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A Revised Picture of the Cu(II)−α-Synuclein Complex: The Role of N-Terminal Acetylation

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Supporting Information

ABSTRACT: α-Synuclein (αS) is an amyloidogenic intrinsically disordered protein implicated in Parkinson's disease, for which copper-mediated pathways of neurodegeneration have been suggested. We have employed nuclear magnetic resonance, circular dichroism, electrospray ionization mass spectrometry, and thioflavin T fluorescence to characterize interactions of Cu^{2+} with the physiological acetylated form (Ac-αS). Significantly, N-terminal acetylation abolishes Cu^{2+} binding at the high-affinity M1-D2 site present in the nonacetylated protein and maintains Cu^{2+} interactions around H50/D121. Fibrillation enhancement observed at an equimolar Cu^{2+} stoichiometry with the nonacetylated model does not occur with Ac-αS. These findings open new avenues of investigation into Cu^{2+}-mediated neurodegenerative pathology suggested *in vivo*.

The link between oxidative stress and protein aggregation in neurodegenerative disorders, including the assembly of α-synuclein (αS) amyloid fibrils in Parkinson's disease (PD), is ever-expanding. Potentially aberrant redox-active metal chemistry in the highly respiratory neural environment may induce formation of harmful oxidants. Endogenous biometals may therefore reinforce oxidative neurodegeneration by dysregulation of metal transport and metabolism. An emerging role for copper in PD is suggested on the basis of its abnormal distribution within the brain. In PD patients, overall depressions of copper levels in the brain and increased levels of copper in the cerebrospinal fluid have been observed. Moreover, spatial imaging detects a complex localization of copper within the PD midbrain.[1]

α-Synuclein is a 140-residue intrinsically disordered protein (IDP) for which an abundance of evidence reveals that the native form undergoes N-terminal acetylation *in vivo*.[2−5] Despite the initial recognition of this cotranslational modification by Anderson et al.,[3] a general awareness of N-terminal acetylation among *in vitro* αS researchers arose when Bartels et al. rediscovered this mammalian modification within the context of a possible helical tetrameric form of the protein.[4] Following this investigation, several *in vitro* studies have focused on the role of acetylation in αS.[6−11] However, the majority of *in vitro* studies on the Cu^{2+}−αS complex predate an interest in the acetylated protein form, and the commonly accepted picture of the Cu^{2+}−αS complex is rooted in a nonacetylated αS model (i.e., nonAc-αS). Multiple lines of evidence reveal that Cu^{2+} binds nonAc-αS at three specific sites (Figure 1A),[12−18] namely the, (1) high-affinity N-terminal M1-D2 site 1, (2) lower-affinity H50 site 2 also located at the N-terminus, and (3) nonstoichiometric low-affinity C-terminal site 3 anchored around D121. Although an extensive literature debate[12] (please see references therein) addresses the relative involvement of sites 1 and 2, one widely accepted model of the highest-affinity complex of Cu^{2+} with nonAc-αS is centered at site 1, where Cu^{2+} is readily anchored by the freely available amino-terminal nitrogen.[12]

In this report, we characterize the impact of N-terminal acetylation within full-length Ac-αS on the conformation and fibrillation of the Cu^{2+}−αS complex using nuclear magnetic resonance (NMR), electrospray ionization mass spectrometry (ESI-MS), visible circular dichroism (CD) spectroscopy, and thioflavin T (ThT) fluorescence amyloid assays. We demonstrate that Ac-αS lacks the highest-affinity amino-terminal Cu^{2+} binding site, maintains low-affinity binding with the H50 and D121 sites, and undergoes altered amyloid formation modulated by the presence of Cu^{2+}. These findings suggest that Ac-αS is the appropriate experimental model for *in vitro* Cu^{2+} binding studies and copper-mediated αS pathology in PD.

NMR has assumed a key role in sequence specific mapping of Cu^{2+} binding, and we employ this approach to examine interactions of Cu^{2+} with full-length Ac-αS. Figure 1A depicts the main putative anchoring residues (M1D2, H50, and D121) at each of the three Cu^{2+} binding sites as described in previous studies employing nonAc-αS.[13−16,19] Normalized $^{1}H-^{15}N$ heteronuclear single-quantum coherence (HSQC) spectroscopy intensities in the presence of 0.5 equiv of Cu^{2+} pinpoint the regions where paramagnetic Cu^{2+} localizes in nonAc-αS and Ac-αS (Figure 1B). Significant differences in signal attenuation are observed at site 1 in the presence of acetylation, while smaller intensity differences are observed at weaker affinity sites 2 and 3. A Cu^{2+} titration shows that the differences seen with 0.5 equiv of Cu^{2+} remain similar up to an equimolar stoichiometry (Figure S1 of the Supporting Information), demonstrating that N-terminal acetylation abolishes the relevance of high-affinity site 1 Cu^{2+} binding in Ac-αS and preserves the binding at sites 2 and 3.

