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ELECTRON PARAMAGNETIC RESONANCE AS A TOOL TO DETERMINE PARAMAGNETIC METAL COORDINATION IN NATIVELY UNSTRUCTURED NEURONAL PROTEINS

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Abstract

Neurodegenerative diseases are defined as diseases wherein nerve cells are damaged or destroyed and are a major health concern. Millions around the world have Alzheimer's and Parkinson's diseases, causing untold suffering and costing billions of dollars annually. Understanding the pathogenesis of these diseases on a molecular level is crucial to developing cures.

Parkinson's disease destroys dopaminergic neurons in the *substantia nigra pars compacta* area of the brain, causing many movement related disorders and ultimately death. A hallmark of Parkinson's disease is the cytosolic, filimental inclusion known as the Lewy Body, which consists mostly of the protein α-synuclein. α-Synuclein is a 140-residue unstructured neural peptide that has been linked to the pathogenesis of Parkinson's disease through genetics and animal models. Genetic mutations arising in Parkinson's are rare, and the disease appears to be instigated more commonly by external influences such as pesticides and heavy metals, in particular copper and aluminum. *In vitro* studies have indirectly demonstrated interaction between α-synuclein and copper ions. However, previous attempts to

characterize the monomeric, unstructured α-synuclein-Cu^{2+} interaction more directly have lead to some confusion and conflicting results regarding the stoichiometry, chelation structures and binding affinity.

Alzheimer's disease is characterized as a dementia and is the most common neurological disorder, primarily affecting the cognitive abilities leading to extreme confusion, aggressive behavior and difficulty speaking. The disease ultimately leads to death within seven years after diagnosis. A hallmark of Alzheimer's disease is extracellular deposits, termed senile plaques, consisting mostly of the aggregated 40-42 residue protein Aβ. These plaques are shown to have elevated levels of copper relative to the rest of the brain and Aβ binds copper strongly in its monomeric form. Indirect evidence has suggested that aggregated Aβ chelates copper with higher affinity than the monomeric form. Direct evidence of this phenomenon, though, is lacking.

In this dissertation we use electron paramagnetic resonance (EPR) to directly investigate how the proteins α-synuclein and aggregated Aβ interact with Cu^{2+} ions. EPR is a technique that is uniquely suited to probe the interaction of natively unstructured proteins with paramagnetic species such as Cu^{2+}. EPR combined with site-directed mutagenesis is a powerful tool for directly determining the protein-paramagnetic species stoichiometry and protein residues involved in copper chelation. Dissociation constant based EPR competition studies directly measure the affinity of the species of interest for the paramagnetic ions. Using these techniques we have determined the stoichiometry, chelation structures and affinities for both unstructured

(chapter 2) and membrane bound (chapter 3) α-synuclein. Furthermore, in chapter 4, EPR competition studies between aggregated Aβ and human serum albumin demonstrate that the aggregated form of Aβ is capable of outcompeting albumin for Cu^{2+} whereas monomeric Aβ is not. This information helps to shed light on both of these proteins disease pathogenesis and perhaps their natural function as well. This knowledge is critical for the development of new therapies for these diseases.

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CHAPTER 1

INTRODUCTION

Neurodegenerative Disease

Overview

Neurodegenerative disease is defined as the damage or destruction of nerve cells. As the nervous system controls the workings of the body and is, indeed, the very conscience, it is easy to deduce that damage to neurons would have deleterious and lasting effects on an individual's well being. The demographics of the United States are such that the median age of the population is increasing (*1*). The combination of the late age of onset of the two most prevalent neurodegenerative diseases (Alzheimer's and Parkinson's disease, respectively) and the increased individual life expectancy of Americans presents a serious health risk for the country. It is estimated that there will be over 19 million people living with neurological diseases such as Alzheimer's and Parkinson's disease in the U.S. alone by 2050 (*2*). The national cost of medical and at-home care can be in the billions, making this a major health and cost concern for the aging population (*3-5*). Additionally, there are the non monetary costs of psychological trauma and stress-fatigue suffered by the caregivers themselves, many of whom will be relatives of the diseased (*6*). Given these facts, it would seem obvious that research into the causes and prevention of these diseases is of great importance. Unfortunately, progress is slow and cures are still nonexistent. Therefore, the focus of this dissertation is to contribute to the current understanding of the proteins that cause Parkinson's and Alzheimer's disease.

Parkinson's Disease

Parkinson's Disease (PD) is a terrible, ultimately fatal, predominantly age-related disorder that is second only to Alzheimer's disease in prevalence among those 60 and older (*7*). PD was first described by the English doctor James Parkinson in *An Essay on the Shaking Palsy* 1817. PD is primarily idiopathic in nature, but its pathology appears to preferentially target and destroy dopaminergic neurons in the *substantia nigra pars compacta* region of the brain. As the neurons in the *substantia nigra* primarily deal with motor control, their destruction manifests itself with symptoms such as slowness of movement, difficulty breathing and swallowing and uncontrolled tremors (*8*). Although the disease has been extensively studied, there are currently no definitive laboratory tests for PD and its symptoms can easily be confused with other neurological diseases and cancer. Identification of PD while the patient is alive is often done by process of elimination based on the symptoms that are manifesting themselves at the time of diagnosis (*9*). The only way PD can be definitively diagnosed is post mortem, where autopsy dissection of the sufferer's *substantia nigra pars compacta* area of the brain may reveal a loss of dopaminergic neurons. The remaining neurons, when stained with melanin staining, tend to show the black inclusions termed Lewy Bodies (LB). LBs consist almost entirely of fibrils of the "misfolded" protein α-synuclein (α-syn).

α-Synuclein

α-syn is a 140-residue natively unstructured neuronal peptide that is expressed in high abundance primarily in the synaptic termini of neurons (*10*). Although it is unstructured in solution the protein does fold upon exposure to certain external

stimuli and the overall peptide can be divided into three sections based upon the structures formed in each. The N-terminal region of α-syn, (residues 9 – 97), possesses a series of 11-residue imperfect repeats with the consensus sequence KTKEGVXXXXX that form an amphipathic alpha-helix when associated with lipid vesicles, a structure similar to exchangeable apolipoproteins (*11*). Encompassed within the N-terminal region is a segment, residues 61-95, referred to as the Non-Aβ Component (NAC), which is dominated by hydrophobic residues and tends to form aggregates leading to the parallel beta-sheet rich fibril structures present in Lewy Bodies (*12*). Interestingly, the name Non-Aβ Component is revealing as aggregates from that segment also contribute approximately 10% of the protein in the senile plaques (primarily Aβ) in Alzheimer's disease patients (*13*). Cloning of the cDNA corresponding to the NAC fragment sequence led to the identification of α-syn. The C-terminal tail of the protein is highly acidic and devoid of secondary structure. However, truncation of this segment produces shortened lag time in fibril kinetics studies, suggesting a possible auto-inhibitory role against α-syn polymerization (*14*). Currently the native function of α−syn is unknown. However, initial studies associated the protein with memory functions in songbirds,(*15*) and recent work has suggested that α-syn may act as a chaperone for the protein's involved in SNARE complexes, assisting in synaptic vesicle fusion (*16*). Furthermore, there is evidence for α-syn copper complexation and redox activity, a trait common to other unstructured neuronal proteins such as Aβ and the prion protein (*17-19*).

The correlation between α-syn and PD has been clearly demonstrated in animal models where α-syn over expression leads to PD-like motor deficits and

intracellular deposits reminiscent of Lewy Bodies. Furthermore, hereditary early onset PD in humans is linked to genetic mutations in α-syn or copy number variation. While there are rare instances of genetic mutations in α-syn causing early onset PD, an interesting aspect of this disease is that it seems to be instigated by long-term exposure to factors such as pesticides and industrial metals rather than genetics or a classical pathogen (*20-23*). Epidemiological studies have demonstrated a correlation between long-term exposure to metals such as aluminum, copper and iron, and fatal PD (*24-26*). In 2001 Uversky et al. showed that α-syn appeared to interact with these metals *in vitro*, (*27*) and since then there have been many studies attempting to further characterize these interactions, particularly with copper ions. Because α-syn's interaction with Cu^{2+} ions is the primary subject of this dissertation and extensively discussed in the next two chapters I will not labor this point further. α-Syn chelates Cu^{2+} with a physiologically relevant binding affinity and that this may or may not be a cause for disease or part of the protein's natural function.

Alzheimer's Disease

Alzheimer's disease (AD) is the most prevalent neurological disorder known to our species, effecting over 26 million individuals worldwide over the age of 60 (*28*). AD is an idiopathic, degenerative disease that is chronic, progressive and has no cure. It is interesting and distressing to note that current predictions place 1 in 85 people suffering from the disease globally (*29*).

AD, unlike PD described above, is characterized as a dementia. That is, it primarily affects the cognitive functions of the brain. Symptoms vary from individual to individual but the earliest outward signs of the disease often manifest themselves as forgetful behavior, or "absent-mindedness." As the disease progresses, however, symptoms worsen and include extreme confusion, aggressive behavior, more severe memory loss and difficulty speaking, swallowing and walking (*30, 31*). AD can progress for years before clinical symptoms manifest themselves and, once they do, life expectancy is commonly limited to less than seven years. Currently AD is the sixth most common cause of death in the United States (*32*).

In addition to the pain and humiliation of the sufferers there is an incredible cost to those charged with their care (*6*). Commonly, sufferers of AD need 24 hour nursing care for several years, which is prohibitively expensive for the families of many AD patients, forcing the families themselves to provide such care (*4, 33*). It is estimated that caring for a person with AD results in thousands of lost work hours per year in the U.S. alone (*34*).

The pathology of AD is not well understood. AD exists in both familial and sporadic forms, though the former accounts for only about 15% of cases (*35, 36*).

Current research implicates so-called "senile-plaques," which are found extracellularly in the brain and consist of mostly the Aβ protein, though other proteins such as the Non-Aβ component of α-syn are also present (*37*). These proteins are "misfolded" into beta sheet structures, which then aggregate, eventually forming the plaques. While the exact mechanism for the formation of senile plaques is unknown, the role of metal ions in promoting the aggregation of Aβ peptides *in vitro* has led to debate and extensive research addressing how metals bind to and induce aggregation of Aβ peptides (*38-44*). This knowledge has provided momentum for the development of metal chelation-based therapies targeted toward treating AD.

The Aβ Peptide

Aβ is a natively un-structured 40-42 residue peptide found primarily extracellularly in Cerebral Spinal Fluid (CSF). Aβ is the result of beta and gamma secretase enzymatic cleavage of the amyloid precursor protein (APP), resulting in Aβ peptides from 40-42 residues in length (*45, 46*). The protein's exact natural function is unknown, but several potential activities have been proposed for Aβ, including activation of kinase enzymes (*47, 48*), protection against oxidative stress (*49, 50*), regulation of cholesterol transport (*51, 52*), and functioning as a transcription factor (*53, 54*). While Aβ is natively unstructured, like α-syn it can form structure in response to certain interactions. The protein can be divided into roughly two sections: 1) The fully soluble, unstructured N-terminal section (residues 1-16) and 2) The highly insoluble, hydrophobic section (residues 17-40 or 42) that is prone to beta sheet formation and aggregation. The Aβ(42) peptide in considerably less soluble than the Aβ(40) and much more prone to aggregation and neurotoxicity (*55*).

Metal ions have been suggested to promote senile plaque formation, and there is evidence that Cu^{2+}-induced Aβ aggregation may be an important factor in the pathogenesis of Alzheimer's disease. Previous work has demonstrated that Aβ coordinates Cu^{2+} with affinities ranging from the nano to picomolar, and elevated concentrations of copper have been measured in the senile plaques (*56, 57*). Interestingly, the use of high-affinity metal ion chelators facilitate the dissolution of these plaques (*58-60*). The Cu^{2+} coordination to Aβ has been discussed in a recent review (*61*). At physiological pH (~7.4), Cu^{2+} (*62*) is coordinated by four ligands, 3N and 1O, with evidence that His6, His13, and His14 are involved, along with possible contributions from Tyr10, the carboxylate group of Asp1, the amide C=O of Ala2, and the N-terminus amine (*39, 40, 63*). However, the studies to date differ on the exact coordinating ligands of monomeric Aβ.

Indirect evidence suggests that aggregated Aβ has a greater affinity for Cu^{2+} ions than it's monomeric form. This aspect may be a key factor in the pathogenesis of AD. In the final chapter of this dissertation, we directly assay the Cu^{2+} binding affinity of aggregated Aβ and propose a copper binding structure that may explain the increase in affinity.

Outline of this dissertation

The majority of the work I've done for my dissertation can be summed up thusly: Determining the Cu^{2+} coordination features and affinities of natively unstructured neuronal proteins involved with neurodegenerative diseases with the primary investigation tools being site-directed mutagenesis and Electron Paramagnetic Resonance (EPR). This dissertation, then, examines three aspects of these interactions via two proteins, α-syn of PD and Aβ of AD. Chapter 2 determines the Cu^{2+} coordination structure, stoichiometry, and affinity of the protein α-syn in its natively unstructured state. Chapter 3 investigates the features and effect of Cu^{2+} association on structured α-syn when the protein is in it's helical, membrane bound state, which is incompatible with the copper coordination structure of α-syn in solution. Finally, in Chapter 4 we investigate how oligomerization effects Aβ Cu^{2+} coordination and affinity using tools and experience gained from work previously done on α-syn. Through this work we hope to create a deeper understanding of the natural functions of these proteins and perhaps also aspects of the pathology of the diseases they are so deeply intertwined with.

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CHAPTER 2

Coordination Features and Affinity of the Cu^{2+} Site in the α-Synuclein Protein of Parkinson's Disease

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Abstract

Parkinson's disease (PD) is the second most prevalent age-related, neurodegenerative disorder, affecting >1% of the population over the age of 60. PD pathology is marked by intracellular inclusions composed primarily of the protein α-synuclein (α-syn). The inclusions also contain copper and the interaction of Cu^{2+} with α-syn may play an important role in PD fibrillogenesis. Here we report the stoichiometry, affinity and coordination structure of the Cu^{2+}-α-syn complex. Electron Paramagnetic Resonance (EPR) titrations show that monomeric α-syn binds 1.0 equivalent of Cu^{2+} at the protein N-terminus. Next, an EPR competition technique demonstrates that α-syn binds Cu^{2+} with a $K_d \approx 0.1$nM. Finally, EPR and Electron Spin Echo Modulation (ESEEM) applied to a suite of mutant and truncated α-syn constructs reveal coordination sphere arising from the N-terminal amine, the amide backbone and side chain carboxyl group of Asp2, and the His50 imidazole. The high affinity identified here, and in accord with previous measurements, suggests that copper uptake and sequestration may be a part of α-syn's natural function protecting cells from Cu^{2+} mediated oxidation. The findings further suggest that the long-range interaction between the N-terminus and His50 may have a weakening effect on α-syn interaction with lipid membranes thereby mobilizing monomeric α-syn and hastening fibrillogenesis.

Introduction

Parkinson's disease (PD) is the second most prevalent neurological disorder after Alzheimer's disease, affecting >1% of the US population over the age of 60 (*1, 2*). PD, an idiopathic neuropathy, is chronic, progressive, and often fatal. Clinical symptoms of PD include diminished motor function, tremors, and speech disorders, attributed to the progressive loss of dopaminergic neurons of the *substantia nigra* (*3*). A hallmark of affected dopaminergic neurons are Lewy Bodies, cytosolic filimental inclusions composed primarily of the protein α-synuclein (α-syn). The correlation between α-syn and PD has been clearly demonstrated in animal models where α-syn over expression leads to PD-like motor deficits and intracellular deposits reminiscent of Lewy Bodies (reviewed in (*4*)). Furthermore, hereditary early onset PD in humans is linked to genetic mutations in α-syn or copy number variation (*5, 6*).

α-Synuclein is a 140 residue, intrinsically disordered protein that is localized primarily to the presynaptic terminals of dopaminergic neurons (Figure 1) (*7*). The segment referred to as the Non Abeta Component (NAC), residues 61-95, is dominated by hydrophobic residues and tends to form aggregates leading to the parallel beta-sheet rich fibril structures present in Lewy Bodies (*8*). Aggregates from the NAC also contribute approximately 10% of the protein in the senile plaques in Alzheimer's disease patients (*9*). The N-terminal region of α-syn, encompassing the NAC (residues 9 – 97), possesses a series of 11-residue imperfect repeats that form an amphipathic alpha-helix when associated with lipid vesicles, a structure similar to exchangeable apolipoproteins (*10, 11*). The C-terminal tail is highly acidic and devoid of secondary structure. However, truncation of this segment produces

shortened lag time in fibril kinetics studies, suggesting a possible auto-inhibitory role against α-syn polymerization (*12*).

