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Phosphorylation of α-Synuclein at Y125 and S129 Alters Its Metal Binding Properties: Implications for Understanding the Role of α-Synuclein in the Pathogenesis of Parkinson's Disease and Related Disorders

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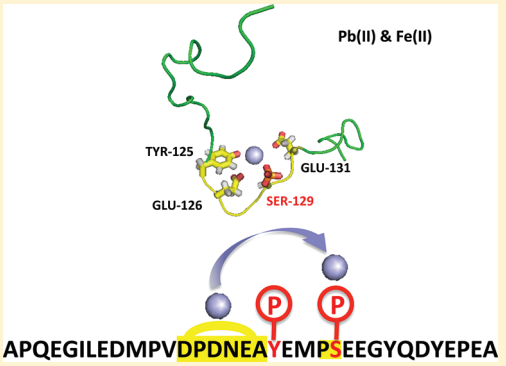
Supporting Information

ABSTRACT: α-Synuclein (α-syn) is a 140-amino acid protein that plays a central role in the pathogenesis of Parkinson's disease (PD) and other synucleinopathies. However, the molecular determinants that are responsible for triggering and/or propagating α-syn aggregation and toxicity remain poorly understood. Several studies have suggested that there are direct interactions between different metals and α-syn, but the role of metal ions and α-syn in the pathogenesis of PD is not firmly established. Interestingly, the majority of disease-associated post-translational modifications (PTMs) (e.g., truncation, phosphorylation, and nitration) of α-syn occur at residues within the C-terminal region (Y125, S129, Y133, and Y136) and in very close proximity to the putative metal binding sites. Therefore, we hypothesized that phosphorylation within this domain could influence the α-syn−metal interactions. In this paper, we sought to map the interactions between the di- and trivalent cations, Cu(II), Pb(II), Fe(II), and Fe(III), and the C-terminal region of α-syn encompassing residues 107−140 and to determine how phosphorylation at S129 or Y125 alters the specificity and binding affinity of metals using electrospray ionization-mass spectrometry (ESI-MS) and fluorescence spectroscopy. We demonstrate that D115-M116 and P128-S129 act as additional Cu(II) binding sites and show for the first time that the residues P128-S129 and D119 are also involved in Pb(II) and Fe(II) coordination, although D119 is not essential for binding to Fe(II) and Pb(II). Furthermore, we demonstrate that phosphorylation at either Y125 or S129 increases the binding affinity of Cu(II), Pb(II), and Fe(II), but not Fe(III). Additionally, we also show that phosphorylations at these residues lead to a shift in the binding sites of metal ions from the N-terminus to the C-teminus. Together, our findings provide critical insight into and expand our understanding of the molecular and structural bases underlying the interactions between α-syn and metal ions, including the identification of novel metal binding sites, and highlight the potential importance of cross-talk between post-translational modifications and metal ion binding in modulating α-syn functional and aggregation properties that are regulated by its C-terminal domain.



KEYWORDS: α-Synuclein, metal ion, binding, C-terminal, phosphorylation, mass spectrometry

α-Synuclein (α-syn) is a 140-amino acid protein that plays a central role in the pathogenesis of Parkinson's disease (PD) and other synucleinopathies. The oligomerization and/or fibrillogenesis of α-syn are thought to be primary causes of the loss of dopamine producing neurons in the *substantia nigra pars compacta* of PD brains and the formation of the cytoplasmic inclusions, known as Lewy bodies (LBs), that are the main pathological feature of the disease.[1−4] However, the molecular determinants responsible for triggering and/or propagating α-syn aggregation and toxicity remain poorly understood. Several studies have demonstrated direct interactions between different metals and α-syn,[5−10] but the molecular mechanisms by which α-syn interactions with metals influence its role in the pathogenesis of PD is not yet firmly established.[11] Metal interactions with α-syn have been reported to influence the structure and propensity for aggregation of α-syn in vitro and in cell culture models of synucleinopathies. Systematic studies by Uversky and colleagues[9] have identified a number of monovalent (Li(I), K(I), Na(I), and Cs(I)), divalent (Ca(II), Co(II), Cd(II), Cu(II), Fe(II), Mg(II), Mn(II), Zn(II)), and trivalent (Al(III), Co(III),

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and Fe(III)) cations that significantly accelerate α-syn fibrillization in vitro under physiological conditions, with Cu(II), Co(II), Al(III), and Fe(III) being the most effective in enhancing α-syn fibrillization. A potential mechanism behind the enhanced amyloid formation is the stabilization of partially folded, on-pathway oligomeric intermediates characterized by an increase in the secondary structure content and changes in the local environment near the tyrosine residues. Indeed, a more recent report by Kostka and colleagues supports this hypothesis. In their study, small oligomers were generated first by incubation with organic solvents. It was shown that Fe(III) specifically induced the formation of larger oligomeric species which resisted SDS-induced dissociation and could form pores as assessed by conductance assays in a planar bilayer setup. Importantly, these oligomers were demonstrated to be on-pathway to amyloid fibril formation.[12] In the case of polyvalent cations, the aggregation mechanism might also include intermolecular bridging between metal anchoring sites. However, the precise mechanism by which metal ions regulate α-syn aggregation may also be unique for each metal ion, as is suggested by the different α-syn fibril morphologies observed with the coincubation of α-syn with various metals.[13]

