Phosphorylation Impacts Cu(II) Binding by ATCUN Motifs

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ABSTRACT: ATCUN (amino terminal Cu(II) and Ni(II) binding) motifs chelate Cu(II) ions strongly. However, the impact of the phosphorylation of neighboring residues on such complexation has not been elucidated. The copper(II) dissociation constants of original and phosphorylated peptides from human histatin-1 and human serum albumin were compared using spectroscopic methods. Phosphorylation markedly weakened Cu(II) binding. Thus, these results indicate that phosphorylation may be a vital mechanism governing metal ion binding.

More than 100 human extracellular proteins and peptides contain an ATCUN motif, which has an amino acid sequence Xaa-Zaa-His, where Xaa is any amino acid residue with a free N-terminal amine, and Zaa is any amino acid residue except proline. Human histatin-1 and human serum albumin (HSA) contain this motif—Asp-Ser-His and Asp-Ala-His, respectively. Cu(II) complexes with these molecules are biologically relevant. Such peptides bind Cu(II) strongly, with subpicomolar to femtomolar dissociation constants. Phosphorylation of serine residues is one of the most frequent post-translational modifications (PTMs). It impacts a range of protein properties, e.g., molecular dynamics, interaction with other molecules or neighboring residues, or the propensity to change the cellular compartment. However, there is no information about the impact of such phosphorylation on Cu(II) binding by the ATCUN motif.

Two hexapeptides, N-termini from human histatin-1 (DSHEKR-am) and HSA (DAHKSE-am), were chosen to test the influence of serine phosphorylation on Cu(II) binding by the ATCUN motif. Spectrophotometric methods revealed the dissociation constants for the original and modified peptides.

First, spectroscopic pH-metric titrations of Cu(II)/peptide solutions in a 0.9:1.0 ratio with UV−vis and circular dichroism (CD) spectra registration were performed (Figures 1 and 2). The appearance of d−d bands characteristic of 4N square planar complexes was detected by both methods. The pKₐ values for the formation of 4N complexes were calculated from the pH dependence of the absorbance and CD signal (Figure 3), as reported earlier (see also the Supporting Information). Interestingly, phosphorylation caused an increase in such pKₐ values by 0.3 pH units for both peptides (4.13 ± 0.04 vs 4.37 ± 0.05 for DSHEKR-am and 4.53 ± 0.04 vs 4.82 ± 0.04 for DAHKSE-am), strongly suggesting that serine phosphorylation decreases the affinity of these peptides for Cu(II).

According to a recently published approach, the competition of the hexapeptides and GGH tripeptide for Cu(II) ions was observed by CD spectroscopy (Figure 4) to examine the influence of serine phosphorylation on Cu(II) binding by the ATCUN motif. Brieﬂy, CD spectra of 0.67 mM hexapeptide and 0.60 mM Cu(II) with variable concentrations of GGH at pH 7.4 (equilibrated for 24 h) were recorded. Each spectrum was decomposed to find the component concentrations: Cu-(hexapeptide) and Cu(GGH) complexes allowing me to compute the relative strength of the hexapeptide with GGH as a reference (Supporting Information).

In detail, the linear dependence is derived from the equation

\[
K_{\text{Cu(peptide)}} = \frac{[\text{Cu(peptide)}][\text{GGH}]}{[\text{Cu(GGH)}][\text{peptide}]}
\]

where [Cu(peptide)] and [Cu(GGH)] are measured by decomposition of experimental spectra, and [peptide] and [GGH] are the total concentrations of each ligand. The slope of this dependence is equal to the number saying how many times

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the formation constant ($K_f$) for Cu(hexapeptide) is higher than for Cu(GGH) (cf. Figure 5 and Supporting Information). The dissociation constant of Cu(GGH) at pH 7.4 was recently determined to be 609.5 fM ($\log K_d(Cu(GGH)) = 12.215 \pm 0.005$). Thus, one can multiply $K_d$ by the relevant slope and obtain the final $K_f$. Based on this approach, the dissociation constants are 2.2 fM ($\log K_f = 14.65 \pm 0.12$) and 38.6 fM ($\log K_f = 13.41 \pm 0.03$) for DSHEKR-am and DP SHEKR-am, respectively. Analogously, 26.9 fM ($\log K_f = 13.57 \pm 0.05$) and 60.8 fM ($\log K_f = 13.22 \pm 0.05$) dissociation constants were found for DAHKSE-am and DAHKpSE-am, respectively. Thus, phosphorylation of nearby serine residues markedly lowers the affinity of the ATCUN motif for Cu(II) ions. The effect is more pronounced for serine residues in the second position in the sequence compared with the fifth position. This seems reasonable, as serine in the second position is much closer to the coordination sphere. Interestingly, copper affinities of nonphosphorylated DAHKSE-am and DSHEKR-am differ substantially from each other. Nevertheless, the value for DAHKSE-am is close to that obtained recently for human serum albumin whole protein ($\log K_f = 13.02 \pm 0.05$). The affinity for DSHEKR-am, in turn, is equal to the one for DTHFPI-am, the N-terminal motif of hepcidin, claimed to be the strongest Cu(II) chelator among ATCUN motifs. Both peptides have an Asp residue in the first position and a Ser or Thr residue in the second. The acidic amino acid residue in the first position and the hydroxyl-containing one in the second position presumably ensure such tight copper binding.