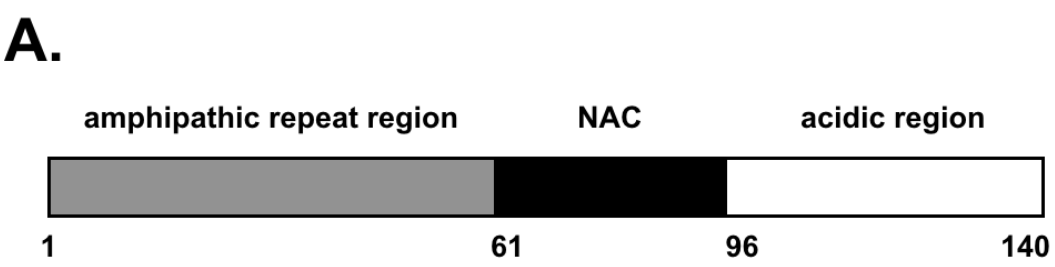

Figure 1. Features of the α-synuclein primary structure identifying (A) the three consensus segments and (B) the amino acid sequence associated with each segment. Residues 9-97, encompassing the amphipathic repeat region and the NAC, form an extended helix when associated with lipid membranes.

While the primary cause of α-syn aggregation in PD is unknown, there is a growing body of evidence implicating environmental factors such as long-term exposure to heavy metals (*13-15*). Lewy bodies have increased levels of iron, zinc and copper relative to other brain tissues (*16, 17*). It is well-documented that α-syn interacts with Cu^{2+}, a prevalent species of the Cerebrospinal Fluid (CSF), leading to

enhanced aggregation and *in vitro* polymerization (*18-21*). Moreover, α-syn-Cu^{2+} nucleates the aggregation of Aβ in extracellular senile plaques (*22*).

The interaction between α-syn and Cu^{2+} may also play a role in the protein's normal physiological function. Other neurodegenerative proteins, such as Aβ in Alzheimer's disease and PrP in the prion diseases take up copper (*23, 24*). Unambiguous functions have not yet been identified in these cases, but the regulation of copper homeostasis and redox activity are common themes. Most α-syn is intracellular, where copper is found predominantly in the Cu^{+} state, but a fraction is secreted to the oxidizing extracellular space that favors Cu^{2+}. Synaptic Cu^{2+} concentrations range from 2 – 200 μM, well in excess of the α-syn-Cu^{2+} affinities measured by most laboratories. Moreover, the *substantia nigra* is part of the *basal ganglia*, which possesses among the highest copper concentrations of the CNS (*20, 25*). Perhaps α-syn is another player in the line of defense against uncomplexed copper, with action localized to the membrane surface. Moreover, Cu^{2+} facilitates oxidation of the N-terminal methionine in α-syn, which hinders in vitro fibril formation (*26*).

Despite the need to clearly characterize the interaction between α-syn and Cu^{2+}, there is still uncertainty regarding the α-syn-Cu^{2+} binding stoichiometry, affinity and coordination structure (reviewed in(*27*)). Potentiometric studies performed on α-syn derived N-terminal peptides suggest that Cu^{2+} is coordinated by nitrogens from the N-terminal amine, the Met1 backbone amide, and the His50 imidazole, and an oxygen from the Asp2 carboxylic acid (*28, 29*). These findings are consistent with Trp fluorescence quenching experiments, which also show a

preference for copper binding to the N-terminal region. Electron paramagnetic resonance (EPR) on full length (140 residue) α-syn suggests a coexistence of structures involving the N-terminus and His50, and that His50 participation may be facile (*30*). NMR line intensity measurements contrast this and suggest instead that there are no long range coordination structures, but rather a series of local Cu^{2+} sites distributed about the N-terminus, His50, and several areas of negatively charged residues in the C-terminal tail (*31*).

Reported affinity measurements also yield variation. Circular dichroism titrations identify two Cu^{2+} sites with dissociation constants of 0.7 μM and 60μM (*32*). Fluorescence quenching probed by a Trp at position four identifies tighter interaction, with a dissociation constant of 100 nM (*33*). Finally, isothermal titration calorimetry (ITC) of exchange with a soluble copper-glycine complex finds a single Cu^{2+} site in wild type α-syn, with a dissociation constant of approximately 0.2 nM (*34*).

While most of the studies above point to the involvement of the α-syn N-terminal domain in Cu^{2+} uptake, the role of other protein segments, most notably His50, remain unclear. And as noted above, reported affinity measurements vary by approximately three orders of magnitude. Here we apply continuous wave (cw) and pulsed EPR to wild type and a panel of mutant α-syn species, at varying stoichiometric ratios. There are three elements to this study. First, through titration studies, we examine the number of Cu^{2+} binding sites, and more generally, how the protein responds to increasing levels of the metal ion. Next, using mutagenesis, we identify the residues responsible for the primary binding sites. Finally, competition

studies are applied to evaluate affinity. While our studies support the involvement of the α-syn N-terminal domain, we find strong evidence for participation of His50, especially at 1:1 copper:protein. Moreover, we find that the affinity at this ratio is very high, with a K_d value consistent with the lowest reported value in the current literature, thus pointing to a physiological role for the α-syn-Cu^{2+} interaction.

Materials and Methods

Proteins and Reagents

The wtα-syn gene cloned into *pRK172/α-synuclein* plasmid vector was a generous gift from the Fink lab at UCSC. The primers for the mutations H50A and Q98Stop were obtained from Invitrogen. Mutations were performed using the GeneTailor™ Site Directed Mutagenesis System (Invitrogen Cat. Nos. 12397-014 and 12397-022). α-syn, α-syn(1-97), and α-syn(H50A) were recombinantly expressed in *Escherichia coli* BL21(DE3) competent cells (Invitrogen, Carlsbad, CA) using an auto-induction procedure of Kim et al. described previously (*35*). Cells were harvested by centrifugation followed by sonication in lysis buffer (50mM NaCl, 20mM Tris, 0.2mM PMSF(phenylmethylsulfonylfluoride), 10%v/v Triton-X100 (Sigma, Switzerland) pH=7.4). Purification was performed using ammonium sulfate precipitation followed by centrifugation, resuspension in 6M guanidine HCl and reverse-phase HPLC. All peptides were synthesized using fluorenylmethoxycarbonyl (Fmoc) methods as described previously (*36*).

Electron Paramagnetic Resonance

Samples were prepared in degassed buffer containing 25mM MOPS buffer and 25%v/v glycerol, where the glycerol served as a cryoprotectant. All continuous wave X-band spectra (ν = 9.44GHz, microwave power in the range of 0.6-5.0 mW, modulation amplitude of 5.0 G, and sweep width 1200G) were collected at approximately 125K, using a Bruker EleXsys spectrometer and an SHQ (Bruker) cavity equipped with a variable temperature controller. Competition assays were

performed as described in text and resultant composite spectra were analyzed using non-negative least-squares (NNLS) in the Matlab program suite. Three-pulse ESEEM measurements were obtained at 20K on a Bruker E580 X-band spectrometer using a dielectric resonator and an Oxford CF 935 cryostat. A $\pi/2$-τ-π-T-$\pi/2$-τ-echo sequence with pulse lengths of 12, 24, and 12 was used. Initial value of τ = 136ns and T was lengthened in 799 steps of 12ns each with 100 samples per step.

Dynamic Light Scattering

Hydrodynamic dimensions were estimated using Dynamic light scattering on a DynaPro Molecular Sizing Instrument (Protein Solutions, Lakewood, NJ) using a 1.5-mm path length 12μl quartz cuvette. All experiments were carried out at 35uM protein in 75mM MOPS/NEM buffer at pH=7.4 after 20min centrifugation at 13000rpm.

Results

Stoichiometry

Copper to α-syn binding stoichiometry was determined by EPR titration at pH 7.4 and referenced against a calibrated copper-EDTA standard. Specifically, Cu^{2+} (delivered as copper acetate) was titrated to a fixed concentration of protein. The integrated cw EPR spectrum (double integral of the 1st derivative EPR signal) is directly proportional to the amount of bound Cu^{2+}. Normalized copper spectra at one, two and four equivalents are characteristic of a type 2 (oxygen and nitrogen) coordination environment, with well-resolved splittings between the parallel hyperfine lines (Figure 2). At one equivalent of Cu^{2+}, the EPR spectrum gives a single set of hyperfine lines, whereas at two or more equivalents, there is a shift towards lower field, along with the emergence of an additional overlapping spectrum. The implications of these spectra are discussed further below. Spectral integration vs added Cu^{2+}, shown in the inset, demonstrates that copper uptake reaches a maximum at two added equivalents. Consistent with saturation of α-syn at two copper equivalents, we observe no change in spectral details between two and four equivalents.

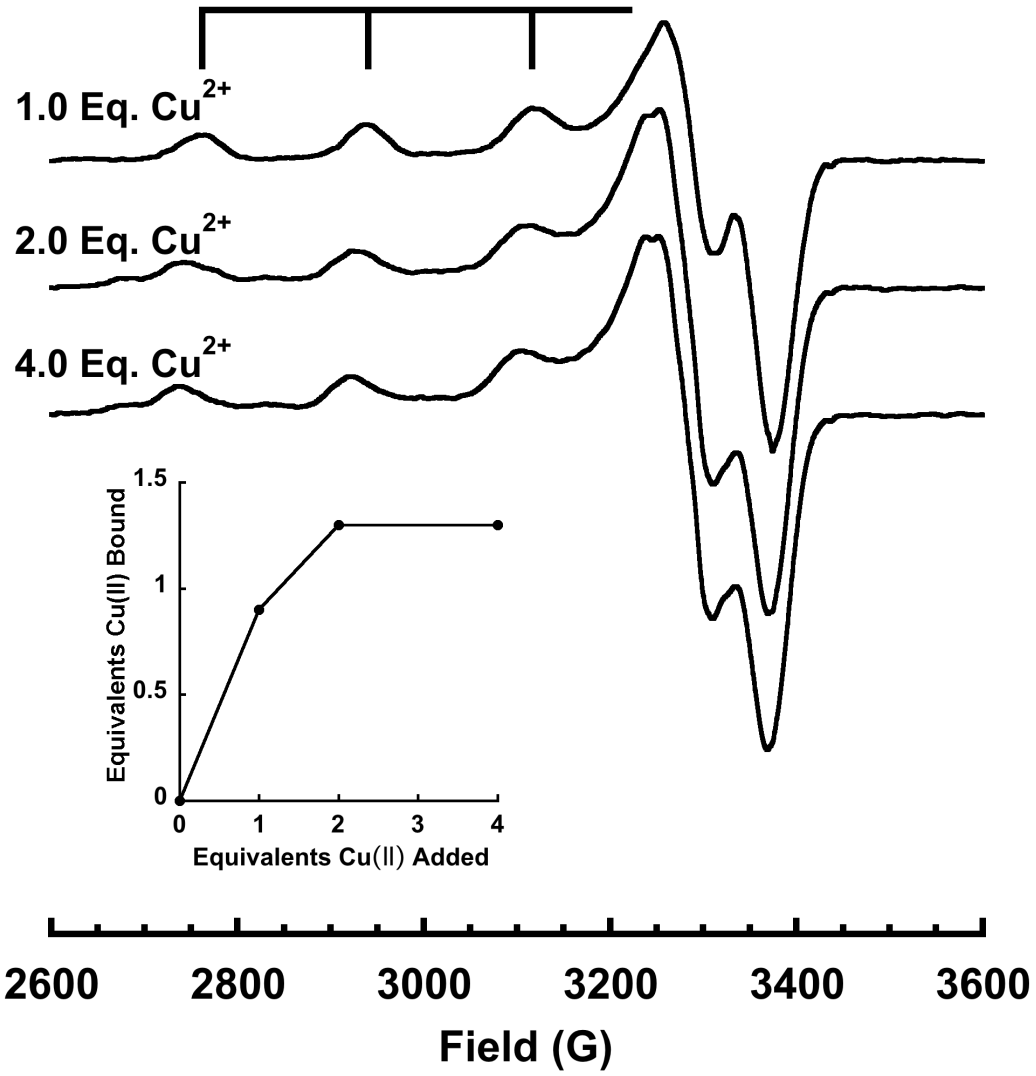

Figure 2. X-Band EPR spectra of α-syn at pH 7.4 with 1, 2, and 4 equiv of Cu^{2+}. Specra were recorded at 111 K, ν = 9.44 GHz, with a sweep width of 1200 G. The inset shows EPR-detected Cu^{2+} as a function of added Cu^{2+} concentration and demonstrates saturation at approximately 2.0 equiv. However, competition studies find that the second equivalent is weakly coordinated.

Although α-syn saturates at 2:1 copper:protein, the integrated spectra reveal only 1.3 bound equivalents (vertical axis of inset in Figure 2). This is in contrast to our previous work with the octarepeat domain of the prion protein, for example, where each added equivalent is directly reflected in the integrated EPR signal. We considered several possible mechanisms that would lead to a lower than expected

signal integral. First, with two bound Cu^{2+} ions, there could be diamagnetic coupling between the sites, giving rise to a singlet ground state. This is observed in multinuclear copper complexes and in proteins that have bridging imidazoles between Cu^{2+} centers. The coexistence of singlet and triplet states gives rise to a strong non-Curie temperature dependence. We examined the integral of α-syn with two added equivalents of copper from 20K to 120K (data not shown) and found typical 1/T dependence, which likely rules out coupled copper centers.

Next, we considered whether α-syn, in the presence of copper, formed dimers or well defined oligomers. For example, an α-syn dimer that saturates at three equivalents of copper would give a maximum copper to protein ratio of 1.5, approximately what is observed in the titration of Figure 2. We used Dynamic Light Scattering (DLS) to assess the protein hydrodynamic radius (R_H) and thus whether α-syn remains monomeric in the presence of copper. Experiments were performed with α-syn in buffer, and with one or two equivalents of Cu^{2+} (data not shown). In all cases, a single peak corresponding to a hydrodynamic radius of 3.3 ± 0.2nm dominated, with no evidence of dimers, trimers or other well defined oligomers. Moreover, the measured hydrodynamic radius is consistent with the expected value for a 140 amino acid random coil polypeptide (*38*).

Finally, we considered whether the second added equivalent is weakly bound and in equilibrium with aquo copper (as EPR silent $Cu(OH)_2$ complexes). To address this possibility, we used the prion-derived peptide HGGGW as a competitor. This well characterized species binds Cu^{2+} with a dissociation constant of 7.0 μM. Addition of 1.0 eq. of HGGGW to a 1.5:1 mixture of Cu^{2+}:α-syn completely

eliminated the α-syn EPR spectrum associated with the second Cu^{2+} equivalent. In its place we observed the characteristic spectrum of the Cu^{2+}-HGGGW complex and, to within experimental error, spectral integration accounted for 1.5 eq. of Cu^{2+}. Consequently, the peptide HGGGW efficiently competes away the second Cu^{2+} eq. from α-syn demonstrating a low affinity interaction characterized by a $K_d >> 7$ μM. We conclude from these experiments that while α-syn takes up two eq. of Cu^{2+}, the second eq. binds with low affinity and is not physiologically important. These results are in good agreement with recent findings by Hong and Simon who used isothermal titration calorimetry to identify a single high affinity copper site in α-syn.

Identification of the Cu^{2+} Coordination Features

To determine the smallest segment of wt α-syn that possesses all the functional groups that directly coordinate Cu^{2+}, we developed a panel of truncated and chemically modified α-syn variants. These include α-syn(1-10), α-syn(1-56), α-syn(1-97), and several N-terminal acetylated species. At a single equivalent of Cu^{2+}, α-syn(1-56) and α-syn(1-97) give spectra that nearly overlap with wild type. However, upon addition of a second Cu^{2+} equivalent, only α-syn(1-97) shows a shift in the parallel region similar to that observed in full-length α-syn. In contrast, while α-syn(1-10) takes up a single equivlent of Cu^{2+}, the bound species gives an EPR spectrum that does not overlap with 1:1 Cu^{2+}: α-syn(1-97) or 1:1 with full-length. Acetylation of any of the α-syn variants completely abrogates copper uptake. Together, these data demonstrate that the α-syn N-terminus participates in Cu^{2+}

coordination, (*39*) but residues beyond the first ten are required to recapitulate the coordination environment of wild type α-syn.

To identify the specific residues required for copper coordination, point mutations were introduced into both synthetically-produced peptides and recombinantly expressed α-syn protein. $A_{||}$ and $g_{||}$ values derived from the EPR spectra (shown in table 1), are consistent with a 3N1O coordination environment, as determined from Peisach-Blumberg correlations. In addition, previous potentiometric studies suggest that Asp2 is involved in Cu^{2+} binding (*28, 29*). Indeed, peptide coordination of metal centers with the 2nd aspartate residue following a free peptide N-terminus give very stable Cu^{2+} complexes (*40*). To directly test for this, we prepared α-syn(1-56) with a D2A point mutation. EPR spectra 1:1 Cu^{2+} complexes with α-syn(1-56) and α-syn(1-56, D2A) reveal differences in the intense perpendicular features at approximately 3300 G and in the parallel hyperfine splittings, in turn suggesting a direct interaction with the aspartate side chain.