Several studies have demonstrated that the C-terminal domain (residues 107−140) plays a central role in regulating the interactions of α-syn with metals. For example, it has been shown to bind several divalent cations, Fe(II), Mn(II), Co(II), and Ni(II), although the low (mM) binding affinities suggest a purely electrostatic, nonspecific mode of interaction.[6] Cu(II), which has been shown to bind primarily to the C-terminus of α-syn, is one of the most potent inducers of α-syn multimerization.[8] In another study, coincubation of α-syn with Mn(II) resulted in the immediate formation of dityrosine cross-links.[9] Although the binding site of Mn(II) to α-syn has not yet been mapped, the fact that most of the tyrosine residues in α-syn (Y39, Y125, Y133, and Y136) are clustered in the C-terminal region strongly suggests that it could be the primary Mn(II) interaction domain. The formation of intermolecular dityrosines has been proposed as a possible event in the initiation of α-syn fibrillization. Oxidation of methionine residues to the sulfoxide form creates additional metal coordination sites that dramatically alter α-syn interactions with metals and its fibrillization in vitro. For example, it has been proposed that intermolecular metal coordination involving two or more methionine sulfoxides could promote α-syn fibrillization via a metal bridging mechanism.[14] The C-terminus of α-syn contains two methionine residues, M116 and M127, which could be involved in such metal interactions. Moreover, their proximity to known phosphorylation sites indicates a potential interplay between C-terminal phosphorylation and methionine oxidation in regulating α-syn interactions with metals.

Interestingly, the majority of disease-associated post-translational modifications (PTMs) (e.g., truncation, phosphorylation, and nitration) in α-syn occur at residues within the C-terminal region (Y125, S129, Y133, and Y136) and in very close proximity to the putative metal binding sites. Despite the detection of most of these modifications within LBs, recent studies from our group and others have shown that phosphorylation at S87 or S129 and ubiquitination alter the conformational flexibility of monomeric α-syn and block, rather than promote, its fibrillization.[15,16] These findings suggest that those α-syn PTMs are not required for α-syn aggregation and LB formation. The high degree of flexibility of the C-terminus in the monomeric and aggregated states of the protein combined with its implication in α-syn interactions with other proteins,[5,17−19] ligands, and metal ions suggests that post-translational modifications within this region could significantly influence these interactions and may contribute to regulating α-syn function in health and disease.

In this paper, we sought to map the interactions between different metal ions and the C-terminal region of α-syn encompassing residues 107−140 and to determine how disease-associated phosphorylations alter the specificity and binding affinity of these metal ions using electrospray ionization-mass spectrometry (ESI-MS), which is a suitable tool for studying bioinorganic complexes.[20−23] This technique provides information on binding sites, possible modifications (oxidations, cross-linking, and truncation), and also relative binding affinities.[24−29] More specifically, we focused on the interactions between Cu(II), Pb(II), Fe(II), and Fe(III) ions and the C-terminal fragment α-syn(107−140) (wild-type, wt) and its phosphorylated forms on residues Y125 (pY125) and S129 (pS129). Moreover, the dissociation constants (K_d) were determined based on tyrosine fluorescence.

RESULTS AND DISCUSSION

Comparison of C-Terminal α-Synuclein Binding to Different Metals. Divalent and trivalent cations (e.g., Cu(II), Pb(II), and Fe(III)) have been shown to act as important regulators of α-syn aggregation and are thought to contribute directly or indirectly to the mechanisms underlying neurodegeneration in PD.[30,31] For example, post-mortem analysis of the brains of PD patients shows a characteristic increase in Fe(III) with respect to Fe(II). Fe(III) has been implicated in selective dopaminergic neuronal death. Youdim and colleagues showed that injection of $FeCl_3$ into the *substantia nigra* of rats results in >90% loss of striatal dopamine.[32] Furthermore, Fe(II) is involved in reactive oxygen species (ROS) production via Fenton chemistry and peroxynitrite formation.[33,34] Tabner et al. demonstrated the accumulation of hydrogen peroxide during the incubation of α-syn in PBS and the production of hydroxyl radicals with the addition of Fe(II).[30] Lead is a well-known toxic heavy metal that is associated with several neurodegenerative disorders, including PD: Coon et al. studied 121 PD patients and showed that occupational lead exposure is a risk factor for PD.[35] Part of the toxic response to Pb(II) exposure is the formation of aggresome-type inclusions in target cells, a process which was shown to be controlled by α-syn, among other proteins.[36] Because copper is redox-active in the brain, it has been hypothesized that dysregulation of copper homeostasis, for example, through abnormal binding to α-syn, could catalyze the formation of noxious ROS and thus contribute to neurodegeneration via increased oxidative stress.[37] α-syn itself can become oxidized by a copper-catalyzed reaction resulting in the formation of SDS-resistant oligomers.[38] Rasia et al. showed that copper accelerates α-syn aggregation under physiological conditions without altering the morphology of its fibrillar structures,[10] and Wang et al. identified α-syn oligomeric species that require the presence of copper to induce cell death.[39]

In this study, we investigated the complexation of C-terminal α-syn(107−140) (wild-type, wt) with metal ions such as Cu(II), Pb(II), Fe(II), and Fe(III) by ESI-MS. As shown in Figure 1a, triply charged wt-Cu(II) complexes were clearly observed at m/z = 1307.7 Th in a high abundance with the addition of a 5-fold concentration of Cu(II). At lower concentrations of Cu(II), α-syn-Cu(II) complexes were not observed. The isotopic distribution of the triply charged ions was also examined and was

