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Coordination of Copper to the Membrane-Bound Form of α-Synuclein

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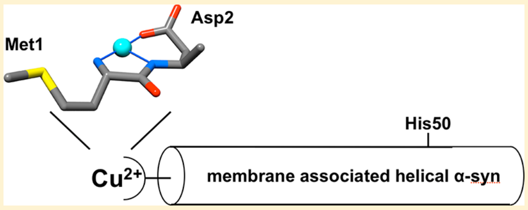
Supporting Information

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 N-terminus of the protein 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 the coordination of copper to the membrane-bound form of α-syn. In this molecular environment, Cu^{2+} binds exclusively to the N-terminus of the protein (Met1-Asp2) with no participation from His50. Copper does not alter the membrane-bound α-syn conformation or enhance the release of the protein 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 effect on the solution form of α-syn.



Parkinson's disease (PD) is a common, age-related neurodegenerative disorder that affects more than 1 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 in ref 5).

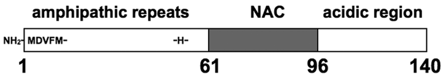


Figure 1. Features of the α-synuclein primary structure identifying the three consensus segments and the amino acids, at the N-terminus and His50, potentially involved in Cu^{2+} coordination. Residues 9–97, encompassing the amphipathic repeat region and the NAC, form an extended helix when associated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

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-Aβ 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 termini of dopaminergic neurons, an area with a large volume of 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 vesicle–cell membrane fusion.[13−15]

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

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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] A BLAST sequence comparison shows that the specific residues required for Cu^{2+} binding, M1, D2, and H50, 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 dysregulation 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 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 in ref 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 in refs 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 the 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 because a helical polypeptide conformation would separate the N-terminus and His50 by approximately 75 Å, 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 the uptake of copper by membrane-bound α-syn and showed that helix content and copper affinity at the N-terminus of the protein are increased in the membrane environment.[53] In the study presented 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 an affinity and a coordination environment similar to those found for the solution form. However, His50 no longer contributes to equatorial coordination regardless of Cu^{2+} concentration, and copper does not influence the distribution of α-syn between the membrane and solution.

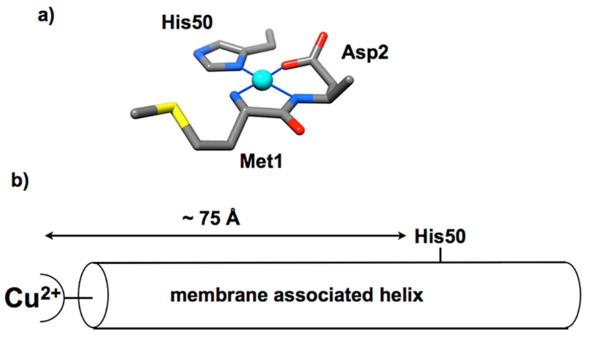

Figure 2. (a) Coordination features of the primary solution Cu^{2+} site of α-syn. Competition studies show that this complex exhibits a dissociation constant of approximately 0.1 nM. (b) α-Syn in its helical form, resulting from membrane association, would result in a separation of His50 from the N-terminus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

MATERIALS AND METHODS

Proteins and Reagents. The wild-type (wt) α-syn gene was cloned into the *pet21* plasmid vector manufactured by Genescript. The primers for the H50A mutation were obtained from Invitrogen (Carlsbad, CA). Mutations were introduced using the GeneTailor Site Directed Mutagenesis System (Invitrogen catalog nos. 12397-014 and 12397-022). α-Syn and α-syn(H50A) were recombinantly expressed in *Escherichia coli* BL21(DE3) competent cells (Invitrogen) using an autoinduction procedure of Kim et al. described previously.[54] Cells were harvested by centrifugation followed by sonication in lysis buffer [50 mM NaCl, 20 mM Tris, 0.2 mM PMSF (phenylmethanesulfonyl fluoride), and 10% (v/v) Triton X-100 (Sigma, Switzerland) (pH 7.4)]. Purification was performed using ammonium sulfate precipitation followed by centrifugation, resuspension in 6 M guanidine hydrochloride, and reverse-phase HPLC (water and acetonitrile). The protein elutes between 63 and 60% acetonitrile on a C18 column (Grace Davidson Discovery Sciences, VYDAC HPLC column, catalog no. 218TP101510).

