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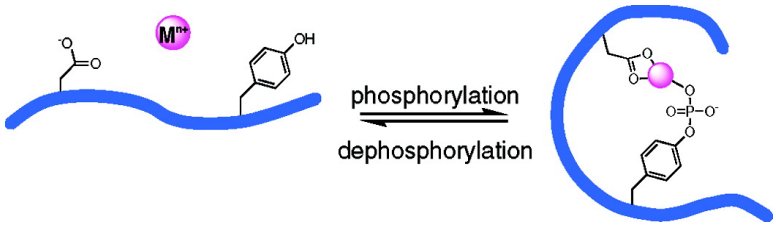

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Phosphorylation of an α-Synuclein Peptide Fragment Enhances Metal Binding

Lucy L. Liu and Katherine J. Franz*

Department of Chemistry, Duke University, P.O. Box 90346, Durham, North Carolina 27708

Received November 9, 2004; Revised Manuscript Received May 26, 2005; E-mail: katherine.franz@duke.edu

The phosphorylation state of a protein can regulate activity, localization, and protein−protein interactions.[1] It also potentially alters a protein's interaction with metal ions, which in some cases is beneficial. For example, egg-yolk phosvitin and milk casein bind dietary iron and calcium, respectively,[2] and dentin phosphoprotein directs calcium biomineralization.[3] In other cases, these interactions may be harmful; for example, Fe^{3+} causes aggregation of hyperphosphorylated tau,[4] and Al^{3+} and Ca^{2+} bind phosphorylated fragments of human neurofilament protein;[5] both proteins are implicated in Alzheimer's Disease.

We are interested in understanding how protein phosphorylation alters metal ion affinity and specificity and how these adducts consequently alter protein conformation. In this report, we demonstrate that the phosphorylation state of a peptide derived from α-synuclein (α-syn), a protein that forms amyloid-type fibrils in Parkinson's Disease,[6] dramatically alters metal binding. We observe multiple metal-binding events on a short peptide, which implies changes in both peptide conformation and charge balance, two important factors that influence fibrillization of the full-length protein.

Metal ions influence the aggregation pathway of α-syn in vitro,[7] most likely by interacting with the acidic carboxyl terminus. Residues 109−140, for example, have been implicated in Ca binding.[7e,f] It has been proposed that metal ions induce partially folded intermediates that affect fibril formation,[7a,d] but few molecular details are known about these intermediates or how phosphorylation influences them. We chose to study the metal-binding properties of the α-syn fragment 119−132 because its arrangement of carboxylate groups is similar to Ca-binding loops (Figure 1) and because it contains two identified phosphorylation sites, Tyr125 and Ser129.[8] Figure 1 shows the full-length sequence of α-syn together with our synthetic peptides, which include **α-syn(119−132)**, its phosphorylated analogues, **pS129** and **pY125**, and several truncated mutants of **pY125** labeled **pY-a**, **pY-b**, and **pY-c**.

We monitored metal binding by using the convenient luminescence properties of Tb^{3+} that make it a useful probe of Ca-binding proteins.[9] To achieve Tb^{3+} luminescence, the coordination environment must provide a sensitizing chromophore and a ligand set that minimizes coordinated waters, which quench emission.[10] Tyrosine[10] and phosphotyrosine[11] are known to sensitize Tb^{3+} luminescence.

If **α-syn(119−132)** were to bind Tb^{3+} in a manner analogous to Ca-binding loops, its tyrosine would coordinate the metal via its backbone carbonyl. If so, **α-syn(119−132)** and **pY125** would respond similarly to Tb^{3+}. As shown in Figure 2, however, only **pY125** displays strong Tb-sensitized luminescence. The absent to weak signals observed for the unphosphorylated peptide and **pS129** indicate little affinity of these peptides for Tb^{3+}. Phosphotyrosine (pTyr) alone provides a weak signal under these conditions, verifying that **pY125** contains a pTyr well positioned among other metal-binding residues. This configuration represents a novel metal-binding site created upon specific phosphorylation, and the results

MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV
GSKTKEGVVH GVATVAEKTK EQVTNVGGAV VTGVTAVAQK
TVEGAGSIAA ATGFVKKDQL GKNEEGAPQE GILEDMPVDP
DNEAYEMPSE EGYQDYEPEA

name	Sequence
Ca-loop	X * Y * Z * # * X * * Z
α-syn 119-132	Ac-D P D N E A Y E M P S E E G
pS129	Ac-D P D N E A Y E M P **pS** E E G
pY125	Ac-D P D N E A **pY** E M P S E E G
pY-a	Ac-D P D N E A **pY** E Nle P S
pY-b	Ac-D P **A** N E A **pY** E Nle P S
pY-c	Ac- N **A** A **pY** E Nle P S

Figure 1. Top: amino acid sequence of human α-syn; residues 119−132 in red. Bottom: sequences of acetyl-capped peptides used in this study and compared to Ca-binding loops. X, Y, and Z are Ca-binding residues (usually E or D); # is a metal-binding backbone carbonyl, and ∗ can be any residue. Phosphorylated residues indicated as pS and pY. Norleucine (Nle) is a hydrocarbon analogue of methionine.

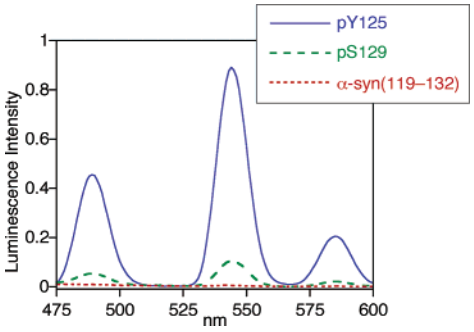

Figure 2. Luminescence emission plots of 2 μM α-syn peptide fragments in the presence of 40 μM Tb^{3+} in 10 mM hepes buffer, 100 mM NaCl, pH 7.0, $\lambda_{ex} = 270$ nm. Residual Tb background signal was subtracted from all spectra.

suggest that it is the phosphate of **pY125** that provides the critical anchor for metal binding. A direct metal−phosphate interaction was confirmed by ^{31}P NMR, which shows a single, sharp resonance at −4.2 ppm for apo-**pY125** that broadens and shifts to −49 ppm upon addition of Tb^{3+} (Supporting Information). In contrast, no shift is observed after addition of Tb^{3+} to **pS129**.

To identify the other metal-binding residues, we examined several mutated versions of **pY125**.[12] Figure 3 displays results of Tb^{3+} titrations of all peptides and pTyr amino acid. For **pY125**, **pY-a**, and **pY-b**, the data reveal three distinct transitions of varying intensity, two that increase and a third that decreases luminescence. Some precipitation was observed above 135 μM added Tb^{3+}, most likely from an insoluble Tb−hydroxide species; therefore, these data were excluded from fitting analysis. For **pY125**, **pY-a**, and **pY-b**, the best fits were provided by a model containing [1:2], 1:1, 2:1, and 3:1 Tb:peptide complexes; a 1:1 model was used for **pY-c** and pTyr. The tight 300 nM dissociation constant (K_D) for 1:1 binding by **pY125** is impressive compared with that of other peptides of this size.[13]

Comparison of the K_D values tabulated in Figure 3 reveals that removing residues corresponding to E130 and E131 (**pY-a**)

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Tb:pep (μM)	K_D(1:1)	K_D(2:1)	K_D(3:1)	K_D(1:2)
pY125	0.31(2)	35(4)	250(30)	0.7(3)
pY-a	0.59(5)	64(4)	500(50)	0.55(6)
pY-b	1.93(6)	420(70)	740(60)	1(2)
pY-c	19.9(4)	-----	-----	-----
pTyr	88.5	-----	-----	-----
pS129				

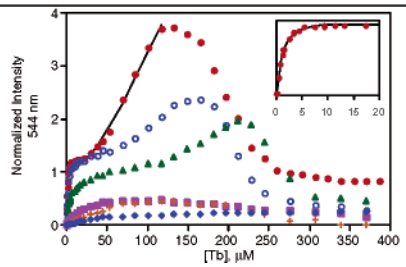

Figure 3. Titration data of Tb^{3+} added to 1 μM peptide in 10 mM hepes buffer, 100 mM NaCl, pH 7.0, $\lambda_{ex} = 266$ nm. Solid line represents the best fit of the data; inset shows the expansion of the pY125 data to 20 μM Tb^{3+}.