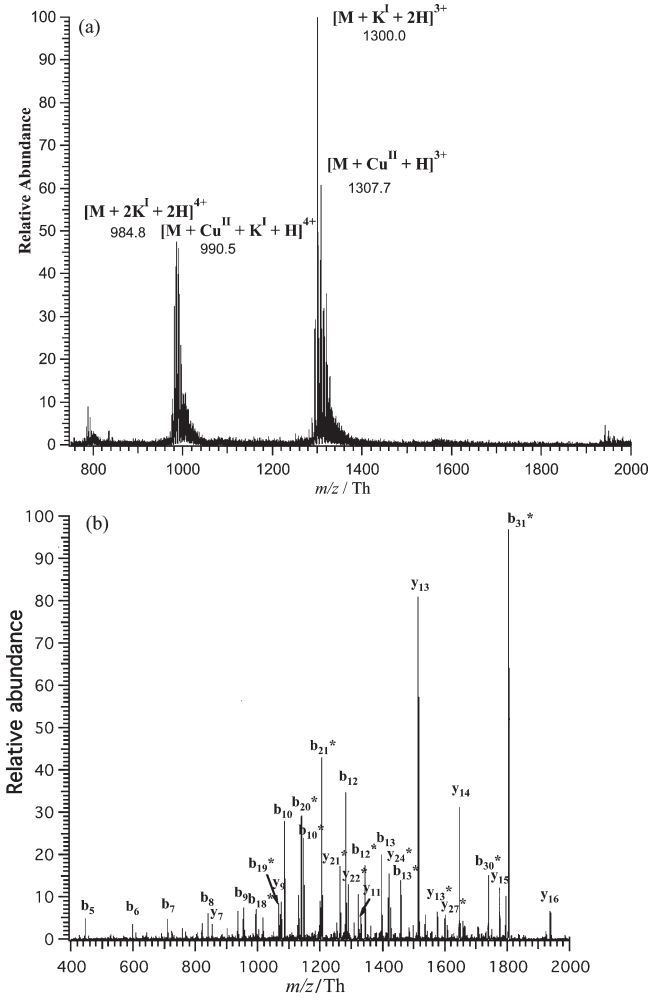

Figure 1. (a) Mass spectrum of C-terminal α-syn(107−140) (5 μM in 50/50 (v/v) MeOH/H_2O) in the presence of 25 μM $CuCl_2$. (b) Tandem mass spectrum of triply charged copper-wt complexes, in which the selected parent ion is at $m/z = 1314.0 \pm 1.7$ Th at 22% of relative collision energy. (Asterisks denote the fragments bound to Cu(II).)

found to match that of the theoretical triply charged wt-Cu(II) complexes. This result demonstrates the existence of Cu(II) complexes to C-terminal α-syn and also suggests that C-terminal α-syn has a relatively weak binding affinity to Cu(II), consistent with previous reports using shorter C-terminal peptide fragments (deleted residues).[10,40]

To determine the binding site of Cu(II) to α-syn(107−140), tandem mass spectrometry (MS/MS) using collision-induced dissociation (CID) was applied. This method allows one to obtain information on structure and to determine binding sites.[20,23] Once selected in a trap, the gas-phase ion (the peptide) is accelerated and gains internal energy by collision with helium gas. The extra internal energy is redistributed along its vibrational modes, which results in the peptide fragmentation. Interestingly, the fragmentation occurs at the peptide bonds, the cleavages of which lead to the generation of b- and y-type fragments (N-terminal and C-terminal, respectively).[41,42] Because metal ions interacting with the peptides prior to their introduction into the mass spectrometer remain bound on the fragments after CID, the residue(s) to which the metal ion binds can be determined by a careful identification of the b- and y-type of ions complexed or not with the metal ion. As shown in Figure 1b, the fragments of triply charged wt-Cu(II) complexes with at least 4% relative abundance were identified and the fragments bound with Cu(II) were assigned (marked with asterisks).

The absence of b_6*, b_7*, and b_8* indicates that the first eight residues are not involved in the binding. The presence of b_9* is difficult to determine because of an overlap between y_8 and b_9*. Then, owing to the coexistence of b_6, b_7, b_8, b_9, and b_{10}*, residues D115-M116 were considered involved in the binding of Cu(II). Both the carboxyl moiety of the aspartic acid side chain and the sulfur of methionine are able to coordinate Cu(II), and hence, it is difficult to exclude either of these residues on the basis of these data. Because b_{10}, b_{12}, and b_{13} along with b_{10}*, b_{12}*, and b_{13}* were produced by CID, the binding site in D115-M116 may not be very stable. The absence of y_7*, y_9*, and y_{11}* excludes the possibility of Cu(II) binding to the last 11 residues. Because of the presence of y_7, y_9, y_{11}, and y_{13}*, residues P128−S129 were found to be involved in the Cu(II) coordination. However, the higher abundance of y_{13} compared to y_{13}* indicates that this binding region (P128−S129) has a lower binding affinity than D115-M116. In addition, the presence of b_{18}*, b_{19}*, b_{20}*, b_{21}* y_{21}*, and y_{22}* in high abundance suggests P120-A124 as another Cu(II) binding region. Together, these results identify three binding sites of Cu(II) within the C-terminus of α-syn, namely, D115-M116, P120-A124, and P128−S129. It has been proposed that the electrostatic interaction between negatively charged carboxylates in the C-terminal region and the positively charged cations contributed to the formation of complexes.[9] The results obtained by tandem mass spectrometry are partly consistent with previous studies by Rasia et al. and Binolfi et al. They utilized $^1H-^{15}N$ heteronuclear single quantum correlation (HSQC) spectra to characterize a second lower-affinity binding motif for Cu(II); C-terminal region D119-E123 showed significant chemical shift changes with the addition of Cu(II) and the strongest change was centered on D121.[6,10]

In these studies, a region with restricted structural elements within this fragment was associated with divalent metal ion binding sites.[6] However, our study demonstrates that D115-M116 and P128-S129 act as additional Cu(II) binding sites. Interestingly, both proline and serine are rarely found in metal binding motifs, and proline has been reported to interfere with metal binding in thioredoxin fold proteins.[43] These findings suggest that phosphorylation at S129 may alter the metal binding affinity and specificity to the C-terminus of α-syn.