The NMR data indicate somewhat enhanced line broadening within the proximity of D121 at site 3 in Ac-αS relative to nonAc-αS (Figure 1B and Figure S3C of the Supporting Information) despite the fact that their C-termini do not differ in sequence or modification. To evaluate whether the Cu^{2+}

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binding profile is altered at the C-terminus due to N-terminal acetylation, we have employed Mn^{2+}, which binds exclusively to the C-terminus, as an alternate paramagnetic probe.[16] The Mn^{2+} attenuation profiles are nearly superimposable (Figure S3A of the Supporting Information) for nonAc-αS and Ac-αS, suggesting that N-terminal acetylation alone does not account for the differences in intensity of the Cu^{2+}-bound Ac-αS complex. Cu^{2+} titration data (Figure S3B,C of the Supporting Information) indicate that enhanced line broadening in region 3a versus region 3b is observed across the Cu^{2+} concentration range, suggesting the Cu^{2+} ion distributes itself differently at site 3 when site 1 is acetylated. Whereas Mn^{2+} binds only sites 3a and 3b, Cu^{2+} binds across the length of the protein with both N- and C-terminal contributions.

Therefore, attenuation differences observed at site 3a may be partially derived from Cu^{2+}-mediated N-terminal interactions. Future mutagenesis studies at Cu^{2+} binding sites will elucidate the extent of independence or cooperativity between N- and C-terminal sites. Consistent with our NMR data, previous studies of short N-terminally acetylated αS peptide models similarly reflect an inability to bind Cu^{2+}.[20,21] The limitation of these peptide model studies is that Cu^{2+} interactions are isolated to single binding sites, making it difficult to account for the complete range of interactions between multiple Cu^{2+} binding sites within the full-length protein. In this respect, our NMR results demonstrate that Cu^{2+} binding in the full-length N-terminal acetylated protein can still occur at sites 2 and 3 in the absence of the participation of site 1. In addition, Cu^{2+} binding may also function as a probe of preexisting long-range contacts in αS, or metal-mediated interactions between Cu^{2+} binding sites distributed throughout the length of the protein.

Visible CD difference spectra of Ac-αS and nonAc-αS in the presence of Cu^{2+} (Figure 1C) indicate, as the NMR data suggest, the inability of Ac-αS to complete a coordination sphere with site 1 atoms. Spectral profiles of the nonAc-αS−Cu^{2+} complex reported previously are characterized by the codevelopment of negative ellipticity arising from a deprotonated backbone nitrogen charge transfer transition at ~300 nm and weak positive ellipticity (d → d) at ~600 nm[19] up to an equimolar ratio, reflective of a 1:1 stoichiometry. While Figure 1C presents expected ellipticity changes for the nonAc-αS−Cu^{2+} complex, the profile of Ac-αS is virtually indistinguishable from the baseline even at a 3:1 copper:protein stoichiometry (Figure S2 of the Supporting Information). Ac-αS does not form a proposed (5,6)-membered ring site[12,22] with Cu^{2+}, as acetylation blocks the anchoring α-amino nitrogen at M1. This may arise from the combined effect of weaker initial anchoring and the inability of a more helical geometry induced by N-terminal acetylation[8,9,11] to provide successive stabilizing ligands from the D2 nitrogen and side chain oxygen. A D2A mutation in a nonAc-αS N-terminal peptide also disrupts stable M1-D2 coordination,[19] thereby supporting the view that high-affinity anchoring of Cu^{2+} to the M1 site requires this suite of ligands.

ESI-MS is used to evaluate the relative populations of Cu^{2+}-bound complexes of nonAc-αS and Ac-αS in a titration from 0 to 10 equiv of Cu^{2+} (Figure 1D,E.). In nonAc-αS, the first equivalent of Cu^{2+} shifts most of the unbound species to a population containing one bound Cu^{2+} atom, whereas in Ac-αS, a significant proportion of the unbound species persists. This reflects the absence of site 1 binding in Ac-αS, which is consistent with the NMR and CD data (Figure 1B,C). A higher number of Cu^{2+} equivalents shifts the population further toward species that contain multiple bound Cu^{2+} atoms. This observation suggests

