

Copper Binding to the N-Terminally Acetylated, Naturally Occurring Form of Alpha-Synuclein Induces Local Helical Folding

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Supporting Information

ABSTRACT: Growing evidence supports a link between brain copper homeostasis, the formation of alpha-synuclein (AS)-copper complexes, and the development of Parkinson disease (PD). Recently it was demonstrated that the physiological form of AS is N-terminally acetylated (AcAS). Here we used NMR spectroscopy to structurally characterize the interaction between Cu(I) and AcAS. We found that the formation of an AcAS–Cu(I) complex at the N-terminal region stabilizes local conformations with α -helical secondary structure and restricted motility. Our work provides new evidence into the metallo-biology of PD and opens new lines of research as the formation of AcAS–Cu(I) complex might impact on AcAS membrane binding and aggregation.

Neurodegeneration in Parkinson disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra and by the presence in multiple brain regions of amyloid fibrillar cytoplasmic aggregates, known as Lewy bodies, containing the protein alpha-synuclein (AS).¹ Although it remains unclear how AS initiates neuronal death, there is growing evidence that supports a role for AS aggregation in the pathological effects associated with PD.² Protein–metal interactions play an important role in AS aggregation^{3,4} and might represent a link between the pathological processes of protein aggregation, oxidative damage in the brain, and neuronal cell loss.^{5–7} Indeed, the role of copper ions in AS amyloid assembly and neurodegeneration became a central question in the pathophysiology of PD.^{8–10}

Recently, abundant evidence revealed that AS undergoes N-terminal acetylation in vivo (AcAS).^{11,12} From several in vitro studies focused on the role of acetylation in AS, it was

demonstrated that the cotranslational modification induces a modest population of α -helical conformation for the first six residues and enhances the lipid binding properties of the protein, whereas no significant differences were observed in the fibrillation kinetics between acetylated and nonacetylated AS.^{13,14} In that direction, it was reported recently that N-terminal acetylation of AS abolishes Cu(II) binding at the high-affinity Met-1 site present in the nonacetylated protein.¹⁵ However, since copper ions are predominantly found in their Cu(I) state in the reducing environment of living cells, it is the characterization of the physiologically relevant AcAS–Cu(I) complexes that becomes fundamental to understand the impact of this interaction on protein conformation and aggregation. Accordingly, we report here the detailed structural characterization of Cu(I) complexes with the full-length acetylated AS protein.

The details of Cu(I) binding to AcAS were explored at an atomic resolution by NMR spectroscopy. We first recorded the ¹H–¹⁵N SOFAST-HMQC spectrum of Cu(II)–AcAS complexes (Supporting Information Figure 1), which confirmed the lack of binding of the metal ion to the Met-1 site at the N-terminus. Then, ascorbate was added as reducing agent to generate the AcAS–Cu(I) complexes.^{16,17} The ¹H–¹⁵N SOFAST-HMQC spectra of AcAS–Cu(I) complexes revealed the occurrence of very large chemical shift changes in a discrete number of residues located at the 1–15 segment of the N-terminal region, with smaller shift perturbations centered on the amide group of His-50 and the region comprising residues 115–130 that contains Met residues at positions 116 and 127 (Figure 1). Then, we investigated the interaction at pH 7.4 and 37 °C using direct ¹³C detection NMR methods,¹⁸ on the basis

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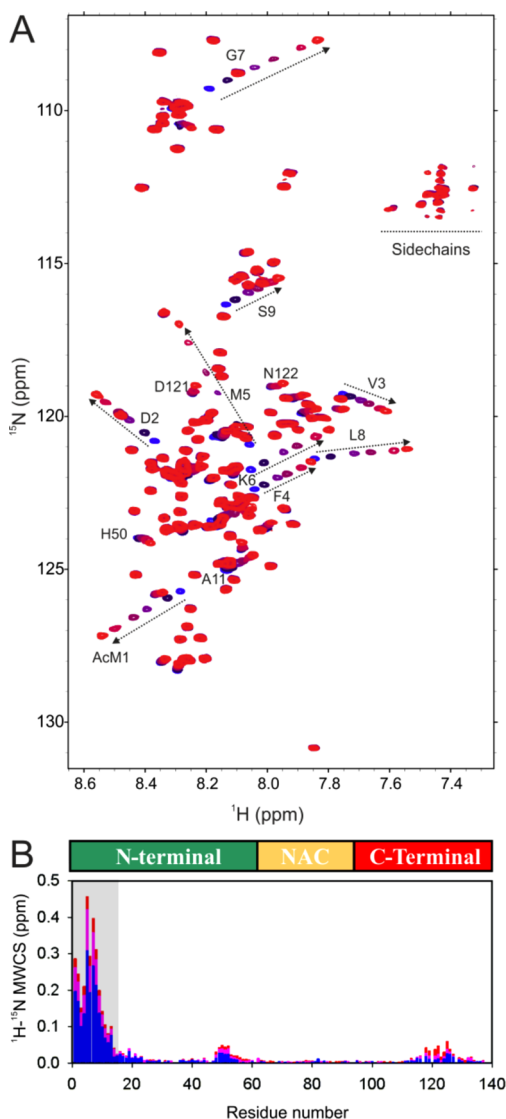