Then, we sought to explore the molecular basis of the complexation of Pb(II) and Fe(II) with C-terminal α-syn(107−140); we conducted similar studies to those described above for Cu(II). Mass spectra of Pb(II) and Fe(II) complexes of α-syn(107−140) were observed with a 5-fold metal ion concentration. Tandem mass spectrometry of triply charged Pb(II)-wt and Fe(II)-wt complexes was carried out as well. By comparing the fragments present in these tandem mass spectra, Pb(II) and Fe(II) complexes produced many similar peptide fragments with and without bound metal ions. Our results suggest that Pb(II) and Fe(II) behaved in a similar way with respect to the binding of α-syn(107−140) and identified D119, P128−S129, and P120-A124 as Pb(II) and Fe(II) binding sites. (The tandem mass spectra for each complex are listed in the Supporting Information.) Consistent with our tandem mass spectrometric data, studies by Binolfi et al. revealed sharp changes in chemical shifts of D121-E123 with the addition of Fe(II) on $^1H-^{15}N$ HSQC spectra and they suggested D119-A124 as a potential

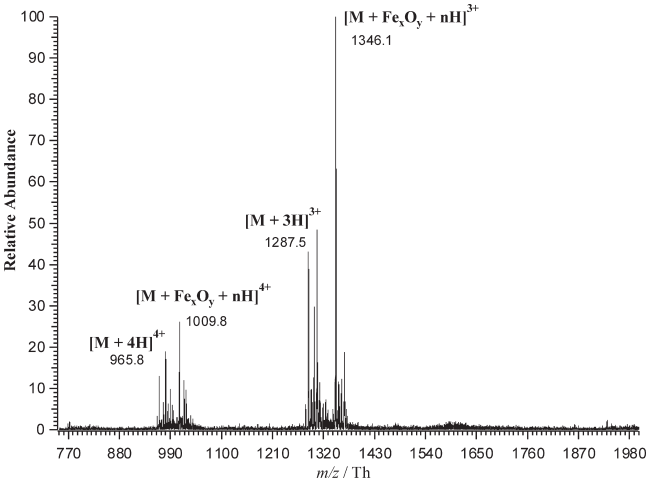

Figure 2. Mass spectrum of C-terminal α-syn(107−140) (5 μM in 50/50 (v/v) MeOH/H_2O) in the presence of 5 μM $FeCl_3$.

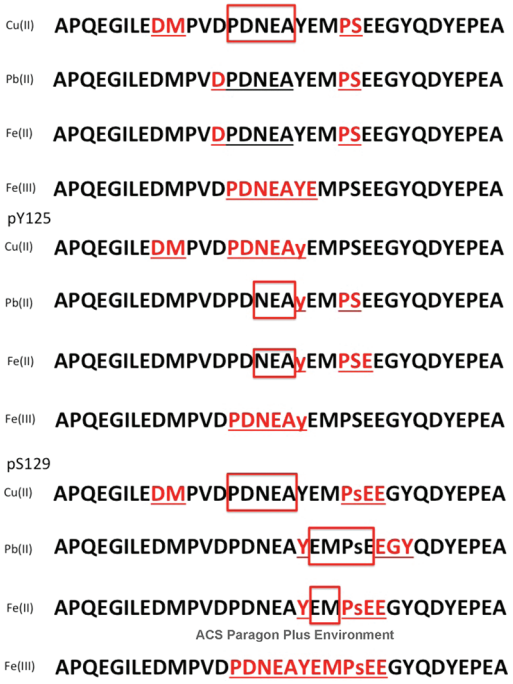

Figure 3. Schematic representation of the binding regions of Cu(II), Pb(II), Fe(II), and Fe(III) to wild type (wt) and phosphorylated C-terminal α-syn(107−140) (pY125 and pS129) obtained from tandem mass spectrometry. The underlined residues were included in the metal binding, and residues in the frame contained a putative binding site.

binding site for Fe(II).[6] Furthermore, we show for the first time that the residues P128-S129 and D119 are also involved in Pb(II) and Fe(II) coordination.

To determine the role of D119 in metal coordination, we synthesized a C-terminal peptide in which D119 was mutated to alanine (D119A). The complexes of D119A with Fe(II) and Pb(II) were observed in the presence of a 5-fold concentration of metal ions, but not in the presence of a one- or two- fold concentration of Cu(II). The relative abundance of the formed complexes was similar to that of wild-type C-terminal peptide, suggesting additional binding sites besides D119. As expected, tandem MS of these complexes (see Supporting Information) revealed that residue A119 in D119A was not involved in the binding to Fe(II) and Pb(II), as corroborated by the absence of b_{13}*. The above observations demonstrate that D119 participates in the binding of α-syn(107−140) of Fe(II) and Pb(II) but is not essential for binding. Also, the metal ion bound fragments of b_{18}*, b_{19}*, b_{20}*, and b_{21}* in the above tandem MS further support the role of P120-A124 in the binding of Fe(II) and Pb(II) and suggest that this region represents a common binding site for divalent metal ions.[6]

The complexation of Fe(III) to C-terminal α-syn(107−140) was then investigated, but wt-Fe(III) complexes were unexpectedly not observed. Instead, as shown in Figure 2, triply charged iron complexes were clearly observed at m/z = 1346.1 Th in a high abundance, in the presence of an equivalent amount of Fe(III). The isotopic signature of single or two iron complexes was compared, and two iron ions were confirmed to be involved in the composition of these iron complexes. An additional shift of +59 Th of this triply charged complex compared to triply charged wt was observed. Considering the high tendency of hydrolysis of Fe(III) in water, the formation of this iron species bound to wt was assigned as $[Fe_2O_4H_2]^+$. Compared to the fact that no complexes of Cu(II), Pb(II), and Fe(II) had been observed with an equivalent amount of metal ions present (i.e., equimolar concentration), the high abundance of hydrated iron oxide complexes implies a much higher binding affinity. Furthermore, tandem mass spectrometry of triply charged hydrated iron oxide complexes was also carried out to identify the binding site of this hydrated iron oxide as P120-M127 (see the Supporting Information). These findings are consistent with the absence of the complexes of Fe(III) and full-length α-syn by ESI-MS that was reported by Zhou et al.[44]

Figure 3 summarizes the binding regions of each metal ion to wild type and phosphorylated C-terminal α-syn(107−140). The underlined residues were clearly identified as binding sites, while the boxed residues represent other potential binding sites. On the basis of the data presented above, the region corresponding to residues D115-M116 was only involved in the Cu(II) binding. D119 was confirmed in Pb(II) and Fe(II) bindings, and the region comprising residues P120-A124 and P128-S129 was involved in the binding of Cu(II), Pb(II), and Fe(II). The binding site of hydrated iron oxide was located within the P120-M127 region, and the loss of two binding sites, at D119 and P128-S129, indicated that Fe(III) binds differently.

