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Interaction of Cu(I) with the Met-X₃-Met motif of alpha-synuclein: binding ligands, affinity and structural features†

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The identity of the Cu(I) binding ligands at Met-X₃-Met site of A α S and its role on the affinity and structural properties of the interaction were elucidated by NMR spectroscopy. We provide evidence that the source of ligands for Cu(I) binding to the Met-X₃-Met site comes from the N-terminal acetyl group and the Met-1, Asp-2 and Met-5 residues. From the study of site-directed mutants and synthetic peptide models of α S we demonstrated the critical role played by Met-1 and Met-5 residues on the binding affinity of the Cu(I) complex, acting as the main metal anchoring residues. While having a more modest impact in the affinity features of Cu(I) binding, as compared to the Met residues, the N-terminal acetyl group and Asp-2 are important in promoting local helical conformations, contributing to the stabilization of these structures by favoring Cu(I) binding.

Misfolding and aberrant self-assembly of proteins are considered key molecular events in several neurodegenerative disorders such as Creutzfeldt–Jakob’s disease, Alzheimer’s (AD) and Parkinson’s disease (PD).^{1,2} Although these structural transformations have been observed for a range of proteins, the mechanisms behind the self-assembly of proteins into fibrillar deposits remain often unknown.

Parkinson's disease, the second most prevalent neurodegenerative disease after AD,³ is characterized by the progressive

Significance to metallomics

Protein-metal interactions play an important role in α S aggregation and might link the pathological processes of protein aggregation, oxidative damage and neuronal cell toxicity. Advances in the bioinorganic chemistry of Parkinson's disease require that details of the binding specificity of Cu(I) to the protein α -synuclein and its conformational consequences to be better understood. In this work we have elucidated identity of the Cu(I) binding ligands at the Met-X3-Met Motif of A α S and its role into the affinity and structural properties of the interaction. Our findings might have both physiological and pathological implications.

degeneration of dopaminergic neurons in the substantia nigra pars compacta affecting motor and non-motor functions.⁴ A hallmark of PD is the intraneuronal aggregation of the protein α -synuclein (α S) into amyloid fibrillar formations.^{5,6} α -Synuclein is an intrinsically disordered protein (IDP) making up 1% of total brain-soluble proteins in humans and may play roles in uptake, storage, recycling of neurotransmitter vesicles and maintenance of dopamine.⁷⁻⁹ The protein comprises 140 amino acid residues (Fig. 1), which constitute the amphipathic region at the N-terminal region (residues 1–60), the hydrophobic non-amyloid- β component (NAC) region (residues 61–95), and the acidic region at the C-terminal region (96–140). In solution, the NAC region tends to be partially shielded from the solvent, induced by transient intramolecular interactions that delay intermolecular aggregation.^{10,11} However, in disorders such as PD or dementia α S may complex with Lewy bodies and adopt conformations that trigger toxicity and neuronal cell death, reflected by the presence of amyloid fibrillar aggregates.^{12,13}

Transition metal ion homeostasis (copper, iron, zinc) plays an important role in neurodegenerative disorders, because

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Site 1 Site 2 Site 3

MDVFMKGLSK AKEGVVAAAE KTKQGVAAEA GKTKEGVLYV GSKTKEGVVH GVATVAEKTQ EQVTNVGGAA

VTGVTAAVAK TVEGAGSIAA ATGVEKKDQI GKNEFGAPQF GILEMPVDP DNEAFMPSE EGYDYDFEFA

Fig. 1 Primary sequence of full-length α S. Met and His residues acting as main anchoring residues in Cu(I) binding sites are highlighted.

With regard to the coordination environment of Cu(I) in site 1 of the α S protein, EXAFS investigations revealed a 2S2O/N coordination sphere for the metal ion, thus indicating the presence of a four coordinated Cu(I) ion, probably with a tetrahedral geometry.³⁷ While the role of the two thioether groups of Met-1 and Met-5 as metal anchoring groups for Cu(I) binding to

We further characterized the binding interaction of Cu(I) with the P1AS variants of Fig. 2 by inspecting the chemical shifts changes induced by Cu(I) binding on the S-CH₃ methyl resonances belonging to Met residues. To detect the characteristic H ϵ proton resonances corresponding to the S-CH₃ groups of Met-1 and Met-5 we performed 1D ¹H NMR experiments (data not shown). Consistent with the results described above, the degree of perturbation induced by one equivalent of Cu(I) on the S-CH₃ resonances of Met-1 decreases in the order: Ac-P1AS ($\Delta\delta$ = 0.14 ppm) > P1AS ($\Delta\delta$ = 0.11 ppm) > D2A P1AS ($\Delta\delta$ = 0.08 ppm), giving further support to the role of N-terminal acetylation and Asp-2 as structural factors promoting Cu(I)

