Interaction of Cu(I) with the Met-X_3-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-X_3-Met site of AcxS and its role into 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-X_3-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 AcxS 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 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 (αS) 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 (resides 1–60), the hydrophobic non-amylloid-β component (NAC) region (resides 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 αS 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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The ability of copper to accept or donate electrons implicates it in the production of reactive oxygen species, an established pathway of PD. This mechanism is a highly selective, site-specific process that involves interactions of the protein with both oxidation states of the copper ion. Added to the abundant evidence revealing that S undergoes N-terminal acetylation in vivo (AcS), it was recently reported that this modification of S abolishes high-affinity Cu(I) binding. Since copper ions are predominantly found in their Cu(I) state in the physiological reducing environment of living cells, characterization of the Cu(I) complex at site 1 might have physiologically relevant implications in helical copper transport proteins, the formation of Ac at the N-terminus of Ac motif (site 1) at the N-terminus and/or the presence of an Asp residue at position 2 and Ac might act as a potential source for the establishment of a Cu-O bond. Based on these data, a similar role was proposed for Asp-2 in Cu(i) coordination to the protein S. In order to assess the role of N-terminal acetylation and Asp-2 on metal coordination to the Met-X-Met site, we investigated the details of Cu(I) binding to the synthetic 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. While the role of the two thioether groups of AcS-Cu(i) complexes at site 1 was demonstrated, the identity of the residues providing the oxygen/nitrogen ligands is less well known. From NMR structural calculations obtained for the complex between Ag(i) and the 1–15 peptide of β-synuclein (βS), an homologue protein of S that coordinates Cu(i) through Met residues at position 1, 5 and 10, it was proposed that Asp residue at position 2 of βS might act as a potential source for the establishment of a Cu-O bond. Based on these data, a similar role was proposed for Asp-2 in Cu(i) coordination to the protein S. Regarding the additional oxygen/nitrogen atom bound to Cu(i) in site 1, it was attributed to a water or acetonitrile molecule.

In this work we sought to delineate the coordination environment and binding specificity of Cu(i) to the Met-X-Met motif of the protein S. The identity of the Cu(i) binding ligands and its role into the affinity and structural properties of the interaction was investigated by the combined application of NMR spectroscopy and the design of site-directed mutants and synthetic peptide models of the protein. Previously, we demonstrated the role of Met-1 and Met-5 as anchoring residues for the binding of Cu(i) to the protein S. In order to assess the role of N-terminal acetylation and Asp-2 on metal coordination to the Met-X-Met site, we investigated the details of Cu(i) binding to the synthetic peptide Ac-DMVFVK (PIAS) and its non-acetylated variants PIAS and D2A PIAS, and compared their Cu(i)-binding features. The backbone amide regions in the 1D 1H NMR spectra of the different peptide species in the absence and presence of Cu(i) are shown in Fig. 2A–F. This spectral region contains well-resolved resonances and thus constitutes an excellent probe to analyze the metal–peptide interaction process. Whereas the amide resonances of Met-1 and Asp-2 cannot be detected in the experiments performed with the non N-acetylated peptide forms because of solvent-exchange effects, the signals from these residues become detectable upon acetylation of the N-terminus (Fig. 2E, F and Fig. S1, ESI†).

As reflected by Fig. 2E and F resonances of amide protons corresponding to Met-1 in the Ac-PIAS peptide were clearly most affected by the interaction with the metal ion than those corresponding to Met-5, indicating that the interaction of the metal ion with Met-1 is favored. Notably, the effects of Cu(i) binding on the amide resonances of Met-5 were more pronounced in the variants PIAS (Fig. 2C and D) and D2A (Fig. 2A and B) compared to the Ac-PIAS peptide, suggesting that acetylation at the N-terminus and/or the presence of an Asp residue at position 2 influences Cu(i) binding at site 1.

We further characterized the binding interaction of Cu(i) with the PIAS 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 H₆ 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-PIAS (Δδ = 0.14 ppm) > PIAS (Δδ = 0.11 ppm) > D2A PIAS (Δδ = 0.08 ppm), giving further support to the role of N-terminal acetylation and Asp-2 as structural factors promoting Cu(i)...
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-X-Met site, we determined the dissociation constants of Cu(i) complexes with the P1AS variants studied. The resonances corresponding to the S-CH$_3$ 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 ($K_{d1}$) 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. 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 αs. To this purpose, we used $^{15}$N isotopically enriched AcxS and the M5I/H50A and D2A mutants. The D2A mutant of αs is not a substrate for the NatB acetylase and thus lacks that post-translational modification, being referred as D2A αs further on.