Figure 1. NMR analysis of Cu(I) binding to AcAS. (A) Overlaid ^1H - ^{15}N SOFAST-HMQC spectra of AcAS in the absence and presence of increasing Cu(I) concentrations. From blue to red, the titration experiments represent the addition of 0, 0.25, 0.5, 1.0, 2.0, and 5.0 equiv of Cu(I). Most-affected residues are labeled. (B) Differences in the mean weighted chemical shift displacements (^1H - ^{15}N MWCS) between free and Cu(I)-complexed AcAS at molar ratios of 1:1 (blue), 2:1 (purple), and 5:1 (red). Gray box identifies the first 15 residues of AcAS sequence. Experiments were recorded at 15 °C using AcAS (50 μM) samples dissolved in buffer A.

that these conditions of pH and temperature would resemble more closely those found intracellularly. A similar profile of chemical shift changes for the AcAS-Cu(I) interactions was obtained from these studies after analysis of $C\alpha$ and CO shift perturbations (Supporting Information Figure 2). Inspection of AcAS side chains through ^1H - ^{13}C HSQC spectra revealed that upon Cu(I) addition the most affected resonances corresponded to those of $\text{H}\epsilon$ - $\text{C}\epsilon$ and $\text{H}\gamma$ - $\text{C}\gamma$ correlations in Met-1 and -5 and to a lesser extent to those belonging to residues Met-116 and -127 and $\text{H}\beta$ - $\text{C}\beta$ from His-50 (Supporting Information Figure 3).

We have previously shown that Cu(I) binding to non-acetylated AS occurs at three independent, noninteracting sites and is mediated by coordination of sulfur atoms from Met-1/

Met-5 (site 1), the imidazole ring of His-50 (site 2), and sulfur atoms from Met-116/Met-127 residues (site 3).^{16,17,19} The results in Figure 1 and Supporting Information Figures 2 and 3 demonstrate that these coordination modes are preserved upon acetylation of Met-1. From the experiments in Figure 1A, the NMR-derived apparent affinities for the Cu(I) complexes at sites 1–3 (Met-1/Met-5 (site 1), His-50 (site 2), and Met-116/Met-127 (site 3)) were $K_{d, \text{app}1} = 12 \pm 4 \mu\text{M}$, $K_{d, \text{app}2} = 50 \pm 6 \mu\text{M}$, and $K_{d, \text{app}3} > 200 \mu\text{M}$, respectively (Supporting Information Figure 4).