Electron Paramagnetic Resonance. Samples were prepared in degassed buffer containing 25 mM MOPS buffer and 25% (v/v) glycerol, where the glycerol served as a cryoprotectant. All continuous wave X-band spectra ($\nu = 9.44$ GHz, microwave power in the range of 0.6−5.0 mW, modulation amplitude of 5.0 G, and sweep width of 1200 G) were collected at approximately 125 K, 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 20 K on a Bruker E580 X-band spectrometer using a dielectric resonator and an Oxford CF 935 cryostat. A $\pi/2-\tau-\pi-T-\pi/2-\tau-$echo sequence with pulse lengths of 12, 24, and 12 ns was used. The initial value of τ was 136 ns, and T was lengthened in 799 steps of 12 ns each with 100 samples per step.

Circular Dichroism. Far-UV CD measurements were performed with an AVIV 60DS spectrophotometer, with a 0.1 cm path length cuvette. Solutions included 10.0 μM protein, 100 mM NaCl, and 20 mM MOPS buffer. Spectra were recorded from 250 to 200 nM with a step size of 1 nm, a bandwidth of 1.5 nm, and an averaging time of 4 s. An average of 20 scans was obtained for each spectrum. Separate buffer spectra were also collected as a reference.

Small Unilamellar Vesicle Preparation. Vesicles were prepared using standard procedures outlined by Langen et al.[7] from a 70% phosphatidylcholine (POPC)/30% phosphatidylglycerol (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, 30 s on/40 s rest for a total of 5 min on time. Lipids were deemed prepared when the 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 the Supporting Information).

Most phospholipids bind Cu^{2+}; however, the specific headgroup plays a significant role in controlling affinity. Lipids with headgroups 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 SUVs and protein and incubated for ~15 min before being flash-frozen with liquid N_2. The lipid composition for the POPC/POPG SUVs was chosen to minimize direct Cu^{2+}−membrane interactions. All samples containing α-syn and SUVs used for EPR experiments were found to have room-temperature CD spectra corresponding to an α-helix. The EPR spectra in Figure 3 show the typical Cu^{2+} hyperfine couplings in the parallel region (2700−3300 G) observed for oxygen/nitrogen rich equatorial coordination and thereby demonstrate that α-syn binds Cu^{2+} in the presence of lipid SUVs. Interestingly, the hyperfine lines in the EPR spectra for 100−200× lipids appear to be broadened in comparison to those of 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-syn(H50A) 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, the EPR spectra appear to correspond solely to the α-syn(H50A) spectrum, suggesting that Cu^{2+} is bound only to the N-terminus of the polypeptide. To ensure that the EPR spectra are the result of Cu^{2+} binding α-syn and not another chelator, such as the bilayer phosphate headgroups, spectra were recorded with Cu^{2+} and SUVs alone for comparison. The lipid−Cu^{2+} spectrum exhibits a B_o downfield shift of ~150 G (compared to the α-syn−Cu^{2+} complex in the presence of lipids) and a different pattern of hyperfine splitting that suggests high O ligand character (see below and Supporting Information). Furthermore, the addition of Cu^{2+} to SUVs 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 SUVs 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.