Figure 2. CD spectra of 0.67 mM peptide and 0.60 mM CuCl$_2$ in the pH range of 3 (red) to 10 (violet). The appearance of $d-d$ bands at 490 and 570 nm with increasing pH is shown for A) DSHEKR-am, B) DpSHEKR-am, C) DAHKSE-am, and D) DAHKpSE-am.

Figure 3. pH dependence of absorbance and circular dichroism of 0.67 mM peptide and 0.60 mM CuCl$_2$ for A) DSHEKR-am, B) DpSHEKR-am, C) DAHKSE-am, and D) DAHKpSE-am. pK$_a$ values ($\pm$SD) for forming 4N square planar complexes calculated from the data are also shown for each peptide.

Figure 4. CD spectra of 0.67 mM peptide and 0.60 mM CuCl$_2$ at pH 7.4 (50 mM HEPES) titrated with GGH. The redshifts of the $d-d$ bands from the investigated Cu(hexapeptide) complex (violet) to Cu(GGH) (black, dotted) during the titration are shown for A) DSHEKR-am, B) DpSHEKR-am, C) DAHKSE-am, and D) DAHKpSE-am.

Figure 5. Determination of the relative Cu(II) binding strength of investigated peptides and GGH, based on the competition experiment shown in Figure 4, calculated for peptides from A) histatin-1 and C) HSA, for nonphosphorylated (blue, dots) and phosphorylated (red, circles) ones. The calculated slopes ($\pm$SD) reflect the relative Cu(II) binding affinities. Speciation diagrams were obtained by decomposing the CD spectra from Figure 4 for peptides from B) histatin-1 and D) HSA. Shown are the data for investigated peptides (squares) and GGH (diamonds), either for experiments with nonphosphorylated (blue, full symbols) or phosphorylated versions (red, open symbols).
Human serum albumin was found to be phosphorylated on Ser5 by Fam20C kinase. However, the extent of this phosphorylation is not known. Although only 2% of ATCUN in HSA is occupied by Cu(II) ions in blood plasma, such saturation is not known for other parts of the organism. Most of the motifs.

The frequency of Ser or Thr in the sequence position 1, 2, 4, or 5 cannot be excluded that may occur transiently or in pathological states. The in vivo nitrogens. Interestingly, the presence of two Asp residues in the ATCUN motif (Asp-Asp-His) is correlated with the highest basicity of the N-terminal amine and, consequently, with the lowest affinity to Cu(II). The basicity of amine in the free serine amino acid is also heightened by phosphorylation of its side chain (9.85 vs 9.25). Therefore, the results presented in this work suggest that phosphate moiety, similarly acidic as the Asp side chain, may weaken the binding of Cu(II) ions by stabilizing the protonated state of the amine and amide nitrogens.

Based on the recent analysis of N-terminal sequences in human proteins, the selection of human proteins with the ATCUN motif and serine or threonine residue within the first five positions was performed and presented in Table S1. The threonine residue was added to this analysis as its phosphorylation probably shows the same impact on Cu(II) binding as serine modification. There are 65 human proteins, either secreted or with N-terminus exposed on the extracellular side of the cell membrane, with ATCUN motifs containing at least one Ser or Thr (Table S1). Seven proteins with such sequences are localized in the endoplasmic reticulum or Golgi apparatus. It sums up to 72 proteins localized in relatively oxidizing compartments, thus, with a higher probability to meet divalent copper ions. Interestingly, more than 100 other proteins are localized in compartments with a more reducing state (Table S1), such as cytoplasm, nucleus, or mitochondrion. The binding of Cu(II) by those proteins is questionable (because intracellularly Cu(I) is more prevalent than Cu(II)) but cannot be excluded that may occur transiently or in pathological states. The frequency of Ser or Thr in the sequence position 1, 2, 4, or 5 throughout the whole selection (223 proteins, Table S1) is relatively uniform with a slight prevalence of serine residues (Figure S7).

The results presented in this work indicate that phosphorylation of serine residues markedly weakens the stability of Cu(II) complexes with the peptides comprising N-terminal sequences of human histatin-1 and human serum albumin. Thus, phosphorylation is perhaps a mechanism of fine-tuning the metal-related functions of these proteins. It may also be a general mechanism valid for other proteins or peptides with ATCUN motifs.

**ASSOCIATED CONTENT**

† Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.inorgchem.1c00939.

Experimental section; details related to decomposition of CD spectra and determination of Cu(II) binding affinity; and list of human proteins with ATCUN motif and serine or threonine residue within first five positions (PDF)

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T.F. conceived, designed, performed the experiments and the analysis, and wrote the article.

**Notes**
The author declares no competing financial interest.

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