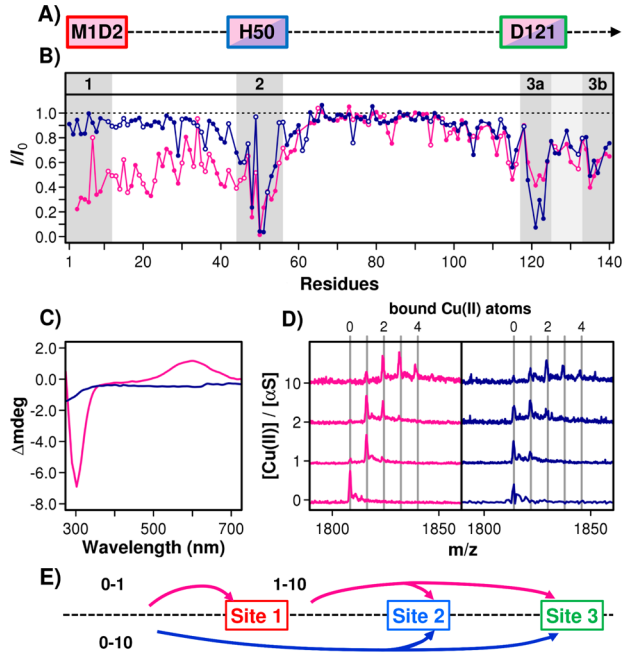

Figure 1. (A) Schematic highlighting the putative main anchoring residues at each Cu^{2+} site, color-coded for their presence in nonAc-αS (magenta) and Ac-αS (dark blue). (B) Ratio of paramagnetic Cu^{2+} $^{1}H-^{15}N$ HSQC attenuation in the presence of 0.5 equiv (200 μM αS and 100 μM Cu^{2+}). (C) Visible CD spectra of 200 μM nonAc-αS and Ac-αS in the presence of 1.0 equiv (200 μM) of Cu^{2+}. (D) ESI-MS profiles of the +8 peak in the presence of 0, 1, 2, and 10 equiv of Cu^{2+} for nonAc-αS and Ac-αS. (E) Schematic illustrating a possible avenue of Cu^{2+} binding reflective of the bound metal ratios observed via ESI-MS. In nonAc-αS, an initial event of 0−1 equiv of Cu^{2+} represents binding at high-affinity site 1, while sites 2 and 3 are occupied at a higher number of equivalents with weaker affinity as observed in Ac-αS.

that sites 2 and 3 contribute to a lesser extent, corroborating the general consensus of nonAc-αS−Cu^{2+} binding published to date.[12] In Ac-αS, the addition of ≥2 equiv of Cu^{2+} shifts the populations toward bound complexes containing one, two, or more Cu^{2+} atoms, in a fashion reflecting a possible lower-affinity binding to sites 2 and 3 alone (Figure 1E).

Much of the attention that Cu^{2+} has received stems from its unique role in accelerating nonAc-αS amyloid formation *in vitro*.[16,23] In view of the lower Cu^{2+} affinity implied by the absence of site 1 binding in Ac-αS, we have conducted ThT fluorescence amyloid assays (Figure 2) to assess whether Cu^{2+} binding at sites 2 and 3 is capable of accelerating amyloid formation. ThT assays conducted with nonAc-αS reveal that Cu^{2+} binding at the lowest stoichiometries accelerates fibrillation

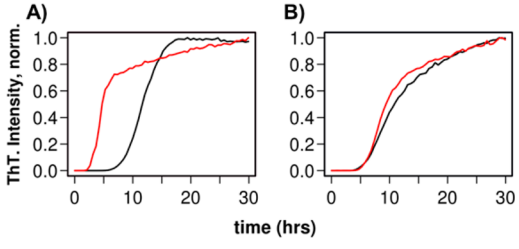

Figure 2. Normalized and averaged ThT fluorescence profiles for nonAc-αS (A) and Ac-αS (B) with 0 (black) and 1 (red) equiv of Cu^{2+} added to the solution.

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in terms of growth rate and lag phase as noted in previous studies.[23] Conversely, ThT assays of Ac-αS reveal that 1 equiv of Cu^{2+} imparts a relatively minor effect on aggregation kinetics, while 2 equiv may more dramatically impact the aggregation kinetics (Figure S4 and the discussion of the Supporting Information). These trends are consistent with the Cu^{2+}-bound population distributions of Ac-αS observed in the ESI-MS experiments, at low and higher stoichiometries, suggesting that weak Cu^{2+} binding at sites 2 and 3 may have a complex effect on aggregation kinetics. Interestingly, the biological relevance of Cu^{2+}−Ac-αS interactions might not derive solely from the acceleration of fibrillation. In fact, previous studies have demonstrated that Cu^{2+}-induced oligomers can represent the toxic species.[24] The mechanism by which the aggregation kinetics are altered by interactions of Cu^{2+} with sites 2 and 3[12,23] requires further investigation into the conformational steps leading to formation of soluble oligomers and ordered fibrils.

In summary, the impact of N-terminal acetylation on Cu^{2+} binding reported herein effectively reshapes the consideration of Cu^{2+}-mediated pathology *in vivo*. In conjunction with recent evidence gleaned from biophysical studies, our findings suggest that the acetylated protein is essential for characterizing Cu^{2+}−αS interactions *in vitro*. While the complexity surrounding coordination of Cu^{2+} to the nonacetylated protein remains, N-terminal acetylation elegantly resolves the issue of the relative contributions of the M1 and H50 binding sites and localizes Cu^{2+}-induced pathology to H50 and the C-terminus within Ac-αS. The results of this report are particularly intriguing in view of the role that copper assumes in localization of the protein[25] coupled with the impact of acetylation on αS membrane affinity and reshaping.[6,11,26] Our revised model may assist in elucidating the mechanisms by which Cu^{2+} induces formation of β-like conformers[27,28] or mediates the balance between protective and pathological intra- and/or interchain interactions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Relevant methodological details and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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