Table 1 EPR Parameters

Protein	$g_{\|\|}$	$A_{\|\|}$ (G)
α-synuclein	2.226	178.6
α-syn(1-10)	2.245	183.3
α-syn(1-56)	2.229	173.2
α-syn(1-97)	2.226	178.6
α-syn(H50A)	2.242	176.6

Next we considered His50. Although distal from the α-syn N-terminus, imidazole is an avid copper binding group and experiments above suggest participation from residues beyond the first ten. EPR spectra from wild type α-syn and α-syn(H50A), each with a single eq. of Cu^{2+}, were compared as shown in (Figure 3). The parallel region for the mutant species exhibits great splitting (larger A_{II}) consistent with replacement of an equatorial nitrogen with an oxygen. Moreover, this spectrum does not overlap with that of wild type with either 1.0 or 2.0 eq. Cu^{2+}.

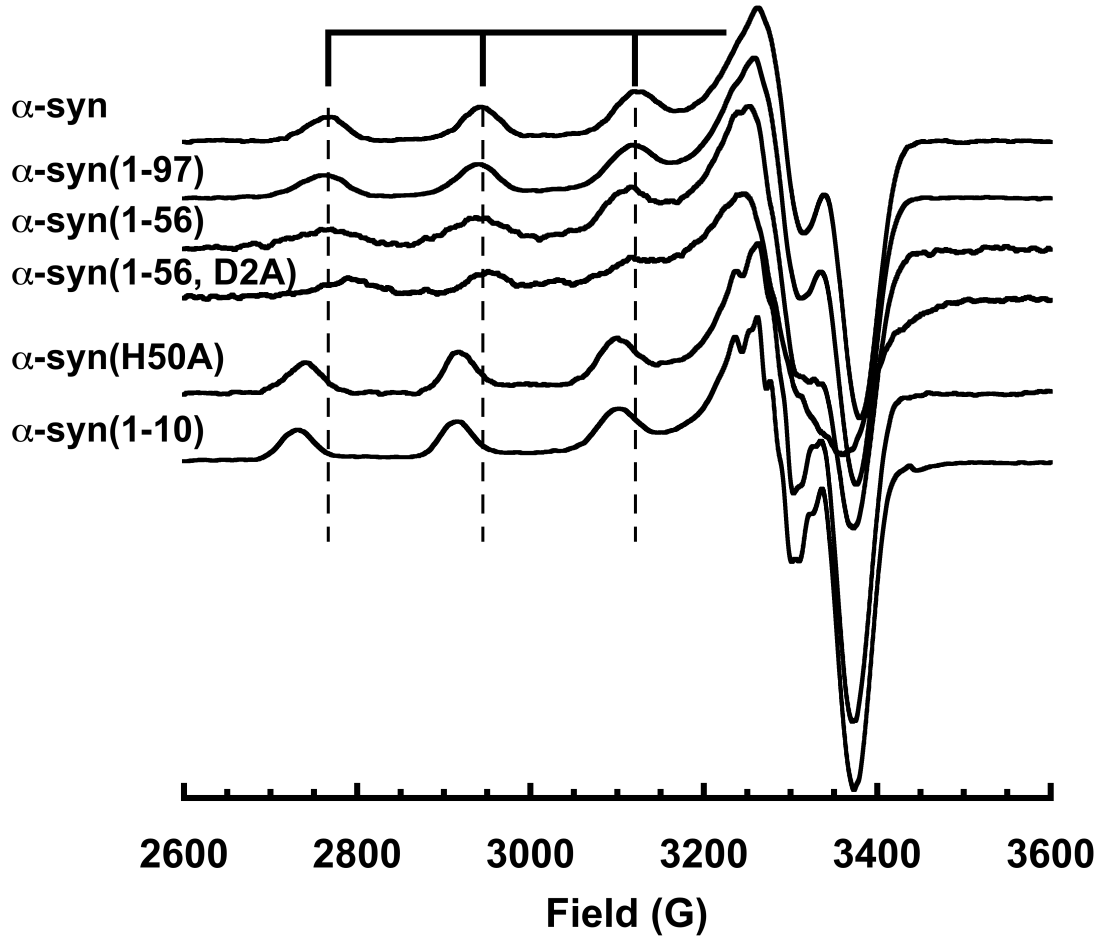

Figure 3. X-Band EPR spectra of α-syn, mutants, and truncated species. Vertical lines correspond to the parallel hyperfine features of wild-type α-syn. α-syn(1-97) gives a spectrum that superimposes on that of the wild type, but all other species exhibit significant variation. α-syn(1-10) and α-syn(H50A) give equivalent spectra, but they are distinct from that of the wild type, demonstrating the involvement of His50 in the coordination sphere.

To further assess the involvement of H50 in Cu^{2+} coordination, we used Electron Spin-Echo Envelope Modulation (ESEEM). ESEEM is a pulsed EPR technique with sensitivity to spin-active nuclei approximately 10 Å of the paramagnetic copper center (*41*). At X-band frequencies, the distal ^{14}N (I = 1) of a coordinated imidazole ring gives characteristic quadrupolar transitions and is diagnostic for interacting His side chains (*42*). The FT ESEEM of α-syn with 1.0 eq. Cu^{2+} shown in figure 4 is typical for imidazole, with three low frequency peaks that correspond to transitions among ^{14}N quadropolar levels in exact cancellation, as well as the ≈ 4 MHz peak from the non-canceled electron spin manifold. We also find that the α-syn(H50A) fails to give an ESEEM spectrum with 1.0 eq. Cu^{2+}. Together, these findings demonstrate unequivocally the equatorial coordination by the H50 imidazole (*36*). The spectra in (Figure 4) further show that the H50 coordinates Cu^{2+} in wild type α-syn and in truncated α-syn(1-97) even in the presence of excess metal ion. Addition of 2.0 Cu^{2+} eq., however, brings out additional low intensity peaks at approximately 2 and 2.8 MHz. Past work from our lab on the octarepeat domain of the prion protein demonstrated that these lines can arise from the ^{14}N of an amide group coordinated through the carboxyl oxygen, as shown in the spectrum for Cu^{2+}-HGGGW. The appearance of these transitions in α-syn suggests that the weakly bound second Cu^{2+} eq. coordinates in a similar fashion.

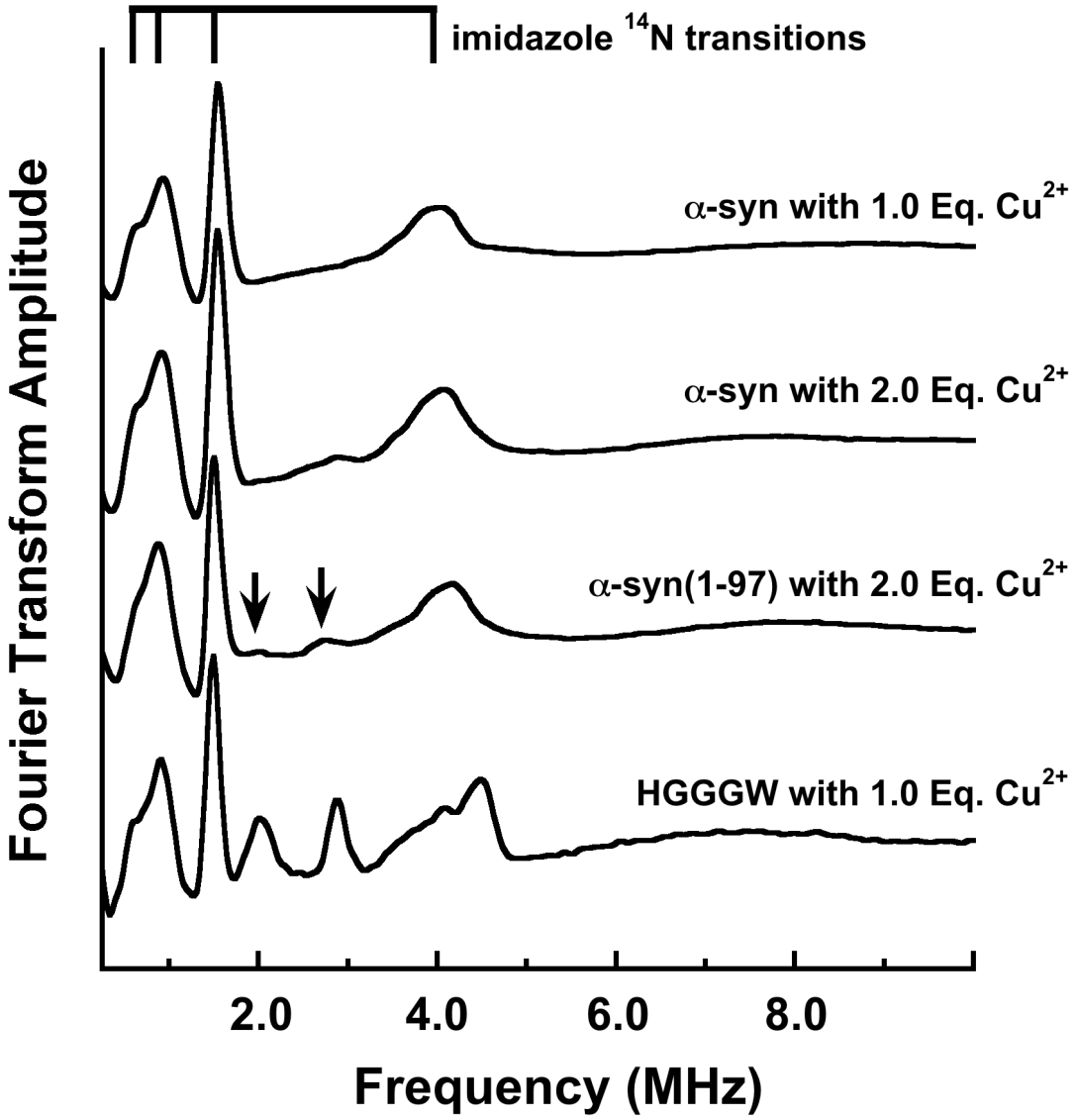

Figure 4. Three-pulse ESEEM spectra of α-syn with 1.0 and 2.0 equiv of Cu^{2+}, α-syn(1-97) with 2.0 equiv of Cu^{2+}, and prion protein sequence HGGGW with 1.0 equiv of Cu^{2+}. These spectra reveal the expected quadrupolar transitions associated with an imidazole remote nitrogen and demonstrate coordination by His50. α-syn(H50A) fails to give an ESEEM spectrum. α-syn(1-97) with 2.0 equiv of Cu^{2+} gives additional weak features at 2.0 and 2.8 MHz (arrows), similar to peaks observed for the HGGGW peptide and assigned to an amide nitrogen coordinated through the backbone carbonyl.

Additional tests were performed to evaluate whether the H50 segment alone of α-syn alone is capable of taking up Cu^{2+} with high affinity. We prepared an acetylated 21 residue peptide corresponding to a segment of α-syn with H50 in the

center (α-syn(39-60)). Titration up to 1.0 eq. Cu^{2+} gave a very weak EPR spectrum (reflecting < 10% of added copper) inconsistent with a bound species.

Binding Affinity

To evaluate the dissociation constant, K_d, of the Cu^{2+}-α-syn complex, we used an EPR competition technique previously developed in our lab (*24*). High affinity competitors that take up Cu^{2+} with a 1:1 stoichiometry are added to a Cu^{2+}/α-syn solution. Both oxidized glutathione and pentaglycine peptides are well characterized chelators and give Cu^{2+} EPR spectra that are distinct from that of the Cu^{2+}-α-syn complex. Spectral decomposition gives the ratio of copper bound to α-syn and specific competitor. Analysis using the known K_d of the competitor determines the α-syn dissociation constant. With this approach, the amount of competitor may be varied to insure that both bound species give resolvable EPR spectra of similar signal strengths. Table 2 shows that wt α-syn binds one equivalent of Cu^{2+} with a K_d of either 0.11 nM or 0.15 nM, as determined from independent experiments with pentaglycine or oxidized glutathione, respectively. These values are approximately five orders of magnitude lower than the > 7 uM K_d found for the second equivalent, as described above. Consequently, these data further support the finding that α-syn takes up only a single eq. of Cu^{2+} with high affinity. To test for H50 coordination, we also performed competition experiments on the α-syn H50A mutant. As determined from both competitors, this species exhibits an approximately four-fold lower affinity than wild type.

Table 2 Dissociation Constants (nM) Determined from Competition Studies

Protein/Competitor	Pentaglycine (K_d = 40 nM)	Oxidized Glutathione[a] (K_d = 0.066 nM)
α-synuclein	0.11 ± 0.03	0.15
α-syn(H50A)	0.40 ± 0.01	0.60

[a]K_d determinations using oxidized glutathione were performed once, and do not have standard errors.

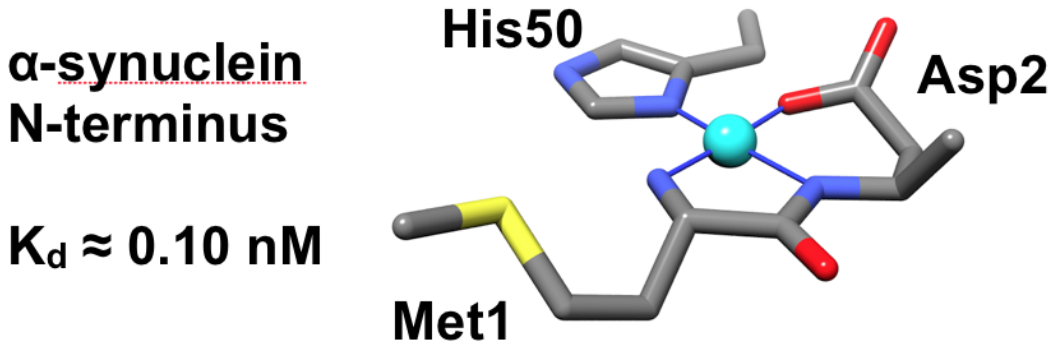

Figure 5. Cu^{2+} coordination structure of α-syn in solution and dissociation constant.

Discussion

Our EPR experiments demonstrate that the Cu^{2+} coordination environment in α-syn involves the N-terminal amine and the carboxylate side chain of Asp2. In addition, ESEEM reveals participation by the H50 imidazole. To account for 3N1O coordination, suggested by evaluation of the magnetic tensor values, we propose involvement of the Asp2 backbone amide nitrogen. Further supporting this assignment, the lack of amide ^{14}N couplings in the ESEEM spectra rule against Met1 backbone carbonyl coordination. Our findings at pH 7.4, suggest a well-defined coordination environment without evidence of structural heterogeneity. The coordination features are shown in Figure 5. Involvement of the N-terminal residues is consistent with studies from peptide based Cu^{2+} coordination complexes with an Asp as the second amino acid (*40*). And while α-syn may take up a second Cu^{2+} equivalent, the interaction is low affinity and likely unimportant to physiological function. In addition, we find that the dissociation constant for the first equivalent is approximately 0.10 nM.

Certain aspects of our findings support previously published work. For example, potentiometric measurements performed on α-syn segments suggest a similar coordination environment, marked by a pH sensitivity expected for N-terminal and backbone nitrogens, and a carboxylate group. Site specific tryptophan fluorescence studies further identify a 1:1 α-syn:Cu^{2+} complex with the N-terminal segment as the primary anchor point. MALDI mass spectrometry finds evidence of two Cu^{2+} binding sites with significantly different affinities, the tighter of which located to the N-terminus with a sub-micromolar dissociation constant. And previous

EPR experiments at pH 5.0 and 7.4 were interpreted to suggest a coexistence of two coordination spheres at the higher pH, distinguished by involvement of His50. While our findings certainly agree with the His-bound species, we do not find evidence for a second pH 7.4 coordination mode. The four-fold enhanced affinity we find with wild type α-syn vs the α-syn(H50A) mutant further supports imidazole coordination, and we do not find features of the α-syn(1-10) or α-syn(H50A) EPR spectra superimposed on wildtype.

Despite emerging consensus on the molecular details of the Cu^{2+} coordination sphere, there remains wide disagreement with regard to affinity. As noted in the Introduction, published values for the dissociation constant range from micromolar to nanomolar. Recently, Hong and Simon used a refined ITC approach whereby Cu^{2+} is added as a glycine complex. With appropriate treatment of the complex equilibria, they determine an association constant based on heat release through a copper titration. Wild type α-syn at pH 7.4 gives an association constant of 4.7×10^9 M^{-1}, corresponding to a K_d of 0.21 nM. Moreover, they find no evidence of a second Cu^{2+} coordination site. These results are in remarkable agreement with ours described here and support a very high affinity, mononuclear site with principal anchor points at the protein N-terminus.

The emerging biophysical evidence strongly suggests that α-syn interacts with Cu^{2+} *in vivo*. The residues involved in copper chelation are highly conserved, and the protein is abundant within the cell and localized in an area of constant, rapid membrane modification at the synaptic cleft where CSF Cu^{2+} levels exceed micromolar concentrations (*43*). The sub-nanomolar α-syn-Cu^{2+} K_d, along with the

abundance of synaptic α-syn, suggests that any Cu^{2+} localized to the extracellular membrane would be tightly complexed. Recent evidence suggests that copper bound to α-syn within neurons of the *substantia nigra* is stabilized in the +2 oxidation state (*44*). An intriguing possibility then is that perhaps α-syn sequesters Cu^{2+} at the membrane and therefore serves as a first line of defense against copper's inherent redox activity.