The Cu(i) complexed states of the AcxS protein and its M5I/H50 AcxS and D2A αs mutant species were analyzed by 2D NMR spectroscopy (Fig. 3 and 4). As reported previously, upon titration of $^{15}$N-enriched AcxS with increasing concentrations of Cu(i), the $^1$H–$^{15}$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 αs 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 AcxS mutant. From these experiments, the conditional affinity for Cu(i) binding at site 1 in AcxS was $K_{d1} = 3.9 ± 1.0$ nM (Fig. 3B), consistent with previous studies. Interestingly, the value reported for the Cu(i)-complex in the non-acetylated protein was $K_{d1} = 7.8 ± 1.0$ nM, in line with the affinity differences found for Cu(i) binding to the N-acetylated and free amine P1AS peptides. The value of $K_{d1}$ for complexation of Cu(i) to the Met-X-Met site in M5I/H50A AcxS and the D2A αs 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 AcxS protein and the M5I/ H50A AcxS and D2A αs 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 AcxS sequence (mean R$_1$ values of 1.5 s$^{-1}$ and 1.9 s$^{-1}$ in the free and complexed protein,
respectively); however, more pronounced deviations were found in the \( R_2 \) values (mean \( R_2 \) values of 2.2 s\(^{-1}\) and 5.8 s\(^{-1}\)) for the free and complexed protein, respectively. These data indicate restricted local sampling in the picosecond to nanosecond time scale for the 1–10 segment of AczS–Cu(i) relative to the free protein, reflecting the loss of flexibility due to the stabilization of local conformations with \( \alpha \)-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 AczS reflect the low affinity interaction and fast exchange of the metal ion at these sites; however, the lower \( R_2 \) values for the D2A \( \alpha \)-S–Cu(i) form might be also reflecting a small increase of \( \alpha \)-helical propensity near the N-terminus, induced by the interaction with Cu(i) at the Met-X\(_2\)-Met site. These results motivate us to evaluate the Cu(i) protein complexes in terms of their conformational properties. \(^3\)J\(_{HN-HS}\) couplings are reliable quantitative reporters of the time-averaged distribution of the backbone torsion angles, \( \varphi \), and are frequently used to probe the propensity of intrinsically disordered proteins to sample different regions of conformational space. Therefore, we measured residue-specific \(^3\)J\(_{HN-HS}\) couplings in both free and copper-bound states of these proteins (Fig. S3, ESI†). With the exception of the decrease in \(^3\)J\(_{HN-HS}\) for the first 10 residues of AczS upon Cu(i) binding, the values measured for the two forms of the M5I/H50 AczS and D2A \( \alpha \)-S proteins were essentially indistinguishable. With an averaged \(^3\)J\(_{HN-HS}\) of 4.5 Hz expected for an ideal \( \alpha \)-helix and a \(^3\)J\(_{HN-HS}\) of \( \sim 7 \) Hz for random coil, our results indicate that a conformational transition toward increased \( \alpha \)-helix structures is clearly observed only for the AczS–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\(_2\)-Met site of AczS. 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 AczS–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 \( \alpha \)-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 \( \alpha \)S 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: Ac-P1AS > P1AS > D2A-P1AS > M5I-P1AS > 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
modification translates into an 76 mV decrease in the reduction potential (53 mV for the M5I mutant), consistent with previous reports. A new finding from this study is that Asp-2 also plays a role in Cu(I) binding; the D2A modification causes a 1.5 fold decrease in binding affinity for Cu(I), which would translate into a decrease of 15 mV in the reduction potential, if this mutation were not to impact Cu(II) binding affinity as it does (Table S1, ESI†). While having a more modest impact in the redox properties of site 1, Asp-2 is certainly also playing a role in stabilizing Cu(i) coordination to αS. Finally, the acetylation of the N-terminal group causes a 1.7 fold increase in Cu(i) binding affinity, which would translate into a ~17 mV increase in the reduction potential of the complex, if it were not to impact Cu(i) 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. 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 αS–Cu complex.

Fig. 4 NMR analysis of Cu(i) binding to the M5I/H50A and D2A protein variants. (A) Overlaid 1H–15N HSQC spectra of M5I/H50A AcαS in the absence (black) and presence (orange) of 1 equiv. of Cu(i). (B) Overlaid 1H–15N HSQC spectra of D2A αS in the absence (black) and presence (orange) of 1 equiv. of Cu(i). For comparative purposes with AcαS–Cu(i), residues well-isolated and involved in Cu(i) binding to site 1 are labeled. Experiments were recorded at 15 °C using 15N isotopically enriched protein (50 μM) samples dissolved in Buffer A.

Fig. 5 15N relaxation parameters of αS variants and its Cu(i) complexes. R1 and R2, relaxation rates of the proteins AcαS, M5I/H50A AcαS and D2A αS in the absence (black) and presence (green) of Cu(i). Experiments were recorded at 15 °C using 15N isotopically enriched protein (200 μM) samples dissolved in Buffer A in the absence and presence of 2 equivalents of Cu(i). The increase of R2 values around His-50 and Met-116/127 residues in AcαS–Cu(i) reflects the fast exchange of Cu(i) at these secondary sites, as previously reported.42,50
Another important impact of the acetylation of αS is the promotion of local helical conformation upon Cu(i) binding.\textsuperscript{42,50} It has been reported that chemical induction of alpha-helical conformation in αS by using fluorinated solvents increases the reduction potential of the αS-Cu complex by ~90 mV, possibly stabilizing the Cu(i) form.\textsuperscript{54} From this shift, an increase in Cu(i) binding affinity of an order of magnitude was estimated for the alpha-helical αS 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 αS 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.\textsuperscript{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 αS-Cu(i) complexation at the Met-X-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 αS-Cu(i) complexes.

Conflicts of interest

There are no conflicts to declare.

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