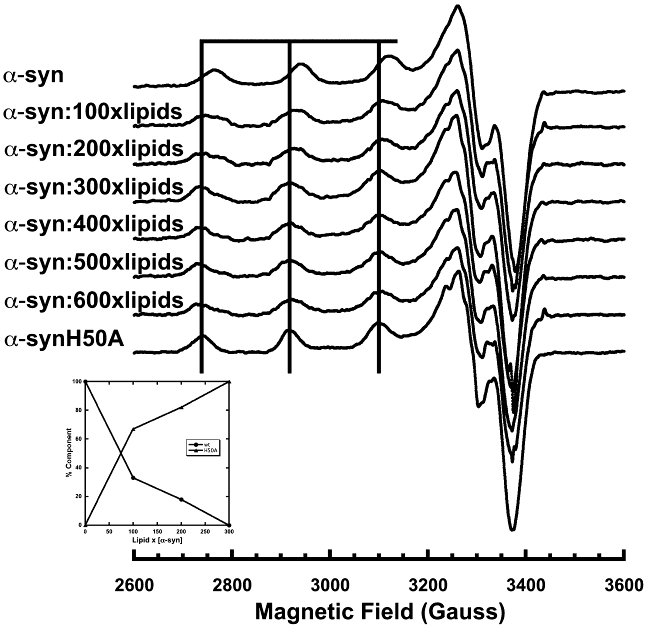

Figure 3. X-Band EPR spectra of α-syn (50 μM) at pH 7.4 with 1 equiv of Cu^{2+} in the presence of an increasing number of molar equivalents of lipid molecules in the form of vesicles. The vertical lines correspond to the parallel hyperfine features of the α-syn(H50A) mutant in solution. α-Syn in the presence of lipids produces a similar if somewhat broadened spectrum that has a downfield shift corresponding to an increase in lipid SUVs. At a lipid:protein ratio of 300:1, the α-syn spectrum essentially overlaps the α-syn(H50A) mutant spectrum. Spectra were recorded at 111 K, ν = 9.44 GHz, with a field sweep of 1200 G.

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:protein ratio. Dichroic peaks with negative intensity at wavelengths of 208 and 222 nm provide a measure of helical structure.[59] As observed in Figure 4a, α-syn achieves its maximal α-helical signal intensity at a lipid:α-syn ratio of 300:1. The mean residue ellipticity (MRE) at 222 nm is approximately 24000 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 upon addition of 1.0× and 10.0× 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).