Metal Binding to Phosphorylated (pY125 and pS129) C-Terminal α-Synuclein Fragments. Phosphopeptides possess strong binding affinities to some metal ions.[45] Thus, phosphorylation may alter metal ion affinity and specificity and further influences the folding, structure, and functional properties of proteins and peptides. As mentioned above, the majority of putative (Y133 and Y136) and disease associated (S129 and Y125) phosphorylation sites in α-syn occur in the C-terminal region spanning residues 120−140, which also contains the regions involved in mediating α-syn binding to metal ions. Therefore, we hypothesized that phosphorylations within this domain could influence the α-syn metal interactions. To date, only a few studies investigating the effect of phosphorylation on the metal-binding properties of α-syn have been reported: Liu and Franz have studied the effect of phosphorylation on metal binding using short fragments of C-terminal α-syn(119−132)[46,47] and showed that the phosphorylation at Y125 dramatically enhanced Tb^{3+} binding.[46] In particular, they indicated that the Y125 phosphorylated

Table 1. Dissociation Constants (K_d) and the Metal Binding Numbers Per Peptide (n) of the Complexes of Metal Ions and Wild Type and Phosphorylated α-syn(107-140)

	Cu(II)		Pb(II)		Fe(II)		Fe(III)	
α-syn (107−140)	K_d/μM	n	K_d/μM	n	K_d/μM	n	K_d/μM	n
wt	203 ± 7	1.9 ± 0.1	240 ± 12	1.7 ± 0.1	72 ± 3	1.5 ± 0.1	52 ± 3	1.8 ± 0.1
pY125	176 ± 6	1.8 ± 0.1	187 ± 5	3.2 ± 0.3	47 ± 6	1.8 ± 0.5	46 ± 2	1.7 ± 0.1
pS129	160 ± 10	1.8 ± 0.2	83 ± 16	1.0 ± 0.2	45 ± 3	2.2 ± 0.3	50 ± 3	1.8 ± 0.1

form of this peptide selectively bound trivalent metal ions such as Fe^{3+} and Al^{3+} over divalent metal ions, and peptide dimerization induced by trivalent metal ions was observed using ESI-MS, suggesting a possible mechanism for phosphorylation and metal-dependent self-assembly of α-syn.[46,47]

Given that the C-terminal region is very dynamic and involves multiple potential metal binding sites that are likely to play an important role in mediating phosphorylation-dependent metal interactions, we thought that it was important to investigate α-syn metal interactions using longer C-terminal α-syn fragments that span the entire C-terminal region 107−140. Therefore, we prepared the monophosphorylated forms (pY125 and pS129) of α-syn(107−140) and determined the effects of the phosphorylation at each residue on the interactions with metal ions.

The complexation of pY125 and pS129 with metal ions, such as Cu(II), Pb(II), and Fe(II), was studied by ESI-MS using the same protocols as for the wild-type peptide. Both m/z and isotopic distributions of the complexes were thoroughly investigated to identify the compositions of these complexes. As observed with the nonphosphorylated peptide, triply charged metal complexes were clearly observed in a high abundance with the addition of a 5-fold excess of metal ions, but not at lower metal concentrations, suggesting that phosphorylation does not have a dramatic effect on the binding affinity of pY125 α-syn(107−140) to these metal ions. In addition, distinct complexes of both phosphorylated peptides and hydrated iron oxide $[Fe_2O_4H_2]^+$ were observed instead of Fe(III)-peptides in the presence of an equivalent concentration of $FeCl_3$.

To determine how phosphorylation changes the binding affinity of α-syn(107−140) to the metal ions in this study, we measured the dissociation constants of the metal ion complexes for both wild type and phosphorylated α-syn(107−140) by a fluorescence quenching (of tyrosine) assay. Tyrosine-containing peptides exhibit a maximal fluorescence emission at 309 nm after an excitation at 280 nm. The presence of metal ions able to bind such peptides in the vicinity of tyrosine residues quenches the fluorescence in a concentration-dependent manner. Table 1 summarizes the calculated disscociation constants and the metal binding capacity per peptide (n) obtained from fluorescence data according to eq 1. Based to the lower K_d of Fe(III) complexes with α-syn(107−140), Fe(III) ions (in fact $[Fe_2O_4H_2]^+$) displayed the highest binding affinity. Consistent with the mass spectrometry results, hydrated iron oxide complexes were only well observed in the presence of an equivalent amount of metal ions. The similar K_d of hydrated iron oxide complexes with either wt or phosphorylated α-syn(107−140) (Table 1) indicates that phosphorylation at either Y125 or S129 does not appear to impact its binding affinity to Fe(III).

