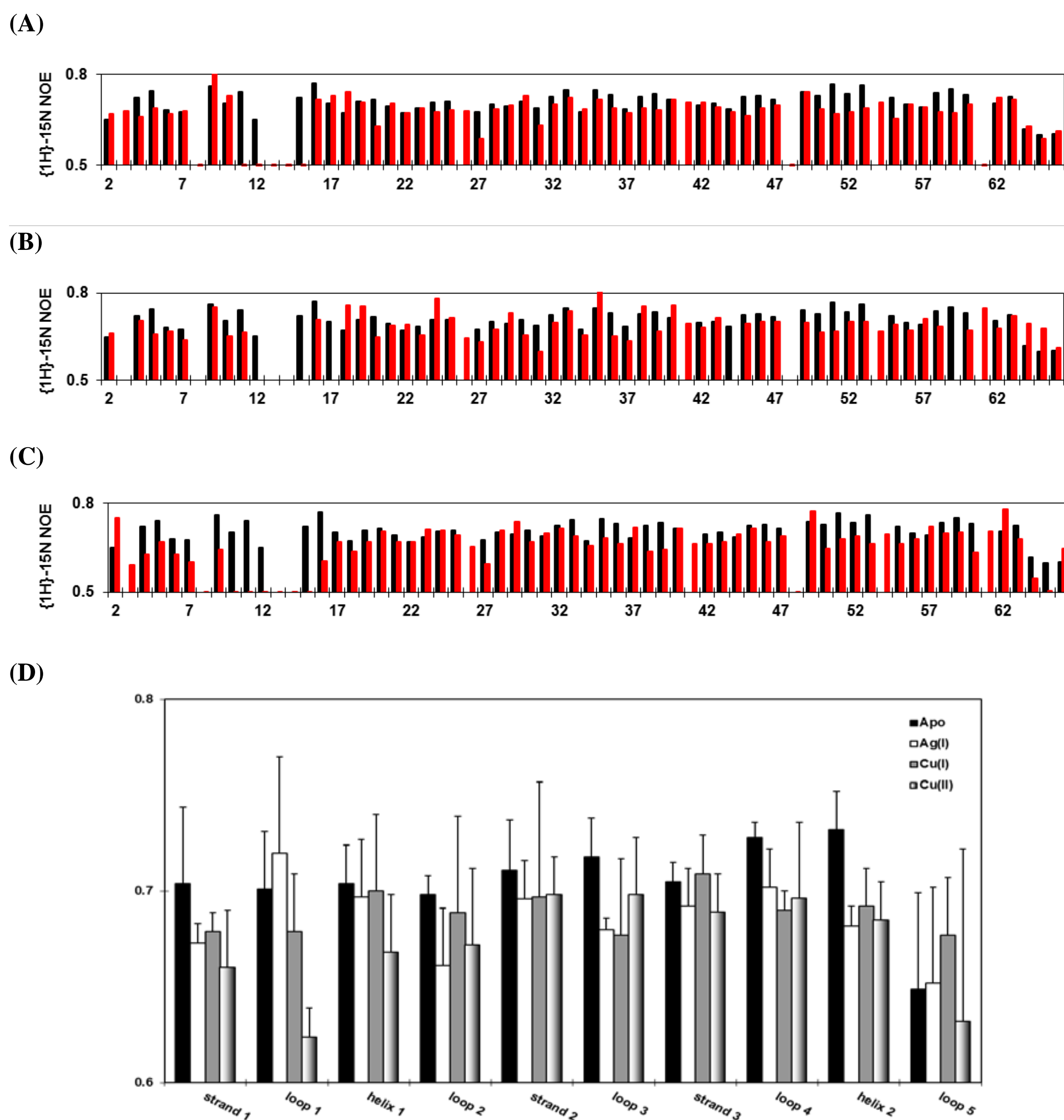

Figure 3. NOE values of metal-bound HpCopP. (A) NOEs of Ag(I)-HpCopP (Red) are compared to those of apo-HpCopP. Y-axis is the NOE value. (B) Cu(I)-HpCopP (Red) (C) Cu(II)-HpCopP (Red) (D) The average NOE value of each secondary structural elements is shown for each sample. Standard deviations are depicted.

summarized in the figure 3. Previously, the pattern of chemical changes upon binding to Ag(I) and Cu(I) was shown to be very similar, suggesting the significant difference between structures of Cu(I)-HpCopP and Ag(I)-HpCopP does not exist. Likewise, the significant difference in NOE values for Cu(I)-HpCopP and Ag(I)-HpCopP was not found, revealing that the mobile property of backbone amides is very similar. Notably, the average NOE of the loop 1 of Ag(I)-HpCopP was higher than that of apo-HpCopP or Cu(I)-HpCopP. This may imply that Ag(I) binding strengthens the structural rigidity around the CXXC motif.

However, the Cu(II)-bound HpCopP showed

significant improvement of backbone flexibility (Figure 3C and 3D). The increased flexibility in the loop 1 and the helix 1 including the CXXC motif was significant and the C-terminal loop 5 becomes more flexible as well. It was reported that oxidation of the CXXC motif structurally affects the loop I (residues 7–13), helix I (residues 16–25), loop III (residues 36–40), and the C-terminus (residues 60–66), resulting in the conformational changes of apo-HpCopP.[14] Current data also support that Cu(II) affects these regions and increase the mobile characteristic of the protein. In addition, the disruption of secondary structures is provoked by Cu(II). The absence of the C-terminal β-strand should contribute the increased flexibility of HpCopP by reducing structural compactness.

In conclusion, dynamic characteristic of backbone amide of HpCopP was successfully monitored. The backbone flexibility significantly depends on the metal-bound state of HpCopP and Cu(II) negatively affects the structural rigidity. The reduced stability of Cu(II)-bound HpCopP resembles the unfolding phenomena. The lack of the C-terminal β-strand probably helps the unfolding by oxidation.

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