The interaction of α-syn intra-cellular with synaptic vesicles is well documented, and new evidence shows that wt α-syn interacts with SNARE complexes, perhaps as a chaperone, participating in membrane fusion (*45, 46*). Although membrane-associated α-syn is largely helical, an extended α-helix structure is incompatible with the polypeptide wrapping back to coordinate Cu^{2+} with the N-terminus and His50. We propose, therefore, Cu^{2+} provides a mechanism for α-syn release from the synaptic vesicle membrane, upon exposure to the extracellular environment, switching from the membrane-induced helical structure to the Cu^{2+}-bound structure shown in figure 5. This same mechanism may also operate in PD. A copper mediated weakening of the interaction between α-syn and cellular membranes could increase the soluble protein fraction thus hastening fibrillogenesis.

In summary, α-syn interacts strongly with Cu^{2+} *in vitro*. Whether this interaction is part of the protein's natural function, a component in the disease process, or both, is the subject for further study. Our results show that monomeric α-syn binds one equivalent of Cu^{2+} with ≈ 0.2 nM affinity. This binding mode creates a protein conformational change bringing the N-terminus and H50 in close proximity. Any additional Cu^{2+} association with the protein is weak, non-specific and not

physiologically relevant. Future work will focus on determining the role of this interaction on α-syn cycling at the cell surface, and the consequences in PD pathogenesis.

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CHAPTER 3

Copper Coordination to the Membrane Bound Form of α-Synuclein

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Abstract

Aggregation of the 140 amino acid protein α-synuclein (α-syn) is linked to the development of Parkinson's disease (PD). α-Syn is a copper binding protein with potential function as a regulator of metal dependent redox activity. Epidemiological studies suggest that human exposure to excess copper increases the incidence of PD. α-Syn exists in both solution and membrane bound forms. Previous work evaluated the Cu^{2+} uptake for α-syn in solution and identified Met1-Asp2 and His50 as primary contributors to the coordination shell, with a dissociation constant of approximately 0.1 nM. When bound to the membrane bilayer, α-syn takes on a predominantly helical conformation, which spatially separates His50 from the protein N-terminus and is therefore incompatible with the copper coordination geometry of the solution state. Here we use circular dichroism and electron paramagnetic resonance (continuous wave and pulsed) to evaluate copper coordination to the membrane bound form of α-syn. In this molecular environment, Cu^{2+} binds exclusively to the protein N-terminus (Met1-Asp2) with no participation from His50. Copper does not alter the membrane bound α-syn conformation, or enhance the protein's release from the bilayer. The Cu^{2+} affinity is similar to that identified for solution α-syn suggesting that copper coordination is retained in the membrane. Consideration of these results demonstrates that copper exerts its greatest conformational affect on the solution form of α-syn.

Introduction

Parkinson's disease (PD) is a common, age-related neurodegenerative disorder that affects over one million individuals in the United States (*1*). PD results from the loss of dopaminergic neurons in the *substantia nigra* region of the brain and produces slowness of speech and movement, uncontrolled tremors and difficult breathing (*2*). While the exact cause of PD is unknown, the neurological protein α-synuclein (α-syn) has been clearly linked to the pathology of PD in both animal and human studies. α-Syn is the primary component of the cytosolic, filamentous inclusions known as Lewy Bodies (LB), a hallmark of the disease ((*3, 4*), reviewed here (*5*)).

The 140 residue α-syn protein is natively unfolded in solution, but its seven imperfect 11-residue repeats (approximately residues 1 – 100, Figure 1)) adopt an amphipathic helical structure when associated with cellular membranes (*6, 7*). The protein also possesses a highly amyloidogenic NAC (non-Abeta component) region and a flexible C-terminal extension that is thought to interact with NAC and inhibit aggregation (Figure 1) (*8*). Although α-syn is found both inside and outside of the cell, it is localized primarily to the presynaptic terminals of dopaminergic neurons, an area of high vesicle traffic (*9*). Most α-syn research focuses on its role in the synucleinopathies, with emphasis on PD (*2-4, 10-12*). The normal physiologic function of α-syn is unknown, but recent work suggests a role in the formation of SNARE complexes that regulate vesicular-cell membrane fusion (*13-15*).

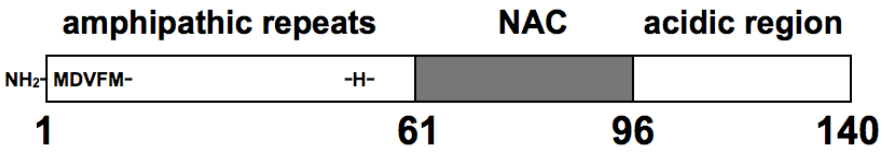

Figure 1. Features of the α-synuclein primary structure identifying the three consensus segments and the amino acids, at the N-terminus and His50, potentially involved in Cu^{2+} coordination. Residues 9-97, encompassing the amphipathic repeat region and the NAC, form an extended helix when associated with lipid membranes.

α-Syn takes up divalent copper ions with high affinity ($K_d \approx 0.1$ nM) (*16, 17*). Although Cu^{2+} is normally found at micromolar concentrations in the cerebrospinal fluid (CSF), epidemiological studies identify a significant link between long-term environmental exposure to copper and an increased incidence of fatal PD (*18-20*). Copper enhances the *in vitro* aggregation rate of the solution form of α-syn, suggesting that elevated Cu^{2+} levels may contribute to PD by accelerating the formation of Lewy bodies (*21-25*). The specific residues required for Cu^{2+} binding are highly conserved across species; the interaction between α-syn and Cu^{2+} may therefore play a role in the protein's normal physiological function. Other neurodegenerative proteins, such as Aβ in Alzheimer's disease and PrP in the prion diseases also take up copper (*26-30*). Unambiguous metalloprotein functions have not yet been identified in these cases, but disregulation of copper homeostasis and redox activity are emerging themes in neurodegenerative disease. α-Syn may serve as a sink for weakly complexed copper, with action localized to the membrane surface.

α-Syn is predominantly an intracellular species, however, the protein is exported to the extracellular space through exosomes in a calcium dependent manner, (*31*) and this process may be a factor in the pathogenesis of the PD and other synucleinopathies ((*32, 33*)). Extracellular α-syn is a component of the senile plaques of Alzheimer's disease, where it contributes approximately 10% of the total protein (*34, 35*). Moreover, elevated levels of α-syn are found in the CSF of patients with PD and related neurological diseases (*36-38*), and studies of tissue grafts with α-syn lesions show that aggregates propagate in a prion-like fashion ((*39*) reviewed here (*40*)).

Intensive work by a number of laboratories has identified Cu^{2+} coordination sites at several locations in the solution form of α-syn (*16, 25, 41-49*). (See recent reviews (*50-52*).) Our lab suggested a single high affinity Cu^{2+} complex ($K_d \approx 0.1$ nM) arising primarily from the N-terminal amine, the backbone nitrogen and side chain carboxyl of Asp2. In addition, the imidazole of His50 simultaneously coordinates this N-terminally bound Cu^{2+} (Figure 2a), resulting in the formation of a large polypeptide loop. In a membrane environment, where most α-syn resides, this type of coordination environment must be altered since a helical polypeptide conformation would separate the N-terminus and His50 by approximately 75Å, as diagrammed in Figure 2b. Here, using small unilamellar vesicles (SUVs), we characterize the copper coordination environment and affinity in the membrane bound form of α-syn. Our work follows the initial findings of Lucas and Lee who provided the first insights into copper uptake by membrane bound α-syn and showed that helix content and copper affinity at the protein N-terminus are increased in the membrane environment (*53*). In our study here, there are three elements. First, using electron paramagnetic resonance (EPR) and circular dichroism (CD), we evaluate the region in α-syn that takes up Cu^{2+} and the consequence of this interaction on the helical structure of membrane-bound α-syn. Next, using mutagenesis and EPR, we identify the residues responsible for the primary binding sites. Finally, competition studies are applied to evaluate affinity. We demonstrate that α-syn in its lipid membrane-bound state remains helical and takes up a single equivalent of Cu^{2+} at its N-terminus, with affinity and coordination environment similar to that found for the solution form. However, His50 no longer contributes to equatorial coordination regardless of Cu^{2+}

concentration, and copper does not influence the α-syn distribution between membrane and solution.

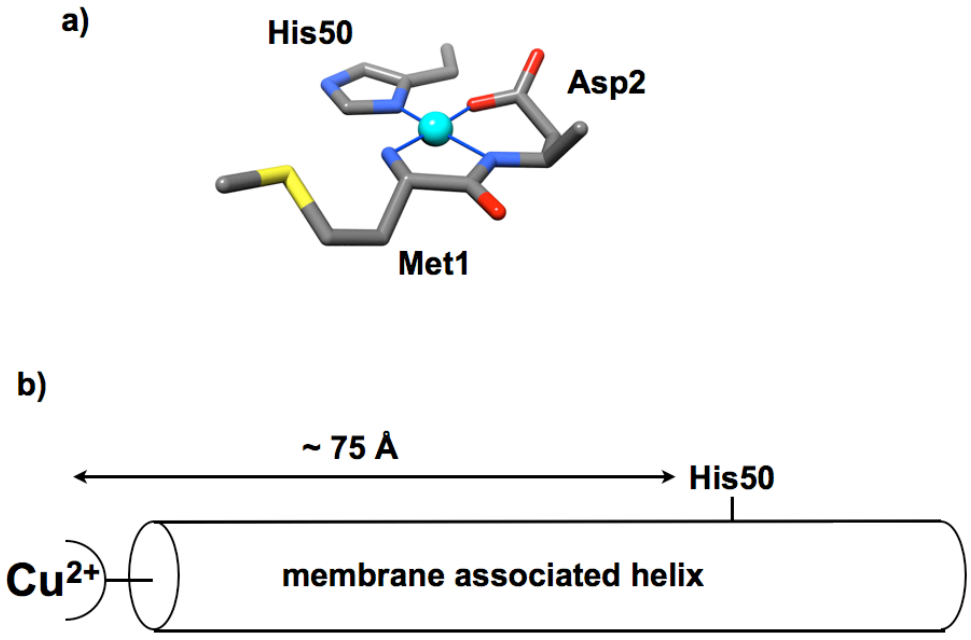

Figure 2. a) Coordination features of the primary solution Cu^{2+} site of α-syn. Competition studies show that this complex exhibits a dissociation constant of approximately 0.1 nM. b) α-Syn in its helical form, resulting from membrane association, would result in a separation of His50 from the N-terminus of approximately 75Å. Consequently, Cu^{2+} coordination details must be altered relative to that in solution.

Materials and Methods

Proteins and Reagents

The wt α-syn gene was cloned into *pet21* plasmid vector manufactured by Genescript. The primers for the H50A mutation were obtained from Invitrogen. Mutations were performed using the GeneTailor™ Site Directed Mutagenesis System (Invitrogen Cat. Nos. 12397-014 and 12397-022). α-syn and α-syn(H50A) were recombinantly expressed in *Escherichia coli* BL21(DE3) competent cells (Invitrogen, Carlsbad, CA) using an auto-induction procedure of Kim et al. described previously (*54*). Cells were harvested by centrifugation followed by sonication in lysis buffer (50mM NaCl, 20mM Tris, 0.2mM PMSF(phenylmethylsulfonylfluoride), 10%v/v Triton-X100 (Sigma, Switzerland) pH=7.4). Purification was performed using ammonium sulfate precipitation followed by centrifugation, resuspension in 6M guanidine HCl and reverse-phase HPLC (water and acetonitrile). The protein elutes between 63-60% acetonitrile on a C18 column (Grace Davidson Discovery Sciences, VYDAC HPLC column Cat.# 218TP101510).

Electron Paramagnetic Resonance

Samples were prepared in degassed buffer containing 25mM MOPS buffer and 25%v/v glycerol, where the glycerol served as a cryoprotectant. All continuous wave X-band spectra (ν = 9.44GHz, microwave power in the range of 0.6-5.0 mW, modulation amplitude of 5.0 G, and sweep width 1200G) were collected at approximately 125K, using a Bruker EleXsys spectrometer and an SHQ (Bruker) cavity equipped with a variable temperature controller. Competition assays were

performed as described previously (*16*) and in the text here, and resultant composite spectra were analyzed using non-negative least-squares (NNLS) in the Matlab program suite. Three-pulse ESEEM measurements were obtained at 20K on a Bruker E580 X-band spectrometer using a dielectric resonator and an Oxford CF 935 cryostat. A $\pi/2$-τ-π-*T*-$\pi/2$-τ-echo sequence with pulse lengths of 12, 24, and 12ns was used. Initial value of τ = 136ns and T was lengthened in 799 steps of 12ns each with 100 samples per step.

Small Unilamellar Vesicle Preparation

Vesicles were prepared using standard procedures outlined by Langen et al. (*7*) from a 70% Phosphotidyl Choline (POPC), 30% Phosphatidyl Glycerol (POPG) mixture (Avanti Polar Lipids). The chloroform was evaporated off with nitrogen gas and the lipids were resolubilized in H_2O and sonicated with a tip sonicator (Fisher Scientific model FB505) at 40% power, 30seconds on/40seconds rest for a total of 5 minutes on time. Lipids were deemed prepared when lipid/H_2O solution appeared clear. Electron microscopy verified the formation of 20 - 50 nm diameter spheres, with no indication of tubes or other unusual structures (see Supplement).

Most phospholipids bind Cu^{2+}, however, the specific head group plays a significant role in controlling affinity. Lipids with head groups that contain free amines, such as phosphatidylserine, bind Cu^{2+} quite strongly, and produce a dominant EPR spectrum (data not shown) (*55-57*). We sought a lipid composition that would allow for α-syn association but without strong Cu^{2+} coordination and found that a

mixture of 70% phosphatidylcholine (neutral) and 30% phosphatidylglycerol (negative charge) produces these desired results.

Results

Membrane Bound α-Syn Binds Cu^{2+}

As discussed above, and emphasized in Figures 1 and 2, simultaneous coordination by the N-terminus and His50 would be incompatible with the lipid bound α-syn helical structure, which separates those polypeptide segments by approximately 75 Å. To evaluate Cu^{2+} uptake in membrane bound, helical α-syn, we used CD and X-band continuous wave EPR. Samples were prepared with lipid SUV and protein and incubated for ~15minutes before flash freezing with liquid N_2. Lipid composition for the POPC/POPG SUVs was chosen to minimize direct Cu^{2+}-membrane interactions. All samples containing α-syn and SUV used for EPR experiments were found to have room temperature CD spectra corresponding to an alpha helix. The EPR spectra in Figure 3 show the typical Cu^{2+} hyperfine couplings in the parallel region (2700 – 3300 Gauss) observed for oxygen/nitrogen rich equatorial coordination and thereby demonstrate that α-syn binds Cu^{2+} in the presence of lipid SUV. Interestingly, the hyperfine lines in the EPR spectra for 100-200X lipids appear to be broadened in comparison to the α-syn in solution spectra, suggesting more than one coordination species at this lipid concentration. To quantify the relative species of protein bound we analyzed the EPR spectra in Figure 3 using a NNLS program with basis sets corresponding to α-syn-Cu^{2+} and α-syn(H50A)-Cu^{2+} spectra. The α-synH50A mutant lacks the His imidazole that contributes to the equatorial coordination observed in the solution α-syn-Cu^{2+} complex, and thus takes up copper only at the N-terminus (*16*). As shown in Figure 3 (inset), at a lipid:protein ratio of 300:1 and greater, the EPR spectra appear to correspond solely to the α-synH50A

spectrum, suggesting that Cu^{2+} is bound solely to the polypeptide N-terminus. To ensure that the EPR spectra are the result of Cu^{2+} binding α-syn and not another chelator, such as the bilayer phosphate head groups, spectra were taken with Cu^{2+} and SUV's alone for comparison. The lipid-Cu^{2+} spectrum exhibits a B_o downfield shift of ~150G (compared to α-syn-Cu^{2+} in the presence of lipids) and a different pattern of hyperfine splitting that suggests high O ligand character (see below). Furthermore, the addition of Cu^{2+} to SUV results in a spectrum that, when doubly integrated to determine the concentration of paramagnetic species bound, consistently shows less than the full amount of added Cu^{2+}. This evidence suggests the POPC/POPG lipid SUV's bind Cu^{2+} very weakly. Indeed when we add α-syn to the lipid-Cu^{2+} solution, the spectra revert to that obtained in the samples shown in Figure 3.