The low K_d of Fe(II) and Fe(III) complexes of both wild type and phosphorylated α-syn(107−140) compared to other metal ions showed that Fe(II) and Fe(III) have similar binding affinities to the peptides. These findings are not consistent with the mass spectrometry results, which showed that Fe(II) complexes of wild type and phosphorylated α-syn(107−140) could not be observed even when a 2-fold excess concentration of Fe(II) ions was used. We assume that the measured K_d for Fe(II) complexes is susceptible to other chemical reactions. The initial excess concentration of Fe(II) ions compared to peptides in a pH 7.4 buffer is not constant during the fluorescence experiment, as Fe(II) is easily oxidized to Fe(III) and hydrolyzed to form a hydrated iron oxide complex, which is a much stronger ligand for α-syn(107−140). Therefore, the binding affinity of Fe(II) should be much lower than the one measured here. Its binding affinity may even approach those of Cu(II) and Pb(II), as the complexes of Fe(II) bound to wild type and phosphorylated α-syn(107−140) could not be observed by mass spectrometry even when a 2-fold excess metal ions was used similar to Cu(II) and Pb(II). The K_d of Cu(II) and Pb(II) for both wild type and phosphorylated α-syn(107−140) was determined to be in the range of 100−200 μM, which indicates weak binding abilities of the peptides. The binding affinities of full-length α-syn to divalent metal ions, such as Ni(II), Co(II), and Fe(II), have also been determined, and the reported ca.1 mM K_d is indicative of similar weak binding abilities.[6] In our studies, the lower K_d value for Fe(II) may be due to the longer C-terminal peptide, and thus, the binding efficiencies of the shorter C-terminal fragments of α-syn to metal ions were higher than the full-length C-terminal region because of differences in the conformational properties for C-terminal peptides of different lengths.[48]

Phosphorylation at both Y125 and S129 did influence the binding affinity for Cu(II), Pb(II), and Fe(II). K_d values of Cu(II), Pb(II), and Fe(II) complexes with phosphorylated α-syn(107−140) were lower than those with wild type, which suggests that the phosphorylations increase the binding affinities for metal ions. Interestingly, the position of the phosphorylated residues did not show a significant difference in the binding affinities, and similar K_d values of the complexes with Cu(II), Pb(II), and Fe(II) were observed for the two phosphorylated forms pY125 or pS129.

Because Fe(II) could be involved in the pathogenesis of synucleinopathies through the formation of ROS by Fenton chemistry, and α-syn has been shown to produce the required H_2O_2 in the presence of catalytic amounts of Fe(II),[30] our results suggest that phosphorylation at either Y125 or S129 could influence Fe(II)-dependent α-syn toxicity via an increased release of hydroxyl radicals. Owing to the proximity of these residues, the present study also highlights the need to study potential cross-talk between phosphorylation sites.

Phosphorylation of α-syn(107−140) Leads to a Shift in Binding Sites of Metal Ions from the N-Terminus to C-Terminus. Similar to the comparisons of the C-terminal α-syn fragments with and without metal bindings present in tandem mass spectra (see the Supporting Information), the binding sites

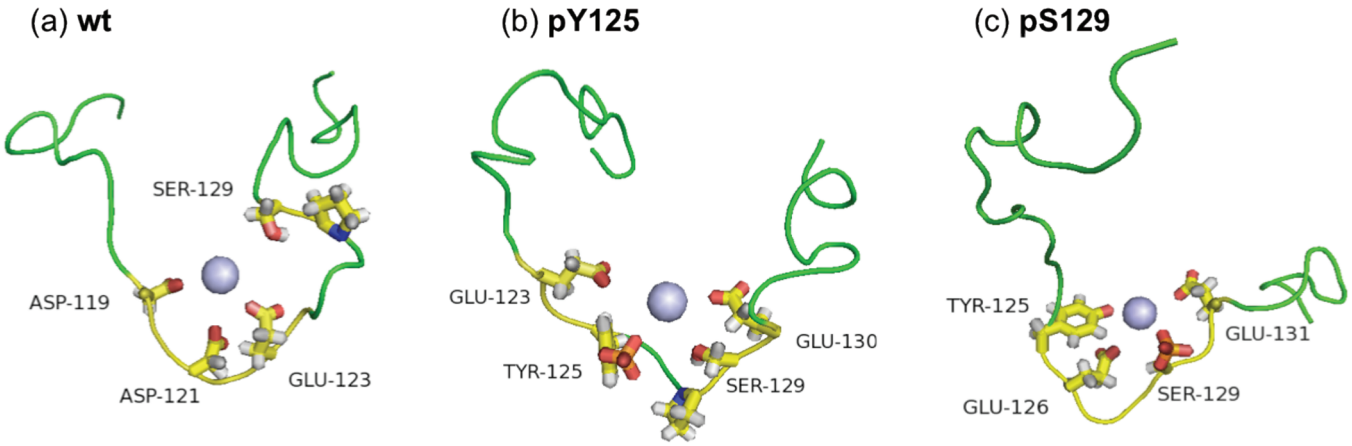

Figure 4. Possible modes of Fe(II) coordinating to wild type (wt) and phosphorylated α-syn(107−140) (pY125 and pS129) schematically drawn using software PyMol.[49]

of Cu(II), Pb(II), Fe(II), and hydrated iron oxide complexes to phosphorylated α-syn(107−140) were identified. Figure 3 clearly shows that the phosphorylations at Y125 and S129 influence the binding sites of metal ions significantly. For example, in Cu(II) binding, residues P128−S129 were involved in the coordination of the wt but not the pY125 peptide. Moreover, compared to the wt binding sites, there was an obvious shift in the Cu(II) binding sites of phosphorylated α-syn(107−140) from the N-terminus to the C-terminus, where the phosphorylated amino acids of both pY125 and pS129 were located. In the cases of the Pb(II) and Fe(II) bindings, the residue D119 participates in the binding of these metal ions to the wt peptide, but not in the binding of both pY125 and pS129. Instead, residue Y125 was only implicated in metal binding of the phosphorylated pY125 and peptides. Furthermore, the different positions of phosphorylated residues on α-syn(107−140) appear to have different effects on the binding sites. Meanwhile, the binding sites of metal ions to pS129 also displayed a similar shift from the N-terminus to the C-terminus compared to the binding sites of pY125. In the case of Pb(II), residues E131-Y133 were involved in the binding of Pd (II) to pS129, but not to pY125. Conversely, the residues within N122-A124 were highly implicated in the binding of Pd(II) to pY125, but not to pS129. In the case of Fe(III) (in fact $[Fe_2O_4H_2]^+$) binding, residues E126-E131 complemented the possible binding sites for pS129 beside these binding regions (P120−Y125) already involved in pY125. Overall, we observed that phosphorylations at Y125 and S129 of α-syn(107−140) lead to the shift of the binding sites of metal ions from the N-terminus to C-teminus.