Overall, the results presented here demonstrate that AcAS is able to interact with Cu(I) with the same binding preferences and affinity features as nonacetylated AS. Notably, the changes observed in the chemical shifts of residues located in the vicinity of the high-affinity Cu(I) binding site were substantially different for the acetylated and nonacetylated proteins,^{16,19} a fact that motivated us to evaluate the AcAS-Cu(I) complexes in terms of their conformational properties. NMR chemical shifts are frequently used to probe the propensity of intrinsically disordered proteins to populate different secondary structure types. We then used $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ secondary chemical shifts (SCS)²⁰ and secondary structure propensity scores (SSP)^{21,22} to compare the secondary structure content of AcAS and its Cu(I) complexed form. As previously reported, the SCS and SSP profiles for the acetylated form of the protein showed a slight increase in the population of α -helical conformation near the N-terminus, strictly limited to the first six residues (Figure 2A,B).^{13,14} Surprisingly, we detected a large increase in α helix content in the first 15 residues of Cu(I)-bound AcAS, as evidenced by large positive deviations of $C\alpha$ and negative deviations of $C\beta$ chemical shifts. Assuming a ~ 3 ppm secondary chemical shift for an ideal α helix, our data is consistent with α -helical conformations sampled ~ 20 and $\sim 70\%$ of the time for the first 6 residues in AcAS and for the first 10 residues in the AcAS-Cu(I) form, respectively (Figure 2A,B). Apart from chemical shifts, $^3J_{\text{HN-H}\alpha}$ couplings are particularly reliable quantitative reporters of the time-averaged distribution of the backbone torsion angles, ϕ .²³ Therefore, we measured a nearly complete set of $^3J_{\text{HN-H}\alpha}$ couplings in both AcAS and AcAS-Cu(I) states. With the exception of the remarkable decrease in $^3J_{\text{HN-H}\alpha}$ for the first 10 residues of AcAS upon Cu(I) binding, the values measured for the two forms of the protein were essentially indistinguishable. The observed decreases, with $^3J_{\text{HN-H}\alpha}$ couplings ranging between 4.0 and 5.6 Hz, are again indicative of a substantially increased α helix conformation in that segment of AcAS-Cu(I) relative to its metal-free form (Figure 2C). With an averaged $^3J_{\text{HN-H}\alpha}$ of 4 Hz expected for an ideal α helix and a $^3J_{\text{HN-H}\alpha}$ of ~ 7 Hz for random coil, the approximate increase in the level of α -helical conformation is consistent with the one estimated by chemical shifts. We also analyzed NOE-based data to evaluate the increase of the α -helical content at the N-terminus of AcAS-Cu(I) relative to AcAS. From 3D ^{15}N NOESY-HSQC edited experiments, we were able to detect strong short-ranged $_{\text{NN}}(i-1, i)$ and medium-ranged $_{\text{aN}}(i-3, i)$ ^1H - ^1H NOE connectivities for the first 10 residues of AcAS-Cu(I), which were absent in the uncomplexed form of the protein (Supporting Information Figure 5A,B). Added to this evidence, which is typically associated with α -helical character,^{24,25} we also quantified the intensity ratios between intraresidue $d_{\text{aN}}(i, i)$ and sequential $d_{\text{aN}}(i-1, i)$ cross-peaks in both the metal-free and metal-complexed states of the protein.¹⁴ As shown in Supporting Information Figure 5C, the increased values measured for the

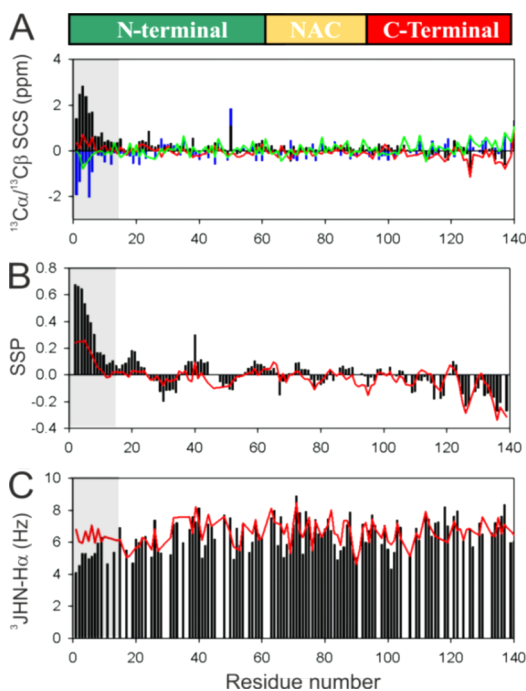


Figure 2. Impact of Cu(I) binding on the structural properties of AcAS. (A) $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ secondary chemical shifts measured for AcAS (red and green lines, respectively) and for its Cu(I)-bound form (black and blue bars, respectively). (B) Secondary structure propensity (SSP) of AcAS (red line) and its Cu(I)-bound form (black bars). $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts were used to calculate the residue-specific SSP scores. Positive values ranging from 0 to 1 and negative values from 0 to -1 represent the propensities to α and β structures, respectively. (C) $^3J_{\text{HN-H}\alpha}$ couplings measured for AcAS (red line) and AcAS–Cu(I) (black bars). Gray box contains the first 15 residues of AcAS sequence. Experiments were recorded at 15 °C using AcAS (400 μM) samples dissolved in buffer A in the absence and presence of 5 equiv of Cu(I).

