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Exploring the Structural Details of Cu(I) Binding to α-Synuclein by NMR Spectroscopy

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Abstract: The aggregation of α-synuclein (AS) is selectively enhanced by copper in vitro, and the interaction is proposed to play a potential role in vivo. In this work, we report the structural, residue-specific characterization of Cu(I) binding to AS and demonstrate that the protein is able to bind Cu(I) with relatively high affinity in a coordination environment that involves the participation of Met1 and Met5 residues. This knowledge is a key to understanding the structural-aggregation basis of the copper-catalyzed oxidation of AS.

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The aggregation of α-synuclein (AS) is a critical step in the etiology of Parkinson's disease (PD).[1] Protein−metal interactions play an important role in AS aggregation and might represent the link between the pathological processes of protein aggregation, oxidative damage, and neuronal cell loss. As reported for the Aβ peptide and the prion protein, AS is also highly susceptible to copper-catalyzed oxidation, a reaction that induces extensive oligomerization and precipitation of these proteins.[2−5] The reaction involves the reduction of Cu(II) by a suitable electron donor and the conversion of molecular oxygen into reactive oxygen species (ROS), which trigger the oxidative modification of the protein.[6] This mechanism is a highly selective, site-specific process that involves interactions of the protein with both oxidation states of the copper ion. Despite the large body of evidence supporting a role for Cu(II)−AS interactions in metal-enhanced protein aggregation,[7−12] the occurrence of an associated Cu(I)/dioxygen chemistry was only recently reported.[13,14] However, detailed knowledge of the structural and binding features of Cu(I) to AS remains unknown. To address such important and unresolved issues of the bioinorganic chemistry of PD, we report here the first structural characterization of the interaction of AS with Cu(I). The details of Cu(I) binding to AS were explored at single-residue resolution by ^{1}H−^{15}N heteronuclear single-quantum correlation (HSQC) NMR experiments. We first recorded the ^{1}H−^{15}N HSQC spectrum of AS in the presence of 1 equiv of Cu(II) (Figure 1A), which shows the previously reported metal-induced broadening effects at residues 3−9 ($K_d = 0.1 \pm 0.01$ μM), 48−52 ($K_d = 35.0 \pm 4.0$ μM), and 119−124 ($K_d \approx 1$ mM).[7−10] Next, ascorbate was added to reduce the AS−Cu(II) complexes (Figure S1 in the Supporting Information). The use of ascorbate as an efficient reductant of Cu(II) sites in metalloenzymes and amyloidogenic proteins is well-documented.[15,16] Indeed, the redox potential of the Cu(II) complex with AS was recently reported ($E = 0.018$ V vs Ag/AgCl), and it was demonstrated that ascorbate ($E = -0.145$ V vs Ag/AgCl) can directly reduce AS−Cu(II) to AS−Cu(I).[14] Furthermore, high levels of ascorbate have been reported in the intracellular milieu of neurons, adding more biological implications to our investigation.[17] Whereas the cross-peaks of residues in the Cu(II)-affected regions completely recovered their intensities in the presence of an excess of ascorbate (Figure 1B), backbone chemical shift changes in a discrete number of residues became evident under these experimental conditions (blue arrows in Figure 1B). Since addition of ascorbate in the absence of Cu(II) caused no changes in the NMR spectral features of AS (data not shown), the observed behavior was attributed to the reduction of Cu(II) to Cu(I) and formation of AS−Cu(I) complexes. The residues exhibiting the largest displacements in the amide resonances were Val3, Phe4, and Met5 (the amide resonances of Met1 and Asp2 were not detected because of solvent-exchange effects), with smaller shift perturbations centered on the amide group of His50 and the region comprising residues 119−129 (Figure 1D). The effects on His50 and 119−129 amide resonances were further pronounced in samples containing 2−3 equiv of added Cu(II) in the presence of an excess of ascorbate (Figure 1E), indicating that residues 3−5 correspond to the high-affinity binding site for Cu(I) in AS. NMR spectral changes induced by the metal ion were abolished upon EDTA addition, confirming the reversibility of the binding process (Figure 1C). Further information on the specificity of Cu(I) binding to AS was gained from monitoring the metal-ion-induced changes in ^{1}H−^{13}C (H)CCH total correlation spectroscopy (TOCSY) experiments, which demonstrated that cross-peaks assigned to connectivities of Met1 and Met5 residues were the most affected by the presence of the metal ion (Figure S2).