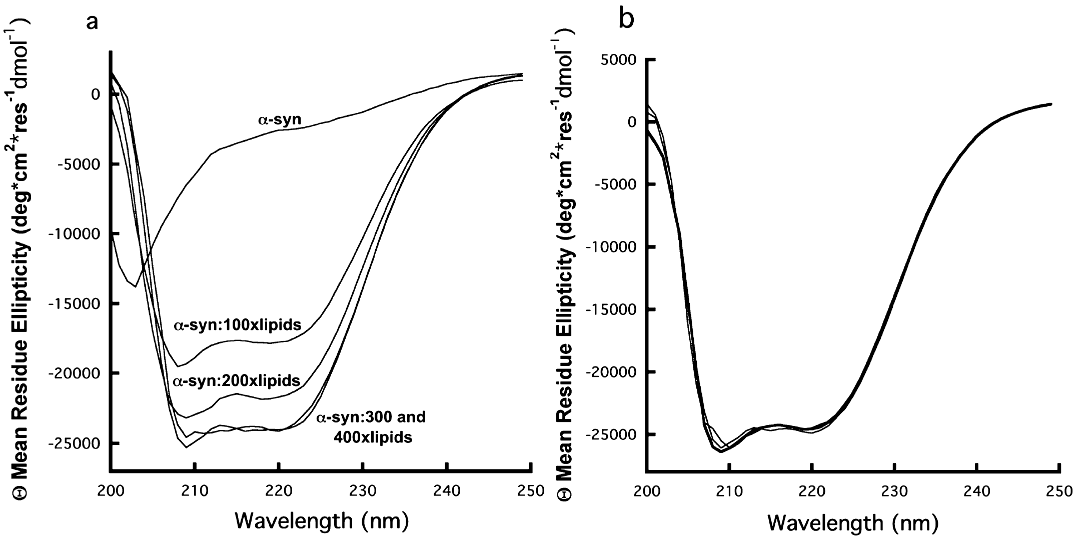

Figure 4. (a) CD spectra of α-syn as a function of addition of lipid in the form of SUVs. From top to bottom, 10 μM α-syn without lipids, 10 μM α-syn with 1 mM lipids, 10 μM α-syn with 2 mM lipids, 10 μM α-syn with 3 mM lipids, and 10 μM α-syn with 4 mM lipids, respectively. The CD signal intensity reaches a maximum at a lipid:α-syn molar ratio of 300:1. (b) Cu^{2+} titration of the 10 μM α-syn–3 mM lipid complex. Cu^{2+} concentrations of 0, 10, and 100 μM 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 SUVs, 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 that is sensitive to spin-active nuclei within approximately 10 Å of 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 equiv of Cu^{2+} shown in Figure 5 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 noncanceled electron spin manifold. Progressive addition of SUVs, measured by lipid concentration, produces a corresponding decrease in the magnitude of the 4.0 MHz peak (see the inset of Figure 5). We also find that lipid SUVs alone and lipid SUVs with α-syn(H50A) (not shown) fail to give an ESEEM spectrum with 1.0 equiv 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.

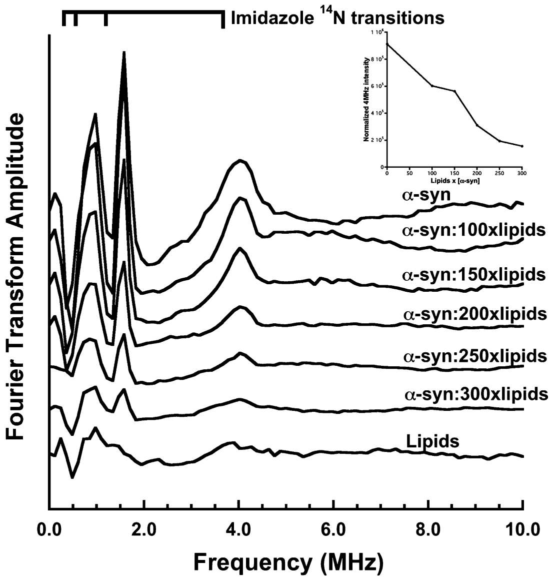

Figure 5. Three-pulse ESEEM spectra of α-syn (50 μM) with 1.0 equiv of Cu^{2+} in the presence of increasing lipid SUV concentrations. The spectra of the samples containing α-syn alone reveal the expected quadrupolar transitions associated with the imidazole remote nitrogen and demonstrate coordination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

Our laboratory previously demonstrated that Met1 and Asp2 form the essential ligands of the Cu^{2+} coordination shell (Figure 2). 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 Supporting Information). Figure 6 shows that the α-syn N-trunc mutation gives a spectrum equivalent to that obtained in the 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.

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

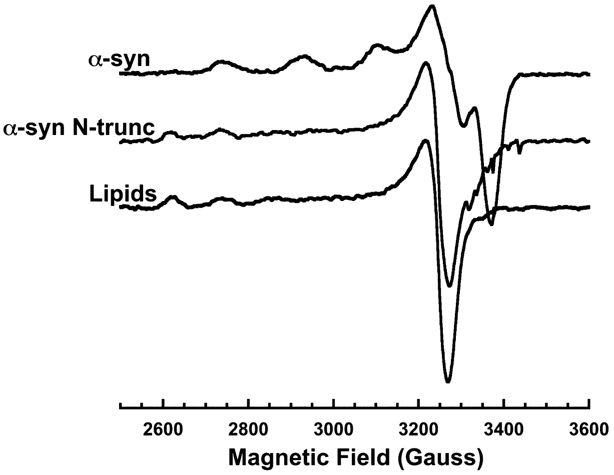

Figure 6. X-Band EPR spectra of α-syn and α-syn N-trunc (both at 50 μM) in the presence of lipid SUVs with 1.0 equiv of Cu^{2+} and the lipid SUVs alone with the same concentration of Cu^{2+}. The α-syn N-trunc spectral features qualitatively resemble those of the lipid SUV spectra, demonstrating the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