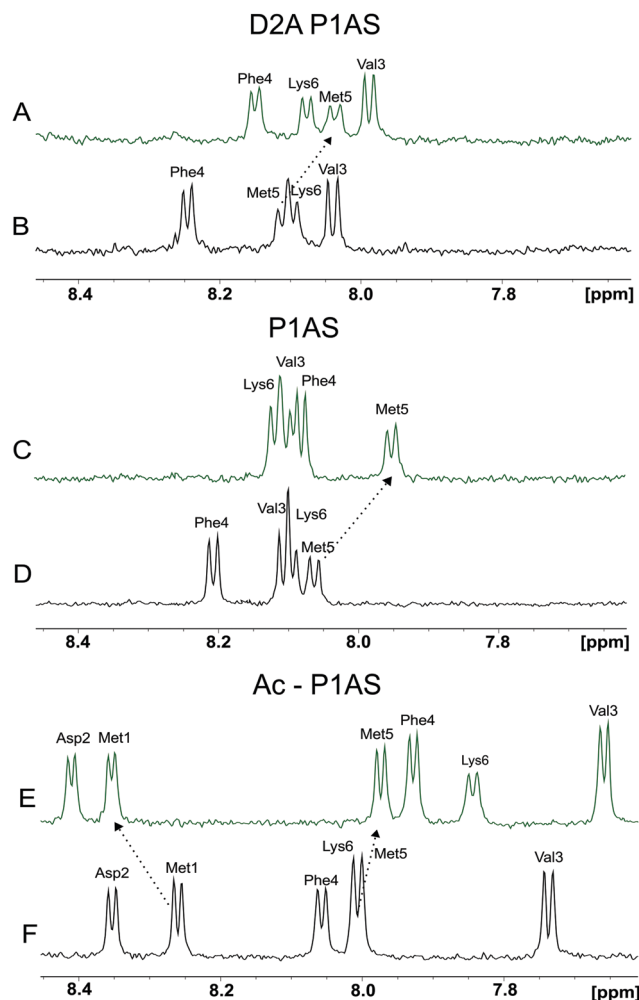


Fig. 2 NMR analysis of Cu(I) binding to α S synthetic model peptides. 1D ^1H NMR spectra (7.5–8.5 ppm) of D2A P1AS (A and B), P1AS (C and D) and Ac-P1AS (E and F) peptides in the absence (black) and presence (green) of 1 equiv. of Cu(I). In all cases, the addition of EDTA abolished the changes induced by the metal ion, confirming the reversibility of the interaction (data not shown). All experiments were recorded on 50 μM peptide samples dissolved in buffer A at 15 $^\circ\text{C}$.

coordination at site 1. Overall, these results confirm that the effects observed on the amide groups of the peptide variants in the presence of Cu(I) reflect the direct interaction of the metal ion with the sulfur atoms of the Met-1 and Met-5 residues, consistent with the binding preference of Cu(I) to coordinate sulfur atoms of Met residues in metalloproteins.

Next, to quantify the impact of these changes on the affinity features of Cu(I) binding to the Met-X₃-Met site, we determined the dissociation constants of Cu(I) complexes with the P1AS variants studied. The resonances corresponding to the S-CH₃ groups of Met-1 and Met-5 were well-resolved over the entire Cu(I) titration experiments and thus well-suited for calculation of the dissociation constant. Fig. S2 (ESI[†]) shows the binding curves of Cu(I) to Ac-P1AS and the P1AS and D2A P1AS variants. The derived conditional dissociation constants ($^cK_{\text{d1}}$) for the complexes of Cu(I) with Ac-P1AS, P1AS and D2A P1AS variants were 4.8 ± 0.7 nM, 8.5 ± 0.5 nM and 13.4 ± 1.0 nM,

respectively. From the estimation of the conditional affinity for the complexes of Cu(I) with the M5I and M1I P1AS peptide variants the values 65 ± 5 nM and 163 ± 10 nM were obtained, respectively.⁴⁹ These results allow us to conclude that: (i) the binding affinity for Cu(I) decrease in the order Ac-P1AS > P1AS > D2A P1AS > M5I P1AS > M1I P1AS; (ii) Met-1 and Met-5 residues act as the main anchoring moieties for Cu(I) binding to site 1, providing S-Cu binding modes; (iii) N-terminus acetyl group and Asp residue in position 2 sequence play a more modest role in terms of Cu(I) binding affinity, acting as potential sources for the establishment of Cu-O binding modes.