According to these results, we then investigated the binding features of Cu(I) to the synthetic peptide ^{1}MDVFMK^{6}, which was previously shown to encompass the independent and noninteractive high-affinity binding site for Cu(II) in AS.[9,10] As shown in Figure 2, cross-peaks assigned to the Hγ−Cγ and Hε−Cε connectivities of Met1 and Met5 residues were the most affected by the presence of the metal ion (Figure 2A,B). Resonances corresponding to Hα−Cα and Hβ−Cβ of Met1; Hβ−Cβ of Asp2, Phe4, and Met5; and Hγ−Cγ of Val3 were also affected, although to a lesser extent, whereas no changes in the signals belonging to Lys6 were observed. The binding features of Cu(I) complexed to the peptide ^{1}MDVFMK^{6}, as indicated by the metal-ion-induced effects on the ^{1}H and ^{13}C chemical shifts of amide and side-chain resonances,

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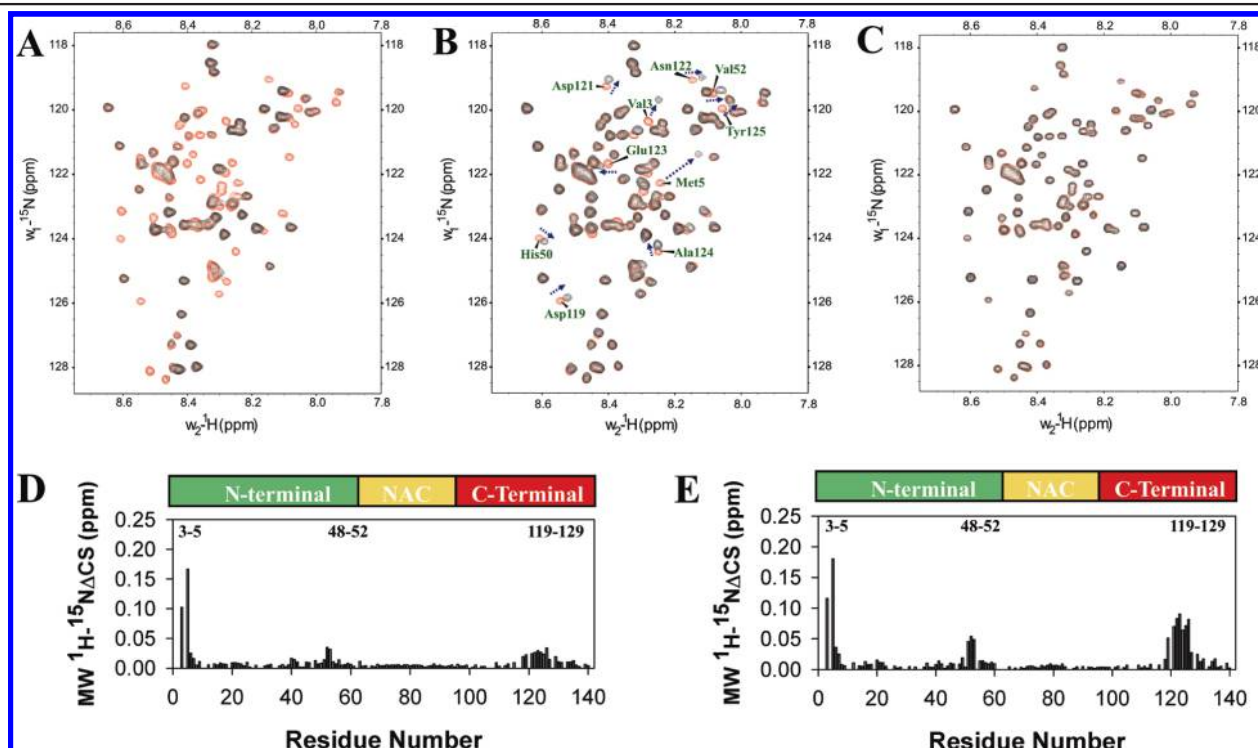

Figure 1. NMR analysis of Cu(I) binding to AS. (A) Overlaid ^{1}H−^{15}N HSQC spectra of AS (300 μM) in the absence (red) and presence (black) 1 equiv of Cu(II). (B) Overlaid ^{1}H−^{15}N HSQC spectra of AS in the absence (red) and presence (black) of 1 equiv of Cu(II) followed by the addition of 35 mM ascorbate. (C) Overlaid ^{1}H−^{15}N HSQC spectra of AS in the absence (red) and presence (black) of equiv of 1 Cu(II) and 35 mM ascorbate followed by the addition of 5 mM EDTA. (D, E) Differences in the mean weighted chemical shift displacements (MW ^{1}H−^{15}NΔCS) between free and Cu(I)-complexed AS at molar ratios of (D) 1:1 and (E) 2:1.