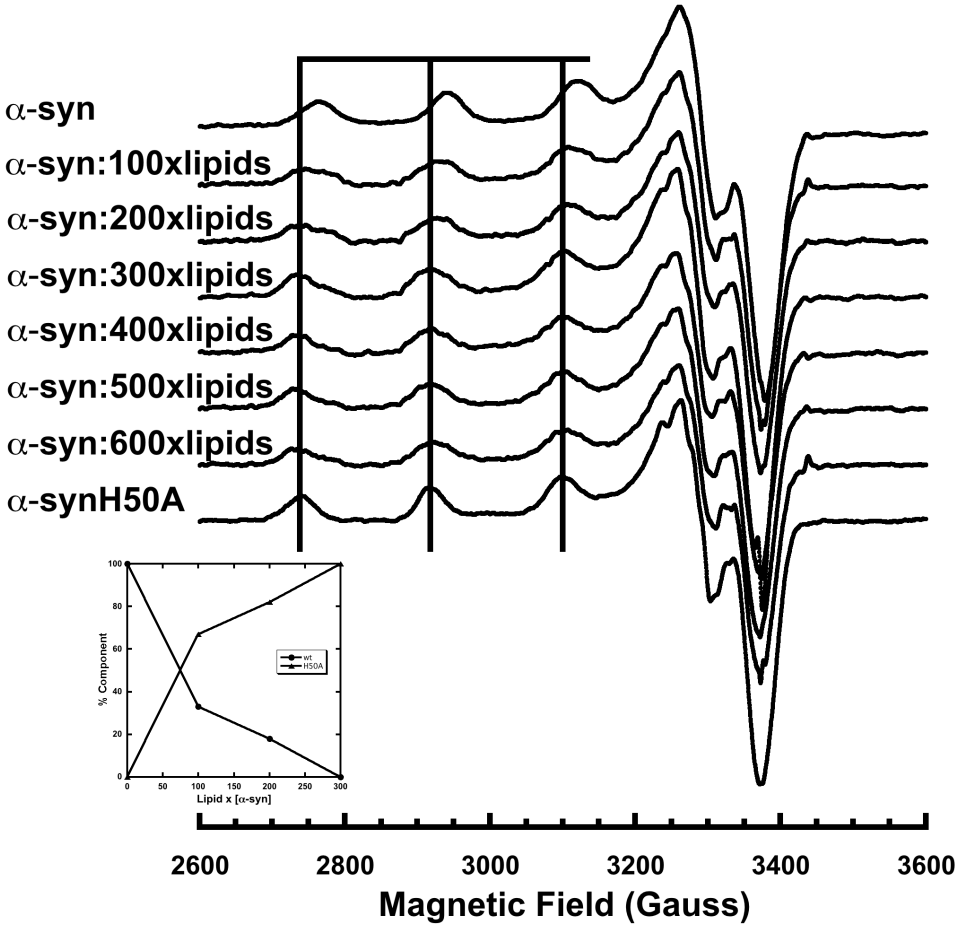

Figure 3. X-Band EPR spectra of α-syn (50 μM) at pH 7.4 with 1 equivalent of Cu^{2+} in the presence of increasing molar equivalents of lipid molecules in the form of vesicles. The vertical lines correspond to the parallel hyperfine features of the α-synH50A mutant in solution. α-syn in the presence of lipids produces a similar if somewhat broadened spectra that has a downfield shift corresponding to an increase in lipid SUVs. At a lipid:protein ratio of 300:1 the α-syn spectra essentially overlaps the α-synH50A mutant spectra. Spectra were recorded at 111K, ν = 9.44 GHz, with a field sweep of 1200G.

Copper Does Not Affect the CD Spectra of Membrane Bound α-Synuclein

In the presence of lipid bilayers residues ~10-90 of α-syn have been shown to form a single α-helix (*7, 58*). The lack of participation of His50 in Cu^{2+} coordination suggests that the α-syn helical structure in the SUV membrane environment should remain intact with copper bound. We tested this directly by evaluating the CD spectra as a function of lipid molecule to protein ratio. Dichroic peaks with negative intensity at wavelengths at 208nm and 222nm provide a measure of helical structure (*59*). As observed in Figure 4a, α-syn achieves it's maximum alpha-helical signal intensity at a lipid:α-syn ratio of 300:1. The mean residue ellipticity (MRE) at 222 nm is approximately 24,000 deg cm^2 $dmol^{-1}$, consistent with that previously reported (*53*). Using the 300:1 lipid:protein ratio we then conducted CD experiments with α-syn and lipids in the presence of Cu^{2+}. Figure 4b shows that there is no change in the CD signal with the addition of 1.0x and 10.0x Cu^{2+}, suggesting that α-syn copper binding has no detectable affect on α-syn helicity in the presence of SUVs. We note that excess Cu^{2+} results in an EPR spectrum showing the N-terminal site, along with a weak spectrum consistent with that observed for copper and lipid alone (Figure 6).

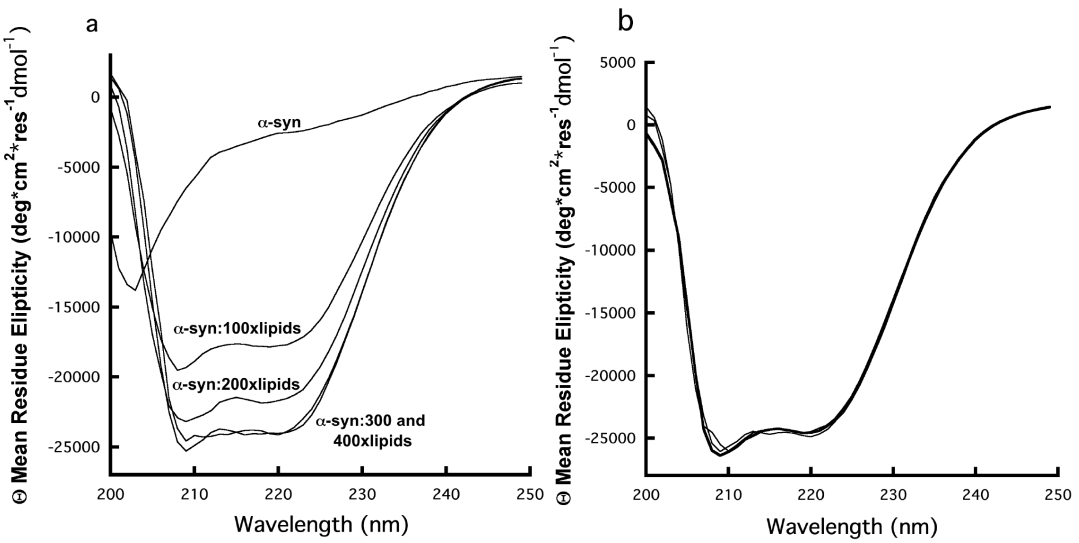

Figure 4. a) CD spectra of α-syn as a function of lipid addition in the form of SUVs. From top to bottom: 10μM α-syn no lipids, 10μM α-syn:1mM lipids, 10μM α-syn:2mM lipids, 10μM α-syn:3mM lipids, 10μM α-syn:4mM lipids. CD signal intensity reaches a maximum at a lipid:α-syn molar ratio of 300:1. b) Cu^{2+} titration of 10μM α-syn:3mM lipid complex. Cu^{2+} concentrations of 0, 10 and 100μM give overlapping α-syn CD spectra.

Identification of Cu^{2+} Coordination Features

To evaluate the Cu^{2+} coordination environment in lipid-bound α-syn, we developed mutations of the key copper-binding α-syn residues previously identified for α-syn in solution. Figure 3 demonstrates using EPR that the greater the concentration of lipid SUV's the more the α-syn copper-binding spectrum shifts downfield to give a spectrum that is within experimental error of that obtained for the α-syn H50A mutant. This evidence, in addition to the aforementioned CD lipid titration spectra, suggests that His50 is not involved in lipid-bound α-syn copper coordination.

To further assess the potential involvement of His50 in α-syn-Cu^{2+} coordination, we used electron spin echo envelope modulation (ESEEM). ESEEM is a pulsed EPR technique with sensitivity to spin-active nuclei within approximately 10Å from the paramagnetic copper center. At X-band frequencies, the distal ^{14}N (I = 1) of a coordinated imidazole ring gives characteristic quadrupolar transitions and is diagnostic for interacting His side chains. The FT ESEEM of solution α-syn with 1.0 equivalent of Cu^{2+} shown in Figure 5a is typical for imidazole, with three low-frequency peaks that correspond to transitions among ^{14}N quadropolar levels in exact cancellation, as well as the ≈ 4.0 MHz peak from the non-cancelled electron spin manifold. Progressive addition of SUVs, measured by lipid concentration, produces a corresponding decrease in the 4.0 MHz peak (see Figure 5 inset). We also find that the lipid SUV alone and lipid SUV with α-syn H50A (not shown) fail to give an ESEEM spectrum with 1.0 equivalents of Cu^{2+}. Together these assays unequivocally

show that the H50 imidazole of α-syn does not coordinate Cu^{2+} when the protein is in its membrane bound, helical state.

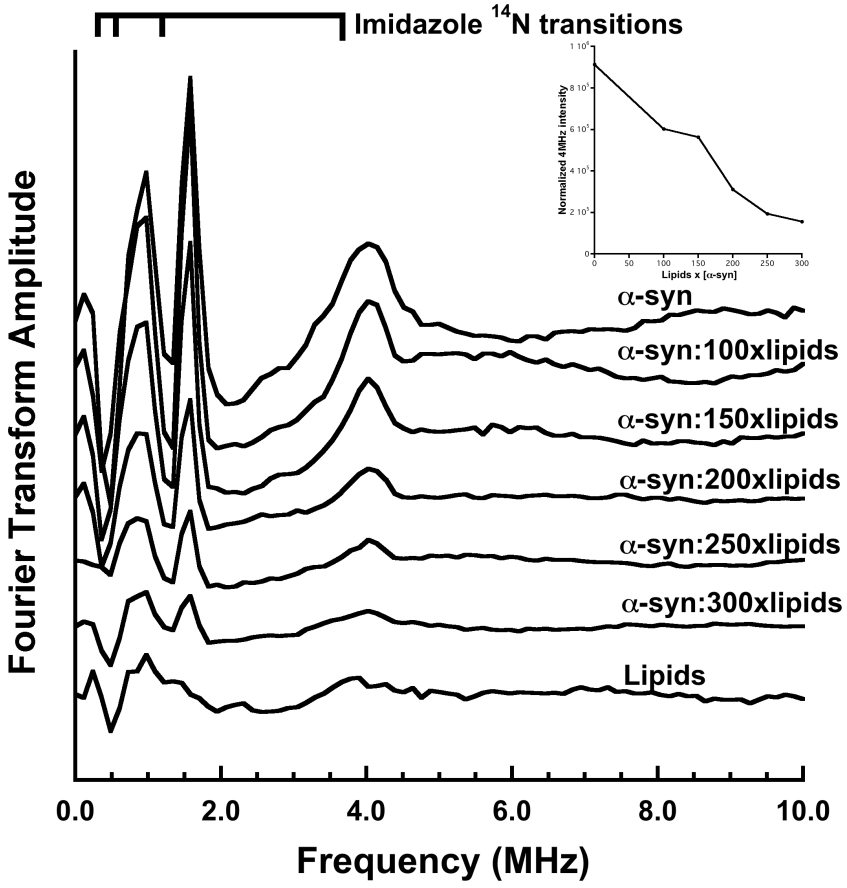

Figure 5. Three-pulse ESEEM spectra of α-syn (50μM) with 1.0 equivalent of Cu^{2+} in the presence of increasing lipid SUV concentration. The spectra of the samples containing α-syn alone reveal the expected quadrupolar transitions associated with the imidazole remote nitrogen and demonstrate coordination by His50 whereas the lipids alone do not. The 4MHz peak is indicative of the non-canceled electron spin manifold from the Cu^{2+}-imidizole far nitrogen interaction. Those samples containing α-syn and lipid SUV demonstrate a decrease in intensity of the 4MHz peak with increasing lipid concentration relative to α-syn in solution. Inset shows normalized 4MHz peak intensity vs. sample lipid concentration.

Our laboratory previously demonstrated that the Met1 and Asp2 form the essential ligands of the Cu^{2+} coordination shell (Figure 2). In order to assess whether this H_2N-M-D- coordination motif remains intact in lipid-bound α-syn, we created a 139-residue truncated D2A mutant protein (α-syn N-trunc). Expression of this mutant in *E. coli* results in cleavage of the N-terminal methionine thereby changing the N-terminal sequence from NH_2-MAVF- to NH_2-AVF- (*60*) (MS verification in the Supplement). Figure 6 shows that the α-syn N-trunc mutation gives a spectrum equivalent to that obtained in lipid alone, demonstrating that the N-terminal sequence NH_2-M-D- is necessary for high-affinity copper coordination when α-syn is in its helical form.

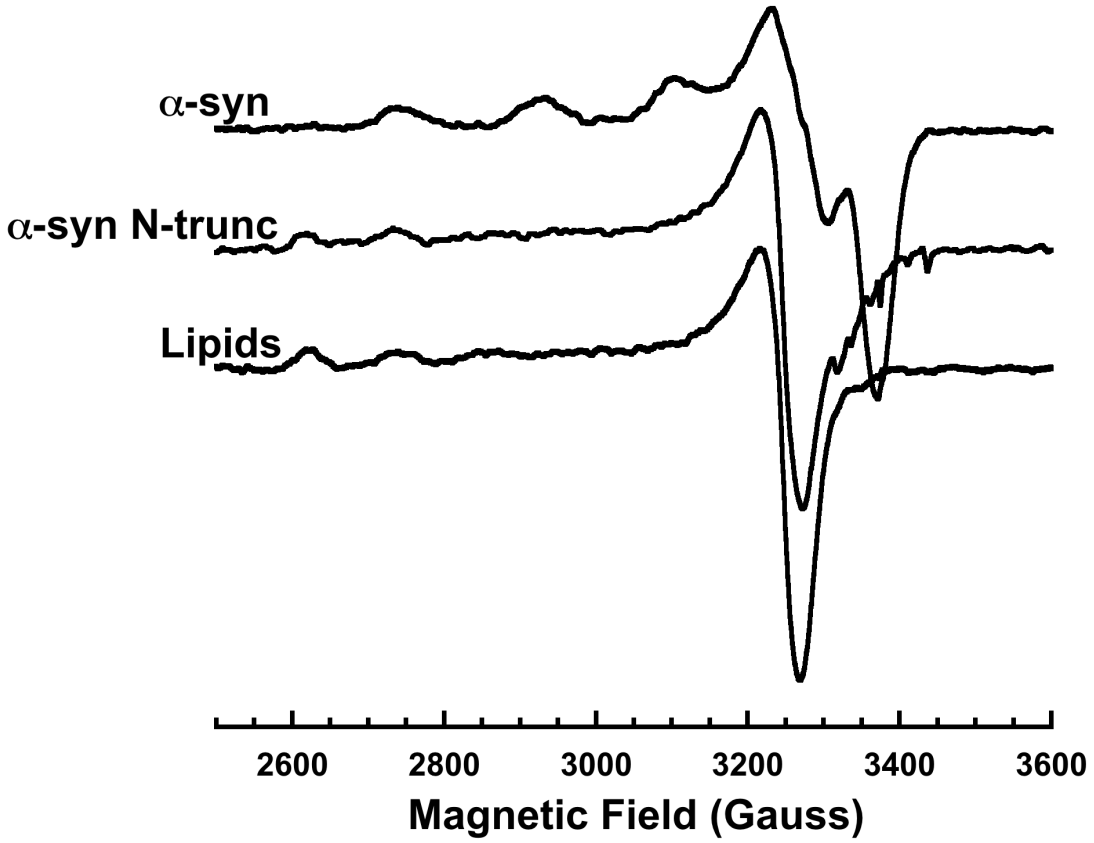

Figure 6. X-Band EPR spectra of α-syn and α-syn N-trunc (both at 50μM) in the presence of lipid SUV with 1.0 equivalent Cu^{2+} and the lipid SUV alone with the same concentration of Cu^{2+}. The α-syn N-trunc spectra features qualitatively resemble the lipid SUV spectra, demonstrating N-terminal sequence involvement in the lipid-bound α-syn coordination sphere.

We considered whether His50 might participate in Cu^{2+} coordination at higher metal concentrations. For example, copper could bridge between histidines on adjacent α-syns on the membrane surface. To test this hypothesis, we titrated up to 10 equivalents of Cu^{2+} and monitored by both EPR and CD. As seen in figure 4, the addition of extra equivalents of copper did not modify the CD spectra, suggesting that if there are higher order structures being formed, they do not disturb the protein's α-helix. EPR of titrations up to 10 equivalents of Cu^{2+} show hyperfine broadening and the introduction of new hyperfines consistent with a mixture of the lipid bound α-syn:Cu^{2+} spectra and the lipid:Cu^{2+} spectra (Supplemental figure 1). We did not observe any additional EPR features consistent with a new species arising from His coordination. We also tested for His coordination at high Cu^{2+} concentrations using ESEEM. The resulting FT ESEEM spectra obtained from samples up to 10 equivalents of Cu^{2+} are low intensity relative to that of equal concentrations of solution α-syn and do not give a prominent 4.0 MHz signal indicative of multiple His coordination (supplemental Figure 2). Consistent with the continuous wave EPR, these data do not support the presence of a high affinity binding site involving His. Consequently, we find that copper partitions primarily between the high affinity site at the α-syn N-terminus and the membrane. Moreover, Figure 6 shows that with the removal of the α-syn N-terminal binding site, the resultant spectrum corresponds to the lipid only spectra, suggesting that if there is histidine involvement in an additional binding site, its affinity for Cu^{2+} is weaker than the lipids (K_d in the millimolar range). These data demonstrate that there is only one site at which copper binds to α-syn, and it is localized to the N-terminus of the protein without participation of His50.