To confirm the findings derived from our analysis of Cu(I) binding to peptide models, we then analyzed the structural and affinity features of the Cu(I) complex with the Met-X-Met motif in the N-terminal region of the protein α S. To this purpose, we used ^{15}N isotopically enriched Ac α S and the M5I/H50A and D2A mutants. The D2A mutant of α S is not a substrate for the NatB acetylase and thus lacks that post-translational modification, being referred as D2A α S further on.

The Cu(I) complexed states of the Ac α S protein and its M5I/H50 Ac α S and D2A α S mutant species were analyzed by 2D NMR spectroscopy (Fig. 3 and 4). As reported previously, upon titration of ^{15}N -enriched Ac α S with increasing concentrations of Cu(I), the ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) spectra retained the excellent resolution of the uncomplexed protein but demonstrated large chemical shift changes in a discrete number of amide resonances belonging to residues involved in Cu(I) binding at site 1 (Fig. 3A). As shown in Fig. 4, the resonances corresponding to amide groups of residues involved in Cu(I) binding to site 1 of the D2A α S protein were affected to a lesser extent by the presence of the metal ion, whereas almost no changes were observed in that set of signals for the M5I/H50 Ac α S mutant. From these experiments, the conditional affinity for Cu(I) binding at site 1 in Ac α S was $^cK_{\text{d1}} = 3.9 \pm 1.0$ nM (Fig. 3B), consistent with previous studies.^{42,50} Interestingly, the value reported for the Cu(I)-complex in the non-acetylated protein was $^cK_{\text{d1}} = 7.8 \pm 1.0$ nM,⁴⁸ in line with the affinity differences found for Cu(I) binding to the N-acetylated and free amine P1AS peptides. The value of $^cK_{\text{d1}}$ for complexation of Cu(I) to the Met-X₃-Met site in M5I/H50A Ac α S and the D2A α S were 63 ± 5 nM and 14 ± 2 nM, respectively. Overall, these data demonstrate that the affinity features observed for Cu(I) binding in the synthetic peptide models are preserved in the proteins.

The structural implications of Cu(I) binding to these proteins were also evaluated in terms of the dynamic properties of their Cu(I) complexed forms. To this purpose, we measured ^{15}N R_1 and R_2 relaxation rates. This set of experiments was first measured on the free state of the Ac α S protein and the M5I/H50A Ac α S and D2A α S mutants (Fig. 5). In all cases, the relaxation parameters showed a similar sequence dependence, with lower values at the termini of the protein and a plateau at the center of the relaxation profile, showing R_1 values between 1.2 and 1.7 s^{-1} and R_2 values between 2.0 and 4.0 s^{-1} (Fig. 5). Complexation with Cu(I) resulted in a slight increase in the R_1 values for the 1–10 segment of the Ac α S sequence (mean R_1 values of 1.5 s^{-1} and 1.9 s^{-1} in the free and complexed protein,