$d_{\alpha\text{N}}(i,i)/d_{\alpha\text{N}}(i-1,i)$ ratios in the first residues of AcAS–Cu(I) relative to AcAS are also indicative of a helical signature at that region.¹⁴ Altogether, the changes in the three independent indicators of helical propensity are consistent with the transition of a transient short α helix for the very N-terminal residues in AcAS toward a longer, more structured and stable α -helical segment in its Cu(I) complexed state.

Next, we characterized the dynamic properties of the Cu(I)-complexed state of AcAS. To this end we measured ^{15}N R_1 and R_2 relaxation rates and heteronuclear (^1H – ^{15}N) NOEs (hetNOEs).²⁶ Whereas structures persisting on supra- τ_c (τ_c = overall tumbling time) can be monitored by chemical shifts and scalar coupling constants (J), ^{15}N relaxation rates and hetNOEs are sensitive to motions faster than the overall tumbling time in the subnanosecond time regime. We first performed this set of experiments on the free state of AcAS, in which R_1 and R_2 relaxation rates and hetNOE data all showed similar sequence dependence: lower values were found at the termini of the protein, whereas the central portion of the relaxation profile formed a plateau with R_1 values between 1.3 and 1.8 s^{-1} , R_2 values between 2.0 and 4.0 s^{-1} , and hetNOEs in the range 0–0.35 (Figure 3). These results clearly indicate that AcAS exists as a highly flexible state,²⁷ in which the population of α -helical conformations remains low, fully consistent with our analysis of chemical shifts, scalar coupling constants and NOE data. Complexation with Cu(I) results in a small increase in the R_1

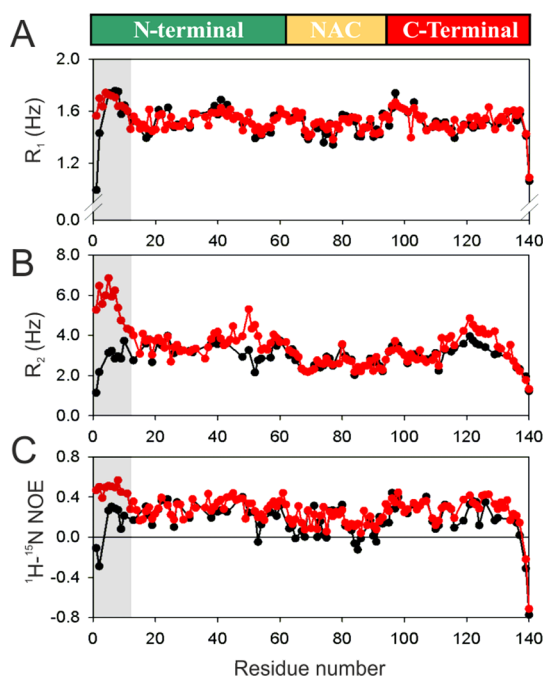


Figure 3. ^{15}N relaxation parameters of AcAS and its Cu(I) complexes. (A) R_1 , (B) R_2 , and (C) ^1H – ^{15}N NOE relaxation data of AcAS in the absence (black) and presence (red) of Cu(I). Gray box contains the first 15 residues of AcAS sequence. Small increases of R_2 values around His-50 and Met-116/Met-127 possibly reflects the fast exchange of Cu(I) at these secondary sites. Experiments were recorded at 15 °C using AcAS (200 μM) samples dissolved in buffer A in the absence and presence of 5 equiv of Cu(I).

values for the first five residues in the protein; however, more pronounced deviations were found in the R_2 values (ranging between 5.0 and 7.0 s^{-1}) and NOE data (ranging between 0.35 and 0.6) for the first 10 residues and progressively smaller values for the following few residues (Figure 3). These values indicated restricted local sampling in the pico- to nanosecond time scale and report on enhanced conformational order at the 1–15 amino acid segment of AcAS–Cu(I) relative to the free protein, consistent with the stabilization of an α helix at this site.