Copper Binding Affinity

To evaluate the dissociation constant, K_d, of the α-syn-Cu^{2+} complex in the presence of lipid SUV, we used an EPR competition technique previously developed in our lab (*61*). High-affinity competitors that take up Cu^{2+} with a 1:1 stoichiometry are added to a Cu^{2+}-α-syn-lipid SUV solution and allowed to come to equilibrium. Both nitrilotriacetic acid (NTA) and triphosphate molecules are well-characterized chelators and give distinct Cu^{2+} EPR spectra that are readily separable from that of the Cu^{2+}-α-syn-lipid SUV complex. Spectral decomposition using non-linear least squares gives the ratio of copper bound to α-syn and specific competitor. Analysis using the known K_d of the competitor determines the α-syn dissociation constant. With this approach, the amount of competitor may be varied to ensure that both bound species give resolvable EPR spectra with similar signal strengths. Table 1 shows that lipid-bound α-syn binds 1eq of Cu^{2+} with K_d values of .11nM and .095nM, determined by NTA and triphosphate, respectively. To further test for H50 coordination we also performed NTA and triphosphate competition experiments on the α-syn H50A mutant in the presence of lipid SUVs. As seen in Table 1 the resultant K_d is 0.19 nM, which differs only slightly from wildtype.

Table 1: Dissociation Constants (nanomolar) Determined from Competition Studies		
protein	Nitrolotriacetic acid (K_d = 0.366nM)	Triphosphate (K_d = 2nM)
α-syn:lipids	0.110 ± 0.005	0.095 ± 0.006
α-syn(H50A):lipids	0.194 ± 0.008	0.196 ± 0.007

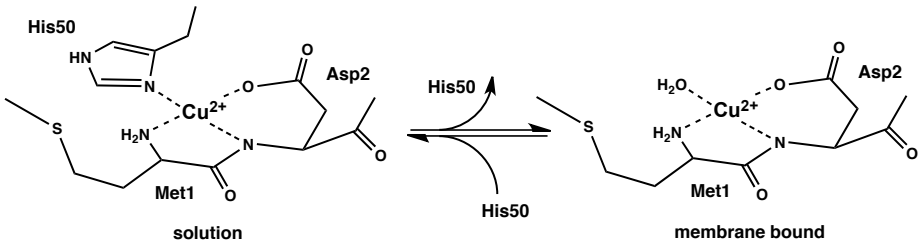

Figure 7. Models of the equilibrium of α-syn copper binding states. Availability of His50 involvement is dependent upon whether the protein is in solution (left) or in the membrane bound helical state (right).

Discussion

Our combined EPR and CD experiments demonstrate that helical, membrane bound α-syn is capable of chelating one equivalent of Cu^{2+} at its N-terminus, with a coordination shell composed of the Met1 amine and the backbone amide and carboxylate residue of Asp2. Furthermore, ESEEM rules out histidine imidazole coordination. Our studies do not identify a fourth atom in the coordination shell, but the downfield shift in the EPR spectrum observed upon addition of lipid is consistent with replacement of a nitrogen with an oxygen, likely from a water molecule. We find the dissociation constant at the maximum possible lipid-bound saturation to be approximately 0.1 nM, similar to that previously reported for α-syn in solution. Together, our results demonstrate that the details of Cu^{2+} coordination depend on whether α-syn is in the solution or membrane bound form, as shown in Figure 7.

α-Syn is found in both solution (inside and outside of the cell) and membrane associated states; it is important to identify cellular factors that influence this equilibrium. Given that His50 coordination to Cu^{2+} in incompatible with the helical, membrane bound form, we wondered whether copper might enhance release from the membrane surface. Our experiments find no evidence of this. Despite addition of up to 10x excess Cu^{2+}, the EPR and CD show no variation pointing to an enhancement of the solution form concentration. Consequently, the energetics driving association between α-syn and the lipid bilayer are substantially stronger than that arising from additional stabilization arising from coordination by His50. However, it is possible that copper coordination to the solution form provides a kinetic barrier to helix formation and consequent membrane association. Our experiments did not address

this. Beyond the dominant, fully helical structure encompassing approximately the first 100 residues, combined NMR and EPR experiments with micelles show that α-syn may also adopt a less ordered structure composed of two helices, separated by a flexible segment (residues 33 – 41) (*62*). Examination of this structure shows that even with additional conformations available in this partially ordered α-syn form, His50 would still not able to come in close contact to an N-terminally coordinated Cu^{2+}.

The equilibrium we identify in Figure 7 suggests that membrane interactions may alter copper mediated redox activity. Aberrant redox reactions at α-syn-copper centers are likely to play an important role in the cellular toxicity associated with α-syn aggregates. Electrochemical properties of α-syn have been carefully evaluated using cyclic voltammetry, as well as reactivity in the presence of ascorbate, dopamine and other relevant species (*63*). Free copper in the presence of ascorbate releases peroxide. At high concentrations, peroxide can react at metal centers producing cytotoxic hydroxyl radicals, thereby contributing to the concentration of reactive oxygen species. The α-syn-copper complex is substantially less reactive than free copper, as measured by both peroxide production and cytotoxicity assays, suggesting that α-syn serves to absorb and modulate adventitious copper (*63*). Interestingly, parallel experiments show that α-syn(1-19), which lacks His50, gives a somewhat different redox profile than the full length protein. Under identical conditions in the presence of ascorbate, α-syn(1-19) produces 10 – 20% more peroxide. Both α-syn species, when complexed with copper, are cytotoxic against neuroblastoma cells, but α-syn(1-19) was more potent at reducing cell viability (*63*). These data suggest that

the copper center in the membrane bound form of α-syn-copper may be more reactive than the solution form.

As described in the Introduction, exposure to high levels of exogenous copper correlates with an increased incidence of PD. Our work here provides insight into the possible initial steps leading to cellular stress and LB formation. We find that copper does not alter the membrane-bound α-syn conformation or contribute to the protein's release from the membrane to solution. However, once released from the bilayer, the simultaneous coordination of the protein N-terminus and His50 limits the distribution of α-syn's random coil states. Although the resulting protein-Cu^{2+} complex is somewhat more protective against the production of reactive oxygen species than the membrane bound form, the loop arising from simultaneous copper coordination of the N-terminus and His50 might expose the amyloidogenic NAC region in a way that results in the observed enhancement of α-syn aggregates (*24*).

In conclusion, we find that membrane bound α-syn tightly binds one equivalent of Cu^{2+} at its N-terminus. This interaction does not affect the helical structure of α-syn or the energetics of membrane association. Indeed, lipid to protein ratio exerts a much stronger influence on α-syn conformation than copper coordination. Consistent with the literature described in the introduction, it is likely that copper uptake is part of α-syn's natural function, with electrochemical properties dependent upon whether the protein is associated with cellular membranes. In PD arising from environmental copper exposure, the solution form of α-syn may be a precursor to LB formation. Future work will focus on how membrane association and

copper uptake are affected by the known inherited mutations in PD, many of which are clustered around His50.

Acknowledgements

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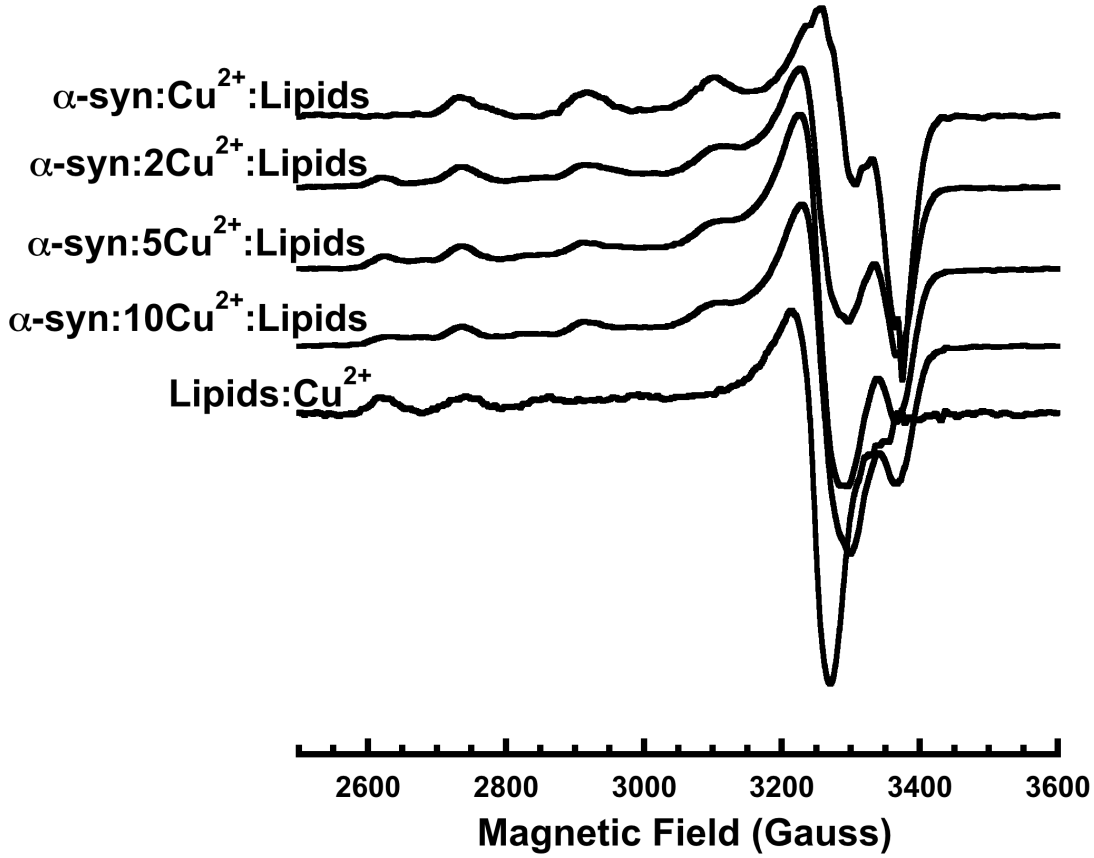
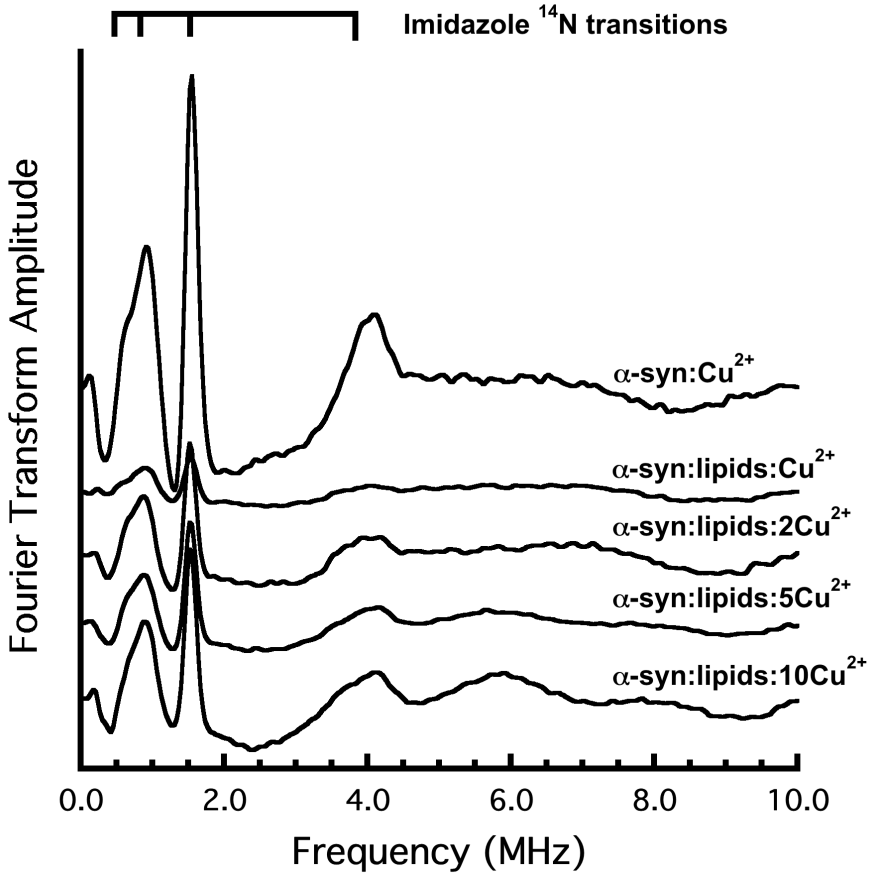
Supplementary Figures

Figure S1



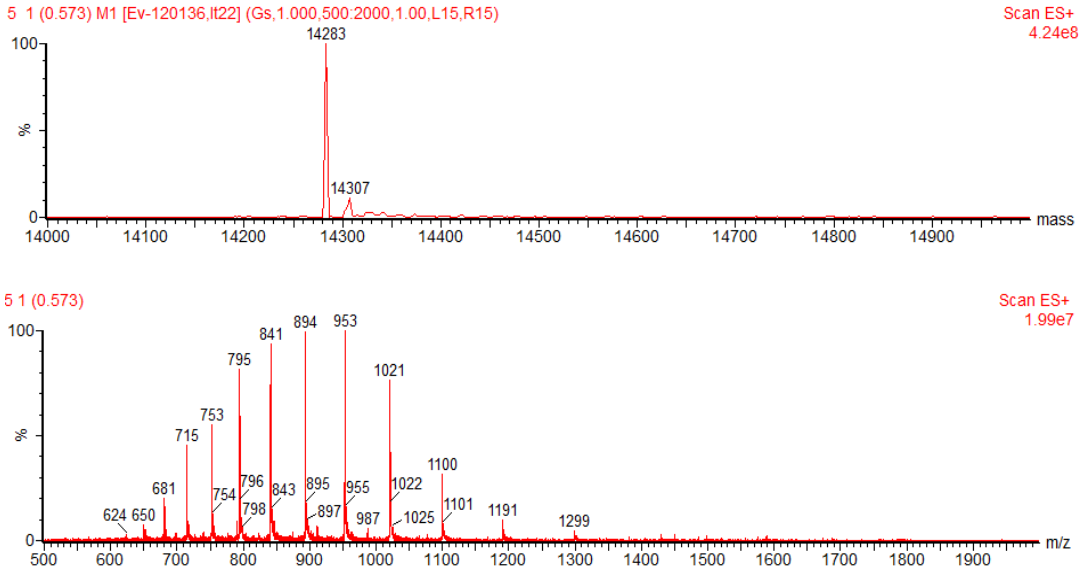
Supplemental Figure 1. X-Band EPR spectra of membrane-bound α-syn (50μM α-syn) as a function of added Cu^{2+}. Copper is increased at a fixed α-syn and lipid composition. With greater than 1.0 equivalent of copper, the spectra exhibit features of a superposition of membrane bound α-syn and lipid copper complexes, consistent with a single membrane-bound α-syn copper binding site with affinity sufficient to outcompete the lipids.