To clearly illustrate the binding shift induced by phosphorylation, the possible binding modes of Fe(II) to peptides according to the results of the tandem MS were deduced and drawn schematically in Figure 4. We assume the coordination number of Fe(II) to be four and to involve the oxygen atoms of side chains, e.g., carboxylates, phosphates, and deprotonated phenols. As shown in Figure 4, the binding site nearest the N-terminus of a wt peptide bound to Fe(II) is D119 instead of E123 in pY125 and E126 in pS129, and the binding site nearest to the C-terminus of pY125 is E130 instead of E131 in pS129 or S129 in wt. Thus, the binding sites of Fe(II) to wt are located closer to the N-terminus than those for phosphorylated α-syn(107−140), and the putative coordinated residues of pS129 were closer to the C-terminus compared to pY125. The relocations of the binding sites and stronger affinities of metal ions to the phosphorylated peptides strongly suggest that phosphorylation at the C-terminus may play an important role in regulating metal ion binding, and both of these modifications could significantly influence α-syn structure and aggregation, and alter its functional and toxic properties.

In the present work, we investigated the binding features of Cu(II), Pb(II), Fe(II), and Fe(III) to C-terminal α-syn(107−140) and the effects of phosphorylation on the metal binding. ESI-MS is a powerful tool to study bioinorganic complexes and provides detailed information on the compositions and structures of the complexes. Here, owing to the analysis of tandem MS data, the sequence D115-M116 was first identified to be involved in Cu(II) binding. Besides the region D119-A124, which is commonly believed to be involved in the divalent metal ions' binding,[6] the sequence P128-S129 was also found to be involved in the binding with Cu(II), Pb(II), and Fe(II). This new observation increased the correlative residues that could induce the conformation change of C-terminal α-syn and could contribute to the effect of metal ions on α-syn aggregation. A hydrated iron oxide complex with wild type and phosphorylated α-syn(107−140) was determined by ESI-MS instead of Fe(III)-peptides complexes, and they displayed a much higher binding affinity than other metal ions, as revealed by intrinsic tyrosine fluorescence. This unexpected observation explained the absence of the complexes of Fe(III) and full-length α-syn by ESI-MS reported by Zhou et al.[44] and makes it necessary to revaluate the so-called "Fe(III)"-induced aggregation of α-syn in vitro. The dissociation constants, measured by intrinsic tyrosine fluorescence spectroscopy, displayed the enhancement of binding affinities of metal ions to phosphorylated α-syn(107−140) compared with wild type. Moreover, we demonstrate here that the phosphorylation at Y125 and S129 influenced not only the binding affinities but also the locations of binding sites. Tandem MS data showed the migration of the binding site of each metal ion toward the site of phosphorylation. These changes in the binding properties of phosphorylated α-syn(107−140) to metal ions may lead to an alteration in the metal-induced conformational changes of the C-terminus of α-syn and its aggregation properties. Our results expand our understanding of the molecular and structural mechanisms underlying the interactions between α-syn and metal ions, including the identification of novel metal binding sites, and highlight the potential importance of cross-talk between post-translational modifications and metal ion binding in regulating α-syn physiological functions and aggregation properties that are

regulated by its C-terminal domain. Indeed, a recent in vivo study by Chen and colleagues highlighted that phosphorylation at Y125 could counteract pS129-dependent neurotoxicity in a *Drosophila* model of Parkinson's disease.[50]

METHODS

Chemicals. Cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$), ferrous sulfate heptahydrate ($Fe(SO_4)_2 \cdot 7H_2O$), ferric chloride anhydrous ($FeCl_3$), lead(II) nitrate ($Pb(NO_3)_2$), *N*-methylpyrrolidone (NMP), and 32% hydrochloric acid were bought from Acros Organics (Geel, Belgium). Methanol (MeOH) was from Riedel-de-Haën (Seelze, Germany). Deionized water (18.2 MΩ·cm) was obtained from a Milli-Q system from Millipore (Bedford, MA). HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate), dimethylformamide (DMF), triisopropylsilane (TIPS), diethyl ether, and acetonitrile were purchased from AnaSpec Inc. (Fremont, California). 1,2-Ethanedithiol (EDT), *N*,*N*-diisopropylethylamine (DIPEA), thioanisole, trifluoroacetic acid (TFA), and dimethylsulfide were obtained from Sigma (St. Louis, MO).

Peptide Synthesis. Solid phase peptide synthesis (SPPS) was performed on an automated CS 336X peptide synthesizer from CS Bio using standard Fmoc protocols including the in situ neutralization protocol developed by Schnölzer et al.[51] α-syn C-terminal peptides (see list below) were synthesized on a Wang resin with the first Fmoc-Ala amino acid preloaded, with a substitution of 0.4−0.5 mmol/g. Syntheses were carried out at 0.2 mmol scale, and coupling steps were performed with 5 equiv of Fmoc-amino acid, 5 equiv of HBTU, and 5 equiv of DIPEA in NMP. Simultaneous peptide side-chain deprotection and cleavage from the Wang resin was performed by treatment with Reagent H[52] (81:5:3:5:2.5:2:1.5 TFA/phenol/H_2O/thioanisole/EDT/Me_2S/NH_4I), twice for 2 h with diethyl ether precipitation after each treatment with Reagent H, in order to generate peptides with a C-terminal acid functionality while avoiding methionine oxidation to the sulfoxide or sulfone form (M116 and M127). After the last diethyl ether precipitation, the crude products were dissolved in 50% aqueous acetonitrile containing 0.1% TFA, and lyophilized. Peptides were then purified by reversed-phase HPLC (Waters, Milford, MA) using either a Vydac or a Phenomenex C18 preparative column (20 × 250 mm, 5 μm particle size, and 300 Å pore size) and a linear gradient of 0−50% of solvent B in solvent A over 40 min at 10 mL/min, where solvent A was water with 0.1% TFA and solvent B was acetonitrile with 0.1% TFA. Peptide elution was monitored by UV absorption at both 214 and 280 nm.