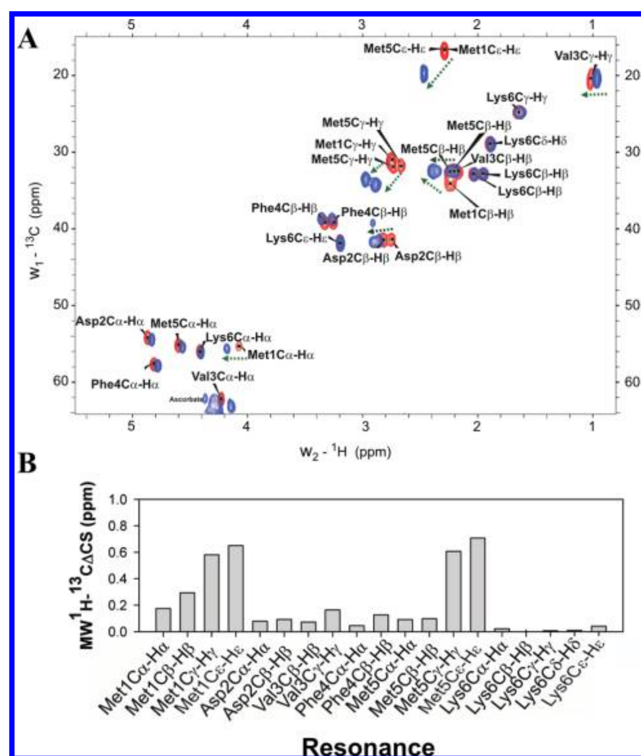

Figure 2. (A) Overlaid ^{1}H−^{13}C HSQC spectra of ^{1}MDVFMK^6 (2.5 mM) in the absence (red) and presence (blue) of 1 equiv of Cu(I). (B) Mean weighted chemical shift displacements (MW ^{1}H−^{13}CΔCS) of ^{1}MDVFMK^6 signals induced by the presence of the metal ion.

were similar to those observed for the high-affinity site in the full-length protein (Figure S3A−E), indicating that the synthetic peptide constitutes an excellent model for characterizing the high-affinity AS−Cu(I) complex. This behavior is also consistent with the absence of interplay between this site and the other identified Cu(I) binding motifs in the full-length protein, suggesting that this site constitute a separate, independent metal binding site. Altogether, these observations support the direct involvement of Met1 and Met5 in Cu(I) coordination to AS, consistent with the well-documented binding preferences of Cu(I) to sulfur atoms of Cys and Met residues in metalloproteins and metalloenzymes.[18,19]

The affinity features of the AS−Cu(I) complex at the ^{1}MDVFMK^6 sequence were determined by NMR spectroscopy. Figure S4 shows the continuous displacement of ^{1}MDVFMK^6 resonances in the amide region of the ^{1}H−^{1}H TOCSY spectra recorded with increasing concentrations of metal ion (see the Supporting Information for experimental details). The amide resonances of residues 3−5 were well-resolved over the entire titration experiment and were thus well-suited for the calculation of the AS−Cu(I) dissociation constant (Figure S4). The NMR-derived affinity ($K_d \approx 2\ \mu$M) is in full agreement with values from previous reports on Cu(I) binding to proteins and peptides involving Met residues as primary metal-anchoring ligands.[16,19]

Since Met residues are one of the preferred targets in proteins under conditions of site-specific metal-catalyzed oxidation, we also investigated the occurrence of oxidative damage in a sample of the ^{1}MDVFMK^6−Cu(II) complex treated with an excess of ascorbate and followed by 5 hs of air exposure. We noted that under our experimental conditions, the Cu(II)/Cu(I) redox cycle can efficiently catalyze the oxidation of the peptide at the Met1 and Met5 positions (Figure S5), as reported previously for AS peptide samples exposed to Cu(II) and hydrogen peroxide.[20] Indeed, it was

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demonstrated recently that the AS−Cu(I) complex can be reoxidized by O_2, producing H_2O_2 as a ROS and inflicting toxicity and neuronal cell death.[14]

This work constitutes the first structural, residue-specific characterization of Cu(I) binding to AS and demonstrates conclusively that the protein is able to bind Cu(I) with relatively high affinity in a coordination environment that involves the participation of Met1 and Met5 residues as the main anchoring moieties. Altogether, the evidence discussed here indicates that in vivo Cu(II)/Cu(I) redox chemistry might be a key event in the pathophysiology of PD. It has also been suggested that copper-induced ROS generation mediates Aβ aggregation.[21,22] Oxidative modification of Aβ has been shown to increase its propensity to aggregate, which has been proposed to increase Aβ oligomer formation and/or plaque deposition in Alzheimer's disease.[2,4,21,23] In this context, one can thus hypothesize that copper binding to the N-terminus of AS might render the protein a relative easy target for oxidative damage and that the ensuing damage might lead to a cascade of structural alterations (e.g., destabilization of long-range interactions between the N- and C-terminus) promoting the generation of a pool of AS molecules more prone to aggregate. Further studies of AS and related amyloid proteins are clearly needed to provide a better understanding of the structural-aggregation and mechanistic basis of the oxidative damage caused by copper-catalyzed oxidation in neurodegeneration.

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Supporting Information Available: Experimental details, supporting results, and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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