In this work, we have characterized the interaction between Cu(I) and the physiological, N-terminally acetylated form of the protein AS. The substantial increase in α helical conformation seen for N-terminally acetylated AS upon Cu(I) complexation, which extends from residues 1–10 as judged by NMR, is reported here for the first time. Moreover, to our knowledge there is no report in literature describing such a dramatic conformational change in AS or AcAS upon interaction with small ligands like metal ions. In fact, the interaction of Cu(I) with AS results only in a small increase of α -helical propensity near the N-terminus (Supporting Information Figure 6), highlighting the role of N-terminal acetylation in promoting local helical conformations upon Cu(I) binding. The increased helicity in AcAS–Cu(I) can be rationalized by stabilization of the helix macrodipole and formation of energetically more favorable hydrogen bond interactions triggered by the removal of the α amino positive charge upon acetylation^{28,29} and by the Cu(I)-induced structural rearrangement of Met-1 and Met-5 side chains, respectively.

Considering that copper concentrations can reach up to 300 μM in synaptic vesicles^{30,31} and that AcAS is highly abundant ($\sim 50 \mu\text{M}$) in brain synaptosomes,³² our results suggest that an AcAS–Cu(I) complex with stabilized helically folded conformations might exist in vivo. Linked to the fact that the Met-X3-Met motif at the N-terminus of AcAS resembles those found in helical copper transport proteins,^{33–35} the formation of AcAS–Cu(I) complex at site 1 might have physiologically relevant implications in processes related to metal transport, membrane binding, or protein aggregation, which are enhanced by increased α -helical content at the N-terminus of the protein.^{14,36,37} Overall, our findings open new avenues of investigations into the metallobiology of PD, reshaping the consideration of copper-mediated pathology in vivo.

■ ASSOCIATED CONTENT

■ Supporting Information

NMR spectra of AcAS–Cu(II) complexes; ¹³C direct detection NMR experiments; ¹H–¹³C HSQC, binding curves, and NOE analysis of AcAS–Cu(I) complexes under different conditions; and ¹³C SCS and SSP analysis for AcAS–Cu(I) complexes. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b01911.