Figure S2



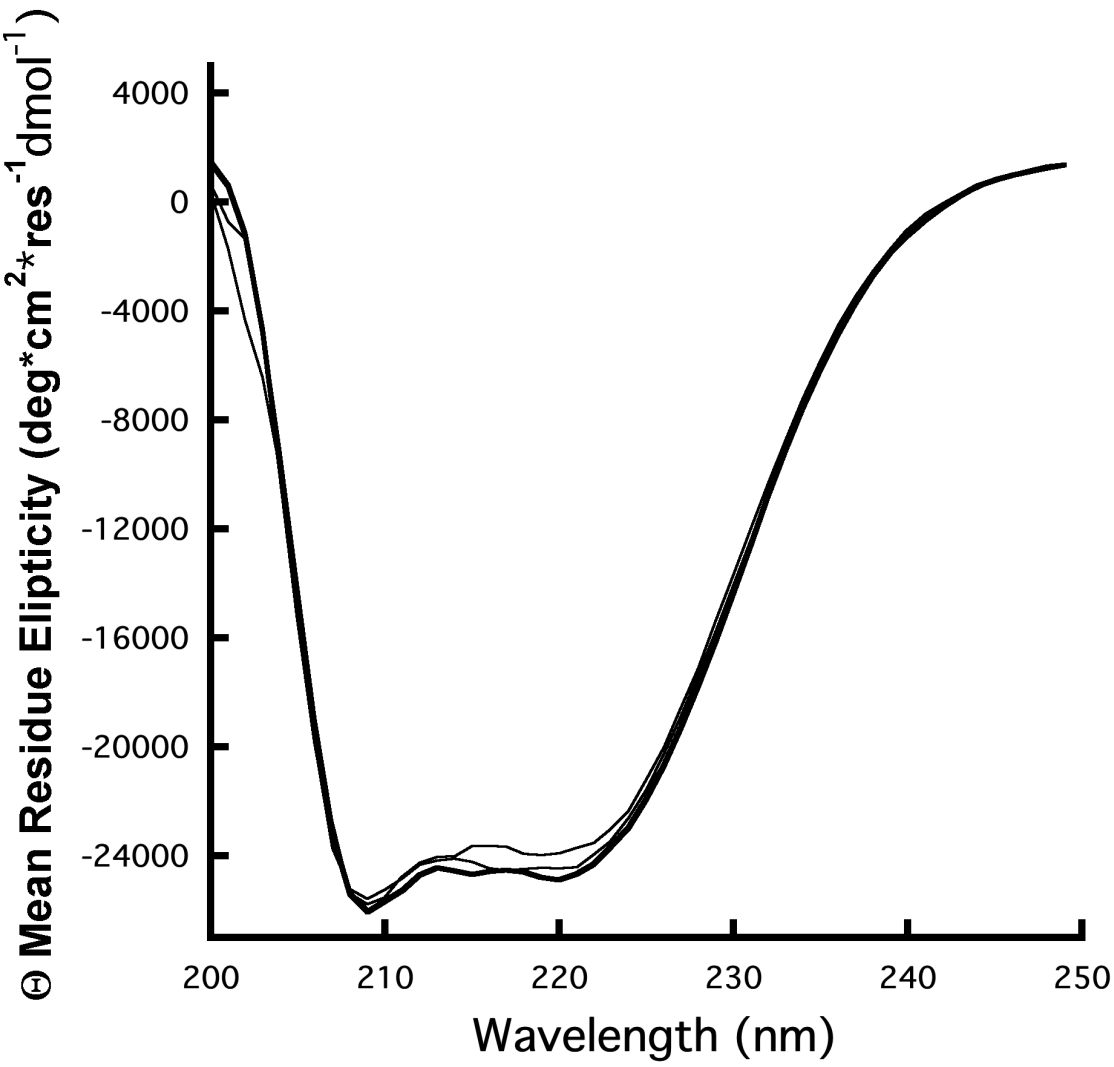
Supplemental Figure 2. Three-pulse ESEEM spectra of α-syn (50μM α-syn, 15mM lipids) with up to 10 equivalents of Cu^{2+}, both in solution and in the presence of lipid SUV. The spectra of the samples in solution and in the presence of SUV with greater than 1 equivalent of Cu^{2+} reveal the expected quadrupolar transitions associated with the imidazole remote nitrogen and demonstrate coordination by His50 whereas the sample with stoichiometric concentrations of α-syn and Cu^{2+} in the presence of SUV do not. While the ^{14}N quadrupolar features of the samples containing lipid SUV with 2, 5 and 10 equivalents of copper suggest a Cu^{2+}-imidizole interaction, these peaks saturate only at copper concentrations well in excess of 250 μM – 500 μM copper, suggesting a very low affinity interaction.

Figure S3



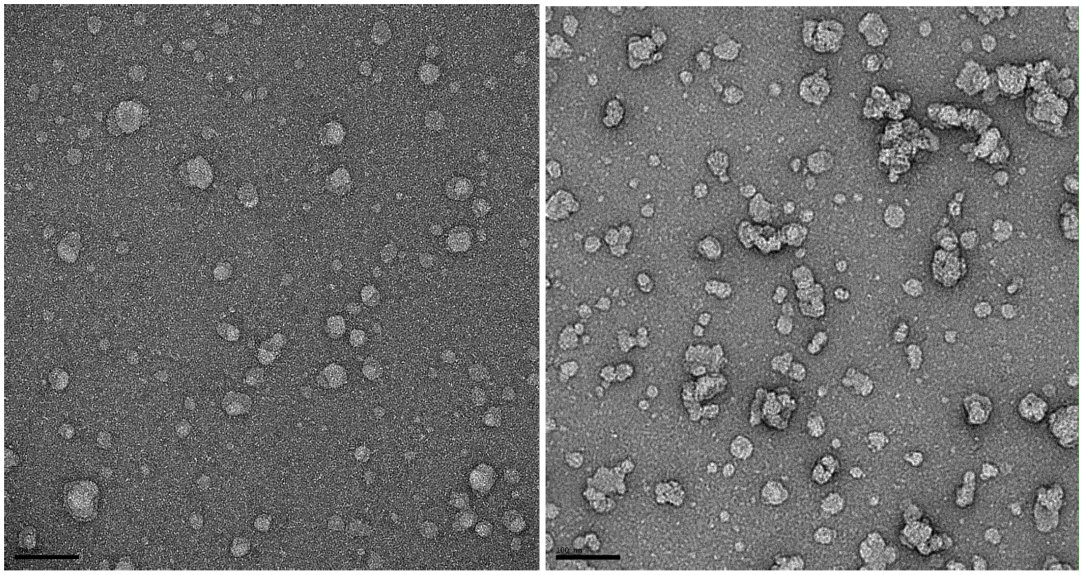
Supplementary Figure 3: Mass spectrometry data for α-syn N-trunc mutant. Deconvolution (above) reveals the expected mass of 14283 as the major species (>90%) of the sample. Wild-type α-syn has a mass of 14456.

Figure S4



Supplementary Figure 4: CD spectra of wild-type α-syn, α-syn N-trunc, and α-syn H50A.

Figure S5



Supplementary Figure 5: Transmission Electron Microscopy images of 70%POPC:30%POPG SUVs both without α-syn (left) and with α-syn (right, lipid:α-syn ratio 300:1). The black bar at lower left is equivalent to 100nM. Most SUVs are in the range 20 – 50 nm, with no indication of tubes or other unusual structures. Samples for electron microscopy were prepared by spotting 3µl onto glow-discharged carbon-coated copper grids followed by staining with 2% (w/v) uranyl acetate solution. Samples were analyzed using a JEOL 1230 microscope operating at 120kV.

CHAPTER 4

Enhanced Binding Strength of Aggregated Amyloid-β Enables Its Copper Sequestration from Albumin: a Pathway to Accumulation of Copper in Senile Plaques

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FORWARD

The following chapter is an excerpt from a larger study that had many contributors, each with different aspects of the project. While the project as a whole was comprehensive in scope and supported the overall conclusion of the paper, I chose to use this topic for my final chapter to illustrate that the EPR techniques I had learned on α-synuclein could be transferred to other unstructured, copper-binding peptides. What is written here is focused essentially only on my contribution to the study.

ABSTRACT

High concentration of copper has been found to coexist with amyloid-β (Aβ) peptides in the senile plaques of Alzheimer's disease (AD) patients. However, the origin of copper and the driving force behind its accumulation remain unknown. Previous studies on the Cu^{2+} binding by Aβ peptides in their monomeric forms yielded only moderate binding affinity constants, which suggest that monomeric Aβ peptides are incapable of seizing copper from other proteins in the extracellular milieu. Using electron paramagnetic resonance spectroscopy (EPR) we demonstrated that Aβ(1–42) aggregates sequester copper from albumin, an abundant copper-containing protein in brain and cerebrospinal fluid. This process was found to not occur in the monomeric Aβ(1–42) peptide or Aβ(1–16). The binding strength of Aβ(1–42) fibrils or amorphous aggregates is enhanced by over two orders of magnitude with respect to that of the Aβ(1–42) monomer. We proposed a Cu^{2+} coordination sphere in the Aβ(1–42) aggregates that cannot be offered by the monomer to explain the enhanced binding strengths. Our work suggests that the long-standing misconception that aggregated and monomeric Aβ(1–42) have similar binding strength to copper is incorrect and may provide new insight into how and why Aβ may accumulate copper *in vivo*, strengthening the hypothesis of copper-induced oxidative stress of AD.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurological disorder known to our species, estimated to be effecting over 26 million individuals worldwide over the age of 60 (*1*). The idiopathic, degenerative disease is chronic, progressive and has no cure. AD, unlike Parkinson's disease described in the previous chapters, is characterized as a dementia (*2*), that is, the disease affects the cognitive functions of the brain rather than motor skills. Symptoms vary from individual to individual but the earliest outward signs of the disease often manifest themselves as forgetful behavior, or "absent-mindedness" (*3*). As the disease progresses, however, symptoms worsen and include extreme confusion, aggressive behavior, more severe memory loss and difficulty speaking, swallowing and walking (*4*). Ultimately the disease is fatal, being the sixth most common cause of death in the United States (*3*). It is interesting and distressing to note that current predictions place 1 in 85 people suffering from the disease globally (*5*).

AD exists in both familial and sporadic forms, though the former accounts for only about 15% of cases (*6*). The etiology of the disease is not known, but the pathological hallmark of AD is the extracellular insoluble protein deposits in the brain known as senile plaques (*3*). Insoluble, natively unstructured Aβ peptides have been shown to be a major component of these senile plaques (*7*). Aβ is the result of beta and gamma secretase enzymatic cleavage of the amyloid precursor protein (APP) resulting in Aβ peptides from 40-42 residues in length with the Aβ(42) peptide being less soluble than the Aβ(40) and much more prone to aggregation and neurotoxicity (*8, 9*).

Although the exact mechanism for the formation of senile plaques is unknown, these aggregates have been shown to contain abnormally high concentration of metals (e.g., Cu^{2+} at ~0.4mM and Fe^{3+} at ~0.9mM) (*10*) and extensive research and debate has been published addressing how metals bind to and induce aggregation of Aβ (*11-17*). Disruptive metal homeostasis has been suggested to be a cause of the metal/Aβ interaction, and a particular attention has been paid to the redox activity of copper-Aβ complexes and their possible linkage to AD pathology (*18, 19*). *In vitro* studies have shown that Cu^{2+} accelerates the Aβ aggregation and alters the Aβ aggregation pathway from fibrillation to amorphous aggregate formation (*20-23*). In animal model studies, feeding rabbits trace amounts of copper induces formation of Aβ plaques and affects their learning ability (*24, 25*). We and others have found that Cu^{2+} bound to both Aβ monomers and aggregates participates in catalytic redox reactions with the production of reactive oxygen species (ROS),(*26-30*) which are known to initiate extensive oxidative stress in AD brain. On the other hand, complexation of Cu^{2+} by Aβ inhibits free radical production from the Fenton-like reactions of free Cu^{2+},(*29, 31*) alluding a possible role of Aβ in protecting neurons from oxidative damage. This knowledge has provided momentum for the development of metal chelation-based therapies targeted toward treating AD.

While the presence of copper in senile plaques has established its connection to possible AD pathogenesis, a remaining mystery is its origin of and the driving force behind copper accumulation in senile plaques. To help explain the accumulation of copper in an AD-afflicted brain, the interaction of copper with Aβ monomers has been extensively studied in vitro (*32-37*). Monomeric Aβ peptides were found to

form Aβ-Cu^{2+} complexes with all the binding sites located in the hydrophilic, N-terminal Aβ(1−16) domain (*33, 38, 39*). The copper binding constant, despite a large variation of the reported dissociation constants (ranging from 10^{-6} to 10^{-10} M), is largely agreed to be around 10^{-9} M (*32, 37, 40-43*). Free copper is very toxic, and therefore is highly regulated in biological milieu (*44*). Given the extremely low free copper concentration in cellular milieu, a prerequisite for senile plaques to accumulate copper is that the overall binding strength of the Aβ peptides should be stronger than some copper-containing biomolecules. As a major copper chelator and transporter protein, albumin is not only the most abundant plasma protein, but also a major protein in cerebrospinal fluid (CSF) (amounts to 80% of the total proteins in CSF) (*45, 46*). Owing to its relatively high copper binding strength, 10−15% of the total copper in plasma are estimated to be withheld by albumin,(*47, 48*) which therefore serves as a viable *in vivo* copper source to Aβ in senile plaques. Interestingly, the binding strength of monomeric Aβ is not as high as the commonly known copper-containing enzymes (e.g., Cu-Zn-superoxide dismutase whose K_d is around 10^{-15} M)(*49, 50*) or copper-transport proteins (e.g., albumin, with a K_d value around 10^{-12} M) (*51, 52*). Indeed, using monomeric Aβ, Faller and coworkers found that in vitro the opposite copper transfer (i.e., from Aβ to albumin) takes place (*53*). This result demonstrates that the binding strength of monomeric Aβ towards copper is perhaps a few orders of magnitude weaker than that of albumin. The inability of Aβ monomers to sequester copper from albumin cannot explain the aforementioned long-standing mystery, which creates doubts about the relevance of copper in the etiology of AD (*32*).

The existence of copper in senile plaques in vivo suggests that Aβ aggregates retain copper in the presence of albumin. The finding that senile plaques can be dissolved by strong copper chelators (*54*) also suggests that copper stabilizes Aβ aggregates. These facts imply that aggregated and monomeric Aβ have different copper binding strengths. While a direct sequestration of copper by monomeric Aβ from albumin is predicted to be impossible in vivo, it is possible that, upon aggregation, Aβ oligomers and aggregates have a greater affinity for copper, and sequester copper from albumin. Nevertheless, thus far there is no direct evidence to support the possibility, nor can a reason be provided to explain the enhanced copper binding strength upon Aβ aggregation.

As shown in the previous chapters, determination of relative copper-binding strengths and dissociation constants by direct competition using electron paramagnetic resonance (EPR) is a robust and well-characterized method. Using this technique we measured the apparent binding constant of Aβ(1–42) throughout its aggregation/fibrillation process for the first time, providing strong evidence that higher-ordered Aβ(1–42) aggregates can compete for copper from some copper-containing proteins. This EPR measurement confirms our contention that aggregated Aβ(1–42), with a different copper binding coordination structure than monomeric Aβ(1–42), possesses a stronger binding strength towards copper.

Table 1 Sequences of the Aβ peptides used in this study

Peptide	Sequence
Aβ(1−16)	DAEFRHDSGYEVHHQK
Aβ(1−42)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAI IGLMVGGVVIA

The hydrophobic segment of Aβ(1−42) is underlined.

MATERIALS AND METHODS

Chemicals and reagents. Aβ(1–42) was obtained from American Peptide Company Inc. (Sunnyvale, CA), and Aβ(1–16) and Aβ(1–16) were purchased from R-Peptides Inc. (Athens, GA). Sodium hydroxide, sulfuric acid, $CuCl_2$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and other chemicals were acquired from Sigma Chemicals (St. Louis, MO). All of the aqueous solutions were prepared using water purified by a Simplicity® Water Purification System (Millipore, Billerica, MA) to a resistivity of 18 MΩ·cm.

Sample preparation. Aβ(1–16) and Aβ(1–16) stock solutions (1–2mM) were prepared by directly dissolving the lyophilized solid samples in Millipore water. They were then diluted with 10mM HEPES buffer (pH 7.4) to different concentrations (0.8–12.5μM). However, for Aβ(1–42), in order to avoid aggregation, the lyophilized Aβ(1–42) powder was first dissolved in 20mM NaOH solution to a final concentration of 500μM and a final pH ~9, and then sonicated for 1min. The resulting solution was then centrifuged at 14000rpm while incubating at 4^{o}C for 30min. Any insoluble particles were separated and discarded, and the supernatant served as the stock solution from which aliquots of Aβ(1–42) solutions were prepared. The Cu^{2+} stock solution was prepared by dissolving 1mM $CuCl_2$ in 1mM H_2SO_4 solution. UV-vis measurements were performed using a Cary 100 UV-vis spectrometer (Varian Inc., Palo Alto, CA**).** The concentration of soluble peptides were determined according to absorbance at 276nm and using extinction coefficient of tyrosine (ε_{276} = 1410 cm M^{-1}) for Aβ(1–16) and Aβ(1–42).

Electron paramagnetic resonance spectroscopy. A sample of 500μM Aβ(1–42) was loaded in an EPR quartz tube (4 mm O.D. and 3 mm I. D.; Wilmad, New Jersey) and incubated for 3 or 6 h at 37 ºC. The solution was then mixed with the Human Serum Albumin (HSA)-Cu^{2+} complex. To ensure that no free Cu^{2+} was present in the mixture of HSA and Cu^{2+}, the Cu^{2+}/HSA ratio was maintained at 0.8. An amount of Cu^{2+}-HSA was added to the pre-incubated Aβ(1–42) so that the final concentrations of HSA, Cu^{2+}, Aβ(1–42) are 100, 80, and 500μM, respectively. Glycerol (10%; v/v) was added for homogeneous freezing and a more uniform dispersion of Aβ(1–42) species in the EPR tube. All solutions were mixed thoroughly, sonicated for 2min and flash-frozen in liquid N_2. For the control experiment, a freshly dissolved Aβ(1–42) was used. Samples prepared were either stored in a –80ºC freezer or immediately measured by EPR. EPR spectra were recorded using an X-band (9.4 GHz) spectrometer (Elexsys E580, Bruker, Germany) equipped with an SHQ resonator and a temperature controller (Bruker). EPR spectra were obtained at 111 K using N_2 gas flow with a microwave power of 9.464mW and a modulation amplitude of 10G. The following EPR parameters were used: time constant of 163.84ms, conversion time of 40.97ms, a receiver gain of 10dB and scans of 64 times.