the membrane surface. To test this hypothesis, we titrated up to 10 equiv of Cu^{2+} and monitored the mixtures 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 equiv of Cu^{2+} shows hyperfine broadening and the introduction of new hyperfine features consistent with a mixture of the lipid-bound α-syn–Cu^{2+} spectra and the lipid–Cu^{2+} spectra (Figure S1 of the Supporting Information). 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 with up to 10 equiv of Cu^{2+} have low intensities 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 (Figure S2 of the Supporting Information). 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 N-terminus of α-syn 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 histidine is involved in an additional binding site, its affinity for Cu^{2+} is weaker than for 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 the 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 SUVs, 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 the solutions 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 a nonlinear least-squares method gives the ratio of copper-bound to α-syn to 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 1equiv of Cu^{2+} with K_d values of 0.11 and 0.095 nM, determined with NTA and triphosphate, respectively. To further test for H50 coordination, we also performed NTA and triphosphate competition experiments with 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 that of the wild type.

Table 1. Dissociation Constants (nanomolar) Determined from Competition Studies

protein	nitrilotriacetic acid (K_d = 0.366 nM)	triphosphate (K_d = 2 nM)
α-syn with lipids	0.110 ± 0.005	0.095 ± 0.006
α-syn(H50A) with lipids	0.194 ± 0.008	0.196 ± 0.007

■ DISCUSSION

Our combined EPR and CD experiments demonstrate that helical, membrane-bound α-syn is capable of chelating 1 equiv 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 maximal 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. (It is important to note that the level of N-terminal Cu^{2+} uptake would be dramatically reduced or eliminated in acetylated α-syn.[62,63])

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 coordination of His50 to Cu^{2+} is 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 an up to 10-fold excess of Cu^{2+}, 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 coordination of copper 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).[64] Examination of this structure shows that even with additional

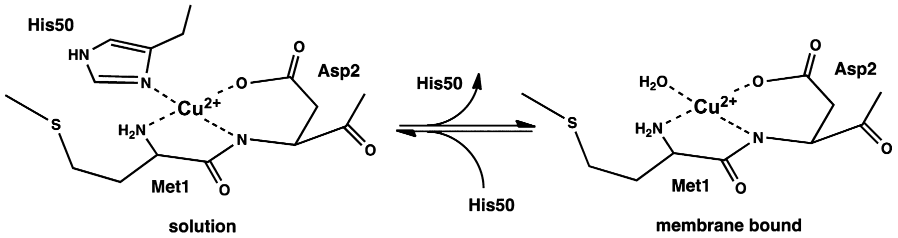

Figure 7. Models of the equilibrium of α-syn−copper binding states. The possibility of His50 involvement is dependent upon whether the protein is in solution (left) or in the membrane-bound helical state (right).

conformations available in this partially ordered α-syn form, His50 would still not able to come into close contact with 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.[65] 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.[65] Interestingly, parallel experiments show that α-syn(1−19), which lacks His50, gives a redox profile somewhat different from that of 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 in reducing cell viability.[65] These data suggest that the copper center in the membrane-bound form of the α-syn−copper complex may be more reactive than the solution form, but this is a tentative proposal because helical α-syn, stabilized by trifluoroethanol, produces lower levels of reactive oxygen species than the unstructured polypeptide.[66]

As described in the introductory section, exposure to high levels of exogenous copper correlates with an increased incidence of PD. This work 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 release of the protein from the membrane to solution. However, once the protein is released from the bilayer, the simultaneous coordination of the N-terminus of the protein 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 1 equiv 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, the lipid:protein ratio exerts a much stronger influence on α-syn conformation than copper coordination. Consistent with the literature described in the introductory section, 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional EPR and ESEEM controls, along with mass spectra of the truncation mutant, and electron micrographs comparing SUVs without and with added α-syn. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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