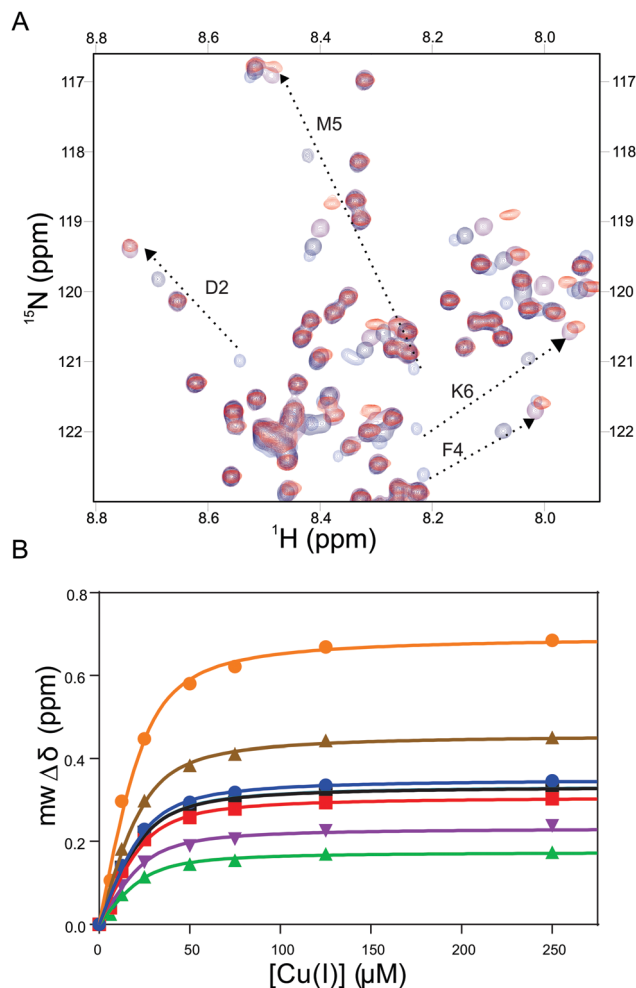


Fig. 3 NMR analysis of Cu(I) binding to $\text{Ac}\alpha\text{S}$. (A) Overlaid ^1H - ^{15}N HSQC spectra of $\text{Ac}\alpha\text{S}$ in the absence and presence of increasing Cu(I) concentrations. From blue to red: 0, 1.0, 3.0 and 5.0 equiv. of Cu(I) . Most-affected residues involved in Cu(I) binding to the Met- X_3 -Met site are labeled. (B) Binding curves of Cu(I) to the Met- X_3 -Met site of $\text{Ac}\alpha\text{S}$ as monitored by the average change in the $\text{mw}\Delta\delta$ for ^1H and ^{15}N of most affected amide resonances: Met-1 (●), Asp-2 (■), Val-3 (▲), Phe-4 (▼), Met-5 (●), Lys-6 (■) and Gly-7 (▲). Curves represent the fit to a model incorporating complexes of Cu(I) into three classes of independent, non-interactive binding sites, using the program DynaFit. Experiments were recorded at 15°C using ^{15}N isotopically enriched $\text{Ac}\alpha\text{S}$ ($50\ \mu\text{M}$) samples dissolved in buffer A.

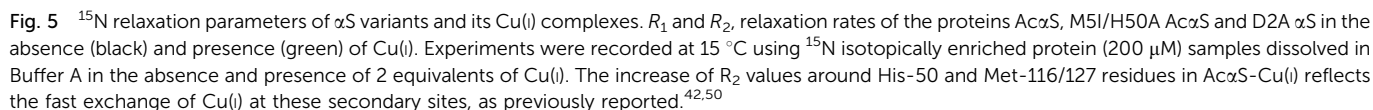
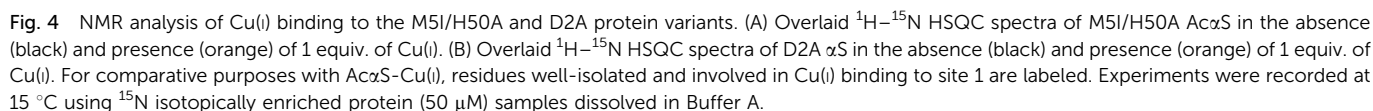
respectively); however, more pronounced deviations were found in the R_2 values (mean R_2 values of $2.2\ \text{s}^{-1}$ and $5.8\ \text{s}^{-1}$ for the free and complexed protein, respectively). These data indicate restricted local sampling in the pico- to nanosecond time scale for the 1–10 segment of $\text{Ac}\alpha\text{S}$ - Cu(I) relative to the free protein, reflecting the loss of flexibility due to the stabilization of local conformations with α -helical secondary structure, as previously published.^{42,50} On the other hand, the slight increase of R_2 values around His 50 and Met-116/127 residues in $\text{Ac}\alpha\text{S}$ - Cu(I) reflects the low affinity interaction and fast exchange of the metal ion at these secondary sites.^{42,50} Interestingly, the backbone dynamic profiles of the Cu(I) complexed states of M5I/H50 $\text{Ac}\alpha\text{S}$ and D2A αS measured under the same experimental conditions do not show changes in their R_1 values relative to

their metal-free states. As shown in Fig. 5, the differences observed between the R_2 profiles of $\text{Ac}\alpha\text{S}$ - Cu(I) , M5I/H50 $\text{Ac}\alpha\text{S}$ - Cu(I) and D2A αS - Cu(I) states are centered at the 1–10 segment. The R_2 values in the 1–10 segment of the D2A αS - Cu(I) are increased to a lower extent compared to the $\text{Ac}\alpha\text{S}$ - Cu(I) state, whereas only a slight deviation is observed for the R_2 values in the M5I/H50 $\text{Ac}\alpha\text{S}$ - Cu(I) form. These differences correlate well with the different affinities determined for the binding of the metal ion at these sites; however, the lower R_2 values for the D2A αS - Cu(I) form might be also reflecting a small increase of α -helical propensity near the N-terminus, induced by the interaction with Cu(I) at the Met- X_3 -Met site. These results motivated us to evaluate the Cu(I) protein complexes in terms of their conformational properties. $^3J_{\text{HN-H}\alpha}$ couplings are reliable quantitative reporters of the time-averaged distribution of the backbone torsion angles, ϕ , and are frequently used to probe the propensity of intrinsically disordered proteins to sample different regions of conformational space. Therefore, we measured residue-specific $^3J_{\text{HN-H}\alpha}$ couplings in both free and copper-bound states of these proteins (Fig. S3, ESI†). With the exception of the decrease in $^3J_{\text{HN-H}\alpha}$ for the first 10 residues of $\text{Ac}\alpha\text{S}$ upon Cu(I) binding, the values measured for the two forms of the M5I/H50A $\text{Ac}\alpha\text{S}$ and D2A αS proteins were essentially indistinguishable. With an averaged $^3J_{\text{HN-H}\alpha}$ of 4.5 Hz expected for an ideal α -helix and a $^3J_{\text{HN-H}\alpha}$ of ~ 7 Hz for random coil, our results indicate that a conformational transition toward increased α -helix structures is clearly observed only for the $\text{Ac}\alpha\text{S}$ - Cu(I) form.