Determination of the dissociation constant (K_d) for aggregated Aβ-Cu^{2+} interaction. In order to further determine the K_d for the Cu^{2+}-aggregated Aβ complex, we used an EPR competition technique previously developed in the Millhauser lab (*55*) and briefly described here. We employed HSA, which has a dissociation constant for Cu^{2+} ~1picomolar and when complexed with copper produces an EPR spectra that is distinct from the Cu^{2+}-aggregated Aβ, as the

competitor for our study. The concentrations of monomeric Aβ and incubation times to induce aggregation were varied while the concentration of HSA was held constant. Spectral decomposition of the resultant EPR spectra gives the ratio of Cu^{2+} bound to both HSA and Aβ (monomeric or aggregated, depending on the incubation time).

RESULTS

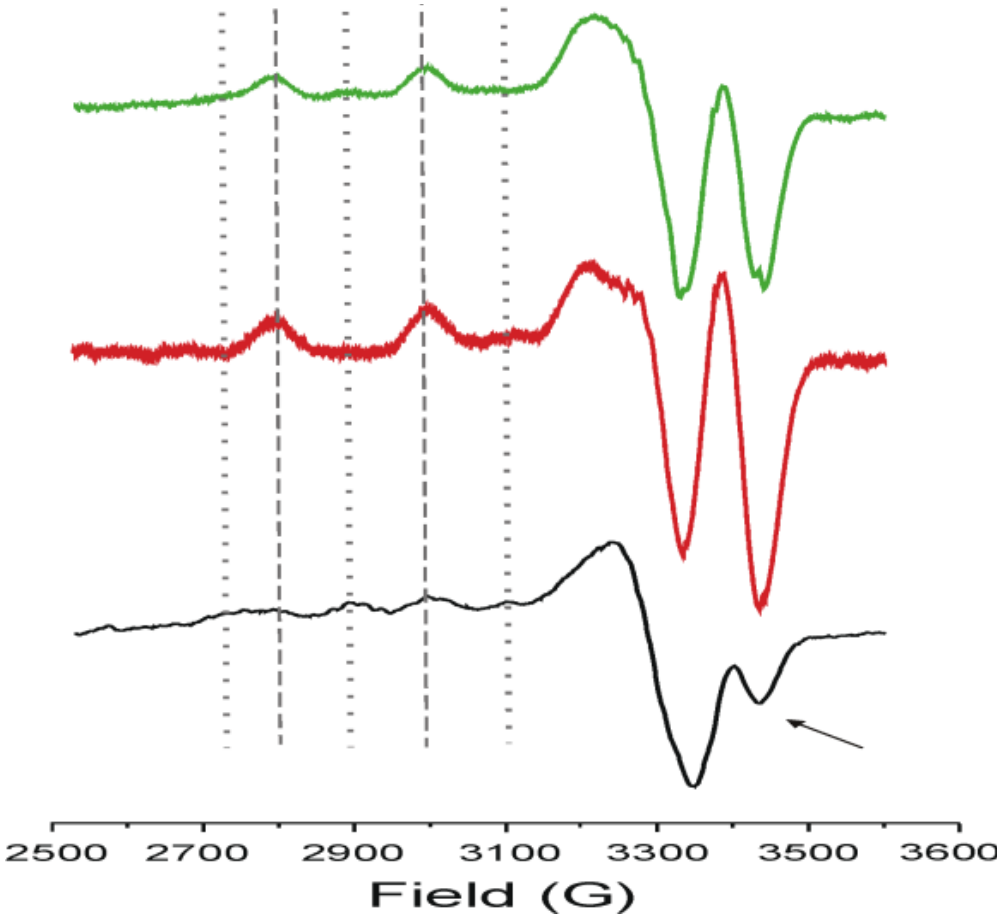

Figure 1. EPR spectra of a preformed Cu^{2+}–HSA complex (100μM) mixed with (A) a freshly prepared 300μM Aβ(1–42) solution; (B) a 300μM Aβ(1–16) solution, and (C) a 300μM Aβ(1–42) solution that had been incubated for 3h. Marked are copper hyperfine peaks of the Cu^{2+}–Aβ complex (dashed gray lines) and Cu^{2+}–HSA (dotted gray lines).

Table 2 EPR Parameters

Sample	$g_{\|}$	$A_{\|}$
Preincubated Aβ(1–42) + HSA-Cu^{2+}	2.255 2.155	168 G 203 G
HSA-Cu^{2+} + fresh Aβ(1–42)	2.155	203 G
HSA-Cu^{2+} + fresh Aβ(1–16)	2.155	203 G

Preincubated (Aggregated) Aβ(1−42) can sequester Cu^{2+} from HSA

We investigated the possible transfer of Cu^{2+} from HSA to higher-ordered Aβ aggregates using EPR. Shown in Figure 1 are EPR spectra of albumin-Cu^{2+} upon being mixed with a freshly prepared Aβ(1–42) solution (A), with an Aβ(1–16) solution (B), and with a solution containing Aβ(1–42) oligomers and protofibrils as the major aggregates (C). The line shape and parameters (Table 2) calculated from spectrum (A) ($g_{||}$ = 2.155, $A_{||}$ = 203 G) are consistent with those of the HSA–Cu^{2+} complex,(*56, 57*) with two large negative peaks at 3317G and 3400G and two large hyperfine peaks. Notice that spectra (A) and (B) are highly comparable, suggesting that neither Aβ(1−16) nor the Aβ(1−42) monomer can compete Cu^{2+} out of HSA, a result predicable from the large difference in the binding constants between monomeric Aβ and HSA. These data are also in line with the report by Faller's group who showed that monomeric Aβ(1–40) cannot seize Cu^{2+} from albumin.(*53*) However, with the addition of a preincubated Aβ(1–42) solution, the negative peak at 3317G increases at the expense of negative peak at 3400G, concurrent with the appearance of three new hyperfine peaks ($g_{||}$ = 2.255 and $A_{||}$ = 168 G; spectrum (C)). The parameters of the new peaks upon the addition of preincubated Aβ(1–42) are consistent with those of the copper complex of aggregated Aβ(1–42). (*35, 36*) This strongly indicates preincubated Aβ(1–42) can sequester copper from albumin-Cu^{2+}.

Determination of the copper binding constant (K_d) of aggregated Aβ(1−42) by competitive EPR binding assay.

In order to quantify the increase in binding affinity for the aggregated Aβ(1−42) we employed an EPR competition assay previously developed in the

Millhauser lab (*55*). High affinity competitors that take up Cu^{2+} with a 1:1 stoichiometry are added to a Cu^{2+}-Aβ aggregate solution. For a successful EPR competitive binding assay, the two Cu^{2+} competing ligands should not have drastically different binding constants. Moreover, ideally their EPR spectra should be distinct from one another the so that quantitative measurements of the Cu^{2+} transfer can be made. Spectral decomposition gives the ratio of copper bound to the species of interest and specific competitor. Analysis using the known K_d of the competitor determines the dissociation constant of species of interest. With this approach, the amount of competitor may be varied to insure that both bound species give resolvable EPR spectra of similar signal strengths. We attempted to observe possible transfer between Aβ(1–42) aggregates and a number of copper-binding molecules (e.g., nitrilotriacetic acid, the oxidized form of glutathione, pentaglycine, and α-synuclein) and found that they did not conform to the above criteria as well as the HSA–Cu^{2+} system.

Prepared with different concentrations and for different incubation times, the Cu^{2+} binding constants of Aβ(1–42) at two different aggregation stages were therefore determined from the EPR competition experiment using HSA–Cu^{2+}. In all cases, Cu^{2+} and HSA concentrations were 49 μM and 61 μM, respectively. Because of the challenges inherent in working with an aggregating protein, an accurate determination of the Cu^{2+} binding constant of Aβ(1–42) by EPR can be difficult to attain. Therefore, the K_d values ranging from $4.5–3.0 \times 10^{-11}$ M (Table 3) obtained for the Aβ(1–42) monomer (freshly prepared solution) may be somewhat overestimated due to the possible presence of aggregated protein. On the other hand, the K_d values

measured from solutions that were incubated for 6 and 24 h (i.e., solutions populated with aggregates of protofibrils and fibrils) could be underestimated value due to the sedimentation of the aggregated Aβ(1–42)–Cu^{2+} undetectable by EPR. This sedimentation problem perhaps explains why the K_d values measured for 6 and 24 h are not consistently different. The decrease of the total copper EPR signal in samples with longer incubation also supports this hypothesis. Nevertheless, there is a clear increase in the binding strength from a freshly prepared Aβ(1–42) sample (i.e., 0 h of incubation) compared to the samples that had been incubated, even though the sedimentation effect is to increase the K_d value in preincubated solutions. Therefore, our EPR measurements demonstrate that the apparent Cu^{2+} dissociation constants decrease with the Aβ(1–42) aggregation time.

Table 3 Dissociation Constants of Aβ(1–42) Determined from Competition Studies

Aβ(1–42) concentration(μM)	Incubation time at 37 °C (h)	K_d (pM)
76	0	30
76	6	4.4
76	24	6.1
306	0	45
306	6	5.0
306	24	4.4

DISCUSSION

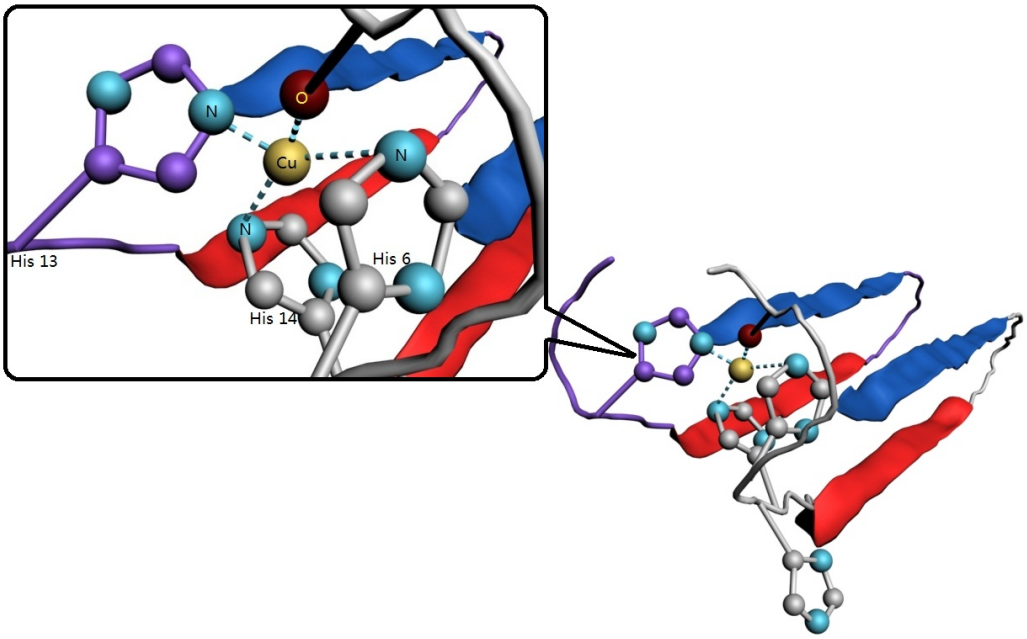

Figure 2. Coordination structure of Cu^{2+} in Aβ(1–42) oligomers and higher-ordered aggregates.

The fact that oligomers (including dimers) and other higher-ordered aggregates of Aβ(1–42) have stronger binding strengths than the monomer must stem from a tighter Cu^{2+} coordination structure that cannot be rendered by the monomer. It has been suggested that His-6, His-13 or His-14, and the N-terminus are the three N-containing moieties that account for the 3N1O coordination sphere identified by EPR in the Cu^{2+} complex of the Aβ monomer (*38, 58*). His-13 and His-14 are located on the opposite sides of the backbone of Aβ and consequently their simultaneous participation in Cu^{2+} binding is sterically hindered (*58*). Previous structural analyses of Aβ(1–42) aggregates have established that dimers are formed via the formation of a β-sheet constituting two pairs of β-strands withheld by hydrogen bonding (*59, 60*). The hydrophilic, metal-binding N-terminus of each Aβ(1–42) molecule is located on

the same side of the β-sheet. Larger oligomers, protofibrils and fibrils can be produced from stacking of the β-sheet-containing oligomers (or "aggregation units"),(*61, 62*) positioning all of the hydrophilic N-termini on the same side of the stacked β-sheets (*61*). Although amorphous aggregates lack higher regularity for ordered β-sheet stacking, they have been shown to contain β-sheets (*63*). Given this evidence, particularly in the context of the fibrils, a plausible reason for the enhanced binding strength towards Cu^{2+} in Aβ aggregates is that more histidine residues are aligned properly to coordinate Cu^{2+}. As illustrated in Figure 2, upon aggregation His-13 in one Aβ molecule and His-14 from another can now concurrently coordinate Cu^{2+}, tightly locking the Cu^{2+} ion in position. Among all of the amino acid residues, histidine is one of the strongest metal-binding ligands, whose participation strengthens the metal binding. For example, in Cu^{2+} complexes of the prion protein, the configuration involving four histidine residues ("component 3") is known to be much more stable than "component 1", in which only one histidine anchors the Cu^{2+} center (*64, 65*).

The structure posited in Figure 2 is supported by several lines of evidence. For example, Pedersen et al. reported that the Cu^{2+} coordination environment in Aβ oligomers and aggregates comprise the Aβ–Cu^{2+}–Aβ linkage, which is absent in the complex formed between Cu^{2+} and an Aβ monomer (*66*). Szalai and coworkers, using X-ray absorption spectroscopy, demonstrated that in the Aβ monomer two histidines (presumably His-6 and either His-13 or His-14) take part in copper coordination, whereas in Aβ oligomers three histidines participate in copper coordination (*34*). We believe that the configuration of bridged Cu^{2+}–His–Cu^{2+} is not likely, because it has

been shown to be non-existent in Cu^{2+}-containing Aβ aggregates (*35*). Finally, the dissolution of senile plaques requires the use of a strong metal chelator,(*54*) suggesting that Aβ fibrils and other aggregates in senile plaques are stabilized by the intermolecular Cu^{2+} binding.

The observation that the Cu^{2+} binding strength is dependent on the Aβ aggregation strength has two major implications. First, it has been long believed that overproduction of Aβ peptides (e.g., in the case of early on-set AD(*67*)) and inefficient Aβ clearance lead to Aβ accumulation/deposition and aggregation. Even when cellular Cu^{2+} regulation is normal, the higher Cu^{2+} binding strength inherent in Aβ oligomers and fibrils would result in Cu^{2+} sequestration from copper-transporting proteins such as albumin, which may explain the high level of copper in senile plaques. Given the significant role of albumin in regulating copper in healthy brain(*44, 57, 68*) and albumin deficiency is shown to be linked to AD development, the Aβ aggregation *in vivo* may eventually lead to disrupted copper homeostasis (*69-71*). Another interesting fact is that 60–90% of the AD cases are associated with cerebral ischemia(*72, 73*), (*74*), which has been shown to decrease the metal binding capacity of albumin (*75*). As a consequence, transfer of copper from albumin to Aβ aggregates is strongly favored. Indeed, treatment of AD patients with plasma exchange, in which albumin is replaced with that from donors, is undergoing clinical trials (*76*). Although albumin has multiple biological functions, the possibility of its loss of copper to aggregated Aβ cannot be overlooked.

The second implication stems from the fact that soluble oligomers of Aβ possess a much stronger copper binding strength than their monomeric counterpart.

A large body of evidence has demonstrated that diffusable oligomers of Aβ are much more toxic than the monomer and fibrils (*77-79*). In a healthy brain, Aβ monomers and oligomers (including dimers) are in an equilibrium and their relative abundances are dependent on how each species is metabolized. When copper homeostasis is disrupted in AD,(*80*) excess copper(*69*) present in the cellular milieu is capable of binding oligomers and stabilizing them. Consequently, the equilibrium is shifted to the direction of dimerization/oligomerization. Because the Aβ–Cu^{2+} complex catalyzes the ROS generation,(*19*)(*27, 29*) the copper-containing oligomers could potentially behave as a mobile ROS generator, leading to an extensive oxidative stress that is commonly found in AD brain (*81, 82*). It is interesting to note that rat Aβ does not have histidine in position 13, yet its sequence in the hydrophobic C-terminus responsible for aggregation is the same as that of human Aβ. Rats do not naturally acquire AD(*83*), which might be related to their aggregates' inability to sequester copper to the extent of aggregates of human Aβ. Furthermore, methylation of histidines in Aβ was found to eliminate copper-mediated Aβ toxicity(*18*), again suggesting the importance of histidines in modulating copper binding and the resultant effect on AD neuropathology.

In summary, this study suggests that the long-standing misconception that aggregated and monomeric Aβ(1–42) have similar binding strength toward copper is incorrect. The Cu^{2+} binding strength elevated by Aβ aggregation may provide new insight into how and why Aβ may accumulate copper *in vivo,* and strengthens the hypothesis of copper-induced oxidative stress in AD.

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