Peptide masses were confirmed by MALDI-TOF-MS (4800 MALDI-TOF/TOF analyzer, ABSciex, Foster City, California) operating in linear negative ionization mode or by ESI-MS on a Thermo LCQ Fleet (Thermo Scientific, San Jose, CA) operated in positive ionization mode (lower-quality data was obtained using the negative ionization mode on that system). Peptide purity was assessed by reinjection of the pure material after lyophilization on a Vydac C18analytical column.

The sequences of the C-terminal α-synuclein(107−140) fragments prepared are

α-syn(107 − 140) (wt)
APQEGILEDMPVDPDNEAYEMPSEEGYQDYEPEA

α-syn(107 − 140) (pS129)
APQEGILEDMPVDPDNEAYEMP**pS**EEGYQDYEPEA

α-syn(107 − 140) (pY125)
APQEGILEDMPVDPDNEA**pY**EMPSEEGYQDYEPEA

mutant α-syn(107 − 140)(D119A)
APQEGILEDMPV**A**PDNEAYEMPSEEGYQDYEPEA

where **pS** and **pY** indicate phosphoserine and phosphotyrosine, respectively. The purified, lyophilized peptides were dissolved in deionized water at a final concentration of 1 mg/mL, which were further confirmed by UV absorption spectroscopy using a Cary 100 Bio UV−vis spectrophotometer (Varian, CH). The molar extinction coefficients used were 2841 $M^{-1} \cdot cm^{-1}$ at 267 nm pY125 and 4470 $M^{-1} \cdot cm^{-1}$ at 280 nm for wt and pS129. These stock solutions were aliquoted and stored at −20 °C. The stock solution of $CuCl_2$ and $Pb(NO_3)_2$ were dissolved in deionized water at a concentration of 10 mM, 100 mM, or 1 M, while the same concentrations of $FeCl_3$ stock solution were prepared in 10 mM HCl solution in deionized water.

Intrinsic Tyrosine Fluorescence. Fluorescence measurements were carried out in a rectangular 1 cm quartz cuvette using an LS 55 luminescence spectrometer (PerkinElmer, Waltham, MA). The excitation was set to 280 nm, and the emission spectra were recorded from 290 to 420 nm (emission slit, 6.0 nm; excitation slit, 4.0 nm; scan speed, 500 nm/min; accumulation, 3). Peptides were dissolved at approximately 5 μM in a 10 mM Tris-HCl buffer at pH 7.4. Metal ions were added from stock solutions (10 mM, 100 mM, and 1 M) to get final concentrations ranging from 0 to 3 mM. All experiments were repeated independently three times.

The maximum tyrosine fluorescence was monitored at 309 nm and corrected from background induced by the Tris-HCl buffer pH 7.4, in the presence and absence of metal salts. The maximum fluorescence was normalized and plotted in log scale as a function of the metal ion concentration. Dissociation constants were obtained by fitting the data (using the software IgorPro 6.0, WaveMetrics Inc., Tigard, OR) according to the following equation:

$$F([M]) = \left(a + b\left(\frac{[M]}{c}\right)^n\right) \Big/ \left(1 + \left(\frac{[M]}{c}\right)^n\right) \quad (1)$$

where [M] stands for the metal ion concentration in μM, *F* for the fluorescence, *a* and *b* for the fluorescence at [M] = 0 and ∞, respectively, *c* for K_d in μM, and *n* for the number of binding sites.

Mass Spectrometry. An LTQ ion trap mass spectrometer (Thermo Scientific, San Jose, CA) was used in the analyses of the peptides and metal−peptide complexes, and all data were collected in positive ionization mode. The commercial heated electrospray ionization interface was used and the heated transfer capillary was kept at 275 °C. The applied high voltage was set at 4.0 kV, and the sample was infused at 3.5 μL/min. The ion optics parameters were kept constant for each experiment. Peptide solutions for the MS analyses were diluted to a final concentration of 5 μM in 50/50 MeOH/H_2O (v/v) before each experiment. Aliquots of metals were added into peptide solutions and gently mixed with a micropipet, and then incubated for at least 10 min at room temperature before being diluted with 50/50 MeOH/H_2O (v/v) to a final peptide concentration of 5 μM before the MS analysis. All solutions were prepared fresh daily. The fragments generated in MS/MS were assigned based on the calculations of the web-based software, MS-Products from UCSF (http://prospector.ucsf.edu/prospector/mshome.htm).

ASSOCIATED CONTENT

Supporting Information. Mass-to-charge ratio peaks on tandem MS spectra of the metal-peptide complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Y.L., M.P., B.F., H.A.L., and H.H.G. were involved in the conception and experimental design of both the project and the experiments, and they participated in the writing and editing of the manuscript. Peptide syntheses and purification were performed by M.P. and B.F. Fluorescence measurements were conducted by M.P. and Y.L. Mass spectrometry measurements were performed by Y.L.

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■ ABBREVIATIONS

α-syn, α-synuclein; wt, wild type; K_d, dissociation constant; MS, mass spectrometry; ROS, reactive oxygen species; CID, collision-induced dissociation

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