These results allow us to conclude that the N-terminal acetyl group and the Met-1, Asp-2 and Met-5 residues provide the binding ligands for the coordination environment of Cu(I) at the Met- X_3 -Met site of $\text{Ac}\alpha\text{S}$. Met-1 and Met-5 residues are critical for the binding affinity of the Cu(I) complex, acting as the main anchoring residues for metal binding. While having a more modest impact in the affinity features of Cu(I) binding at this site, as compared to the Met residues, the N-terminal acetyl group and Asp-2 are important in promoting local helical conformations, contributing to the stabilization of these structures by favoring Cu(I) binding. Thus, the increased helicity in $\text{Ac}\alpha\text{S}$ - Cu(I) can be rationalized by stabilization of the helix macrodipole and formation of energetically more favorable hydrogen bond interactions triggered by the removal of the α -amino positive charge upon acetylation, by the role of Asp-2 diminishing the dipole moment to its N-terminus and by the Cu(I) -induced structural rearrangement of Met-1 and Met-5 side chains, respectively.^{51,52}

Our study demonstrates that perturbing the coordinating residues involved in Cu(I) binding at site 1 of αS has an effect also in the redox properties of the complex. Specifically, for the series of peptide and protein variants studied here, the trend in Cu(I) binding affinity follows: $\text{Ac-P1AS} > \text{P1AS} > \text{D2A-P1AS} > \text{M5I-P1AS} > \text{M1I-P1AS}$. Consistent with our results and with previous findings,⁴⁹ the Met residues play a key role in Cu(I) coordination and affinity features, having Met-1 a more important role in stabilizing Cu(I) binding. In terms of the redox properties of the site, the decrease in Cu(I) binding affinity observed for the M1I





translate into a ~ 17 mV increase in the reduction potential of the complex, if it were not to impact Cu(II) binding affinity. However, it is important to note that acetylation also has a drastic impact in Cu(II) coordination, since it abolishes metal binding at this site.⁴⁶ Thus, the combined effect of acetylation of the N-terminal group is expected to stabilize Cu(I) while significantly destabilizing Cu(II), contributing to a significant increase of ~ 258 mV of the reduction potential of the site, as compared to the non-acetylated form (Table S1, ESI[†]). Overall, these results underscore the important role that acetylation and the Asp-2 residue play together to stabilize the reduced form of the α S-Cu complex.

Another important impact of the acetylation of α S is the promotion of local helical conformation upon Cu(I) binding.^{42,50} It has been reported that chemical induction of alpha-helical conformation in α S by using fluorinated solvents increases the reduction potential of the α S-Cu complex by ~ 90 mV, possibly stabilizing the Cu(I) form.⁵⁴ From this shift, an increase in Cu(I) binding affinity of an order of magnitude was estimated for the alpha-helical α S form, as compared to the unstructured state. In line with this, a more recent work reported that Cu(I) binding to the 1–15 fragment of α S in the presence of SDS micelles resulted in a dissociation constant two orders of magnitude smaller than that found for the complex with the full protein in aqueous buffer.⁴⁷ On the other hand, our data does not show such a dramatic change in Cu(I) affinity upon induction of α -helical conformations by complexation of the metal ion to the N-acetylated form of the protein. Although this could be attributed to a shorter length and/or the more dynamic (transient) properties of helical structures induced by α S-Cu(I) complexation at the Met-X₃-Met site, as compared to that induced by the fluorinated solvent or SDS micelles, studies performed under more physiologically relevant conditions are clearly needed to evaluate the role of local conformations over the affinity and redox potential properties of α S-Cu(I) complexes.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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