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■ Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Goedert, M.; Spillantini, M. G.; Del Tredici, K.; Braak, H. *Nat. Rev. Neurol.* **2012**, *9*, 13.
- (2) Lashuel, H. A.; Overk, C. R.; Oueslati, A.; Masliah, E. *Nat. Rev. Neurosci.* **2013**, *14*, 38.
- (3) Binolfi, A.; Quintanar, L.; Bertocini, C. W.; Griesinger, C.; Fernandez, C. O. *Coord. Chem. Rev.* **2012**, *256*, 2188.
- (4) Binolfi, A.; Rodriguez, E. E.; Valensin, D.; D'Amelio, N.; Ippoliti, E.; Obal, G.; Duran, R.; Magistrato, A.; Pritsch, O.; Zweckstetter, M.; Valensin, G.; Carloni, P.; Quintanar, L.; Griesinger, C.; Fernandez, C. O. *Inorg. Chem.* **2010**, *49*, 10668.
- (5) Bolognin, S.; Messori, L.; Zatta, P. *NeuroMol. Med.* **2009**, *11*, 223.
- (6) Brown, D. R. *Metallomics* **2011**, *3*, 226.
- (7) Gaggelli, E.; Kozlowski, H.; Valensin, D.; Valensin, G. *Chem. Rev.* **2006**, *106*, 1995.
- (8) Gorell, J. M.; Johnson, C. C.; Rybicki, B. A.; Peterson, E. L.; Kortsha, G. X.; Brown, G. G.; Richardson, R. J. *Neurotoxicology* **1999**, *20*, 239.
- (9) Kozlowski, H.; Brown, D.; Valensin, G. *Metallochemistry of Neurodegeneration: Biological, Chemical, and Genetic Aspects*. RSC Publishing: Cambridge, U.K., 2007.
- (10) Pall, H. S.; Blake, D. R.; Gutteridge, J. M.; Williams, A. C.; Lunec, J.; Hall, M.; Taylor, A. *Lancet* **1987**, *330*, 238.
- (11) Bartels, T.; Choi, J. G.; Selkoe, D. J. *Nature* **2011**, *477*, 107.
- (12) Fauvet, B.; Fares, M. B.; Samuel, F.; Dikiy, I.; Tandon, A.; Eliezer, D.; Lashuel, H. A. *J. Biol. Chem.* **2012**, *287*, 28243.
- (13) Kang, L.; Moriarty, G. M.; Woods, L. A.; Ashcroft, A. E.; Radford, S. E.; Baum, J. *Protein Sci.* **2012**, *21*, 911.
- (14) Maltsev, A. S.; Ying, J. F.; Bax, A. *Biochemistry* **2012**, *51*, 5004.
- (15) Moriarty, G. M.; Minetti, C. A.; Remeta, D. P.; Baum, J. *Biochemistry* **2014**, *53*, 2815.
- (16) Binolfi, A.; Valiente-Gabioud, A. A.; Duran, R.; Zweckstetter, M.; Griesinger, C.; Fernandez, C. O. *J. Am. Chem. Soc.* **2011**, *133*, 194.
- (17) Miotto, M. C.; Rodriguez, E. E.; Valiente-Gabioud, A. A.; Torres-Monserrat, V.; Binolfi, A.; Quintanar, L.; Zweckstetter, M.; Griesinger, C.; Fernandez, C. O. *Inorg. Chem.* **2014**, *53*, 4350.
- (18) Bermel, W.; Bertini, I.; Felli, I. C.; Pierattelli, R. *J. Am. Chem. Soc.* **2009**, *131*, 15339.
- (19) Miotto, M. C.; Binolfi, A.; Zweckstetter, M.; Griesinger, C.; Fernandez, C. O. *J. Inorg. Biochem.* **2014**, *141*, 208.
- (20) Wishart, D. S.; Sykes, B. D. *Methods Enzymol.* **1994**, *239*, 363.
- (21) Marsh, J. A.; Singh, V. K.; Jia, Z.; Forman-Kay, J. D. *Protein Sci.* **2006**, *15*, 2795.
- (22) Tamiola, K.; Mulder, F. A. *Biochem. Soc. Trans.* **2012**, *40*, 1014.
- (23) Serrano, L. *J. Mol. Biol.* **1995**, *254*, 322.
- (24) Hill, R. B.; Flanagan, J. M.; Prestegard, J. H. *Biochemistry* **1995**, *34*, 5587.
- (25) Wuthrich, K. *NMR of Proteins and Nucleic Acids*. Wiley-Interscience: New York, 1986.
- (26) Boehr, D. D.; Dyson, H. J.; Wright, P. E. *Chem. Rev.* **2006**, *106*, 3055.
- (27) Bussell, R., Jr.; Eliezer, D. *J. Biol. Chem.* **2001**, *276*, 45996.
- (28) Hol, W. G. *Adv. Biophys.* **1985**, *19*, 133.
- (29) Tyanova, S.; Cox, J.; Olsen, J.; Mann, M.; Frishman, D. *PLoS Comput. Biol.* **2013**, *9*, e1002842.
- (30) Hopt, A.; Korte, S.; Fink, H.; Panne, U.; Niessner, R.; Jahn, R.; Kretschmar, H.; Herms, J. *J. Neurosci. Methods* **2003**, *128*, 159.
- (31) Scheiber, I. F.; Mercer, J. F.; Dringen, R. *Prog. Neurobiol.* **2014**, *116*, 33.
- (32) Wilhelm, B. G.; Mandad, S.; Truckenbrodt, S.; Krohnert, K.; Schafer, C.; Rammner, B.; Koo, S. J.; Classen, G. A.; Krauss, M.; Haucke, V.; Urlaub, H.; Rizzoli, S. O. *Science* **2014**, *344*, 1023.
- (33) Guo, Y.; Smith, K.; Lee, J.; Thiele, D. J.; Petris, M. J. *J. Biol. Chem.* **2004**, *279*, 17428.
- (34) Ohrvik, H.; Nose, Y.; Wood, L. K.; Kim, B. E.; Gleber, S. C.; Ralle, M.; Thiele, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, E4279.
- (35) Schushan, M.; Barkan, Y.; Haliloglu, T.; Ben-Tal, N. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10908.
- (36) Anderson, V. L.; Ramlall, T. F.; Rospigliosi, C. C.; Webb, W. W.; Eliezer, D. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 18850.
- (37) Ghosh, D.; Singh, P. K.; Sahay, S.; Jha, N. N.; Jacob, R. S.; Sen, S.; Kumar, A.; Riek, R.; Maji, S. K. *Sci. Rep.* **2015**, *5*, 9228.