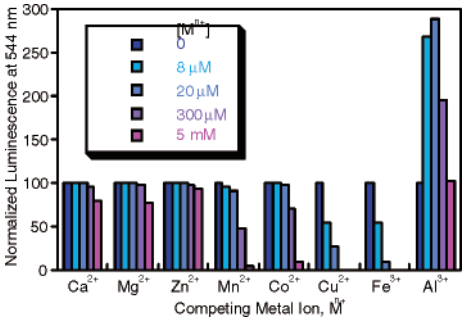

Figure 4. Tb luminescence response upon addition of various concentrations of competing metal ions to 1 μM pY125 peptide in the presence of 8 μM Tb^{3+}.

compromises the 1:1 binding affinity by less than a factor of 2, whereas replacing D121 with nonligating alanine (**pY-b**) reduces the affinity 6-fold compared to **pY125**. Furthermore, the second binding site of **pY-b** is 12-fold weaker than **pY125**. The **pY-c** mutant has lost its ability to stabilize 2:1 or 3:1 species, but its increased affinity over pTyr establishes that glutamate E126 helps to stabilize a 1:1 Tb:peptide complex. Taken together, these data indicate that D121 and E126 combine with pY125 to adopt a phosphotyrosine-dependent 1:1 metal-binding site. In addition, E130 and E131 are important for binding additional Tb^{3+} ions.

The loss in emission at high [Tb] is not due to phosphate hydrolysis, as evidenced by HPLC and mass spectral analysis of **pY125** following incubation with 500 μM Tb^{3+}, revealing only intact phosphopeptide. The decrease may be due to lanthanide self-quenching[14] as a third Tb^{3+} associates with the peptide.

Further evidence for 1:1, 2:1, and 3:1 Tb:peptide species comes from mass spectrometry, which reveals all three metal adducts for **pY125** but not for **α-syn(119−132)** (Supporting Information). Mass spectrometry also provides an alternative assessment of binding affinity. From spectra of samples of varying Tb:pep ratios, we calculate a 1:1 K_D value for **pY125** of 0.26(5) μM, in agreement with results from luminescence titrations. A similar analysis of **α-syn(119−132)**, however, provides numbers ranging from 12 to 2 μM. This variability suggests that the observed gas-phase adducts do not reliably represent solution-phase behavior; therefore, only a lower limit K_D can be estimated for **α-syn(119−132)**. Notably, this range is consistent with values for typical Ca-binding loops.

Metal ion selectivity of **pY125** was assessed by measuring the change in Tb^{3+} luminescence upon addition of a second metal ion, as shown in Figure 4. Displacement of Tb^{3+} causes a loss in signal, as observed at elevated concentrations of Ca^{2+} and Mg^{2+}. A change of signal could also result from heterometallic species formed with paramagnetic ions such as Fe^{3+} or Cu^{2+} capable of luminescence quenching, or a diamagnetic ion like Al^{3+} capable of inducing a change in coordination around Tb^{3+} that improves luminescence. The behavior of Fe^{3+} is especially interesting because it has been detected in Lewy Bodies.[15] Other Tb-binding peptides do not show Fe^{3+}-dependent quenching under similar conditions,[13a] suggesting that our result arises from a specific interaction between **pY125** and Fe^{3+}.

In conclusion, these studies highlight that a single and specific phosphorylation site appropriately surrounded by other metal-binding residues has a dramatic influence on the metal-binding properties of a peptide. Importantly, the affinity for metal is regulated by phosphorylation, which raises intriguing questions about the potential role of these interactions in biology.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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