Copper–Ubiquitin Binding

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Ubiquitin Stability and the Lys 63-Linked Polyubiquitination Site Are Compromised on Copper Binding**

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Several neurodegenerative diseases are believed to share a common molecular mechanism involving protein misfolding and aggregation,[1,2] and an increasing number of observations indicate that metal ions are capable of accelerating these processes.[3–6] Failure to eliminate misfolded proteins can lead to the formation of toxic aggregates and cell death.[7] Moreover, it has been reported recently that β-amyloid, a major component of the extracellular plaques associated with Alzheimer's disease, starts to accumulate in intracellular compartments.[8] In eukaryotic cells the ubiquitin (Ub) proteasome system (UPS) is the main pathway for eliminating misfolded proteins.[9] Proteins are earmarked by the covalent attachment of a polyUb chain, which is specifically recognized by the proteasome. PolyUb chains can have diverse structures depending on the Lys residue of Ub involved in chain elongation.[10] As an example, Lys 63-linked chains are known to activate cell signals in several pathways: tolerance of DNA damage, inflammatory response, protein trafficking, and ribosomal protein synthesis.[11] Biological investigations have demonstrated that exposure of the UPS to increasing amounts of metal ions affects its main proteolytic activity, suggesting a close relationship between the age-dependent increase in metal ion concentration in the brain, UPS failure, and disease.[12] The link between UPS impairment and disease is also supported by the observation that protein aggregates within affected cells often contain Ub.[13]

Here, differential scanning calorimetry (DSC) experiments were carried out on different metal/Ub solutions with the aim of assessing whether some cations may interact specifically with Ub and affect its stability. The effect of different cations on the thermal stability of Ub is reported in Figure 1. Cu^{II} addition decreases the thermal stability of Ub both in terms of unfolding temperature (T_m) and enthalpy (ΔH). In contrast, addition of other cations—Zn^{II}, Ni^{II}, Al^{III} and Cd^{II}—to protein samples does not affect the thermal unfolding of Ub, thus confirming a specific effect of Cu^{II} on its thermal stability.

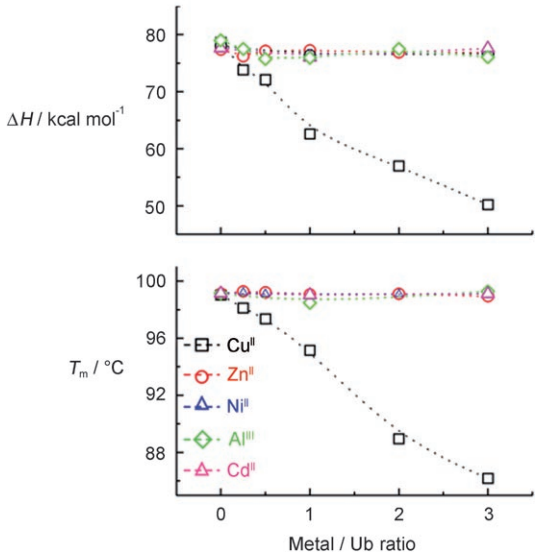

Figure 1. Effect of different cations on the thermal stability of Ub. Plots of ΔH and T_m values of Ub as a function of metal/Ub ratio.

The stoichiometry of the Cu^{II}/Ub binding was assessed by electrospray mass spectrometry (ESI-MS; see the Supporting Information). At Cu^{II}/Ub ratios up to 5:1, ESI mass spectra indicate binding of a first and a second Cu^{II} ion to the protein (see Figure S1 in the Supporting Information), however with fourfold-different affinities. The affinity constant for the first Cu^{II} anchoring site was determined to be approximately $10^7\,M^{-1}$ from spectrophotometric measurements (see Figure S2 in the Supporting Information).

The EPR spectrum of the complex at a Cu^{II}/Ub ratio of 1:1 is characterized by the parameters $g_{\|} = 2.30$ and $A_{\|} = 159 \times 10^{-4}\,cm^{-1}$, which are consistent with a tetragonal N_1O_3 (type II) Cu^{II} site (see Figure S3 in the Supporting Information). Such a Cu^{II} coordination environment is also supported by the presence of a weak absorption band at 680 nm in the visible spectrum, assignable to the d–d electronic transition. The

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unpaired electron of a type II Cu^{II} ion is characterized by relatively long electronic relaxation times (10^{-8}–10^{-9} s). Therefore its coupling with nuclear spins has dramatic effects on the nuclear relaxation and consequently on the NMR signal widths.[14] Indeed, addition of substoichiometric amounts of Cu^{II} salts to Ub results in the disappearance of a number of amide cross peaks (i.e. residues 1–3, 16–18, and 64, mapped in green in Figure 2) in the ^{1}H,^{15}N heteronuclear single-quantum coherence (HSQC) spectra (see Figures S4 and S5 in the Supporting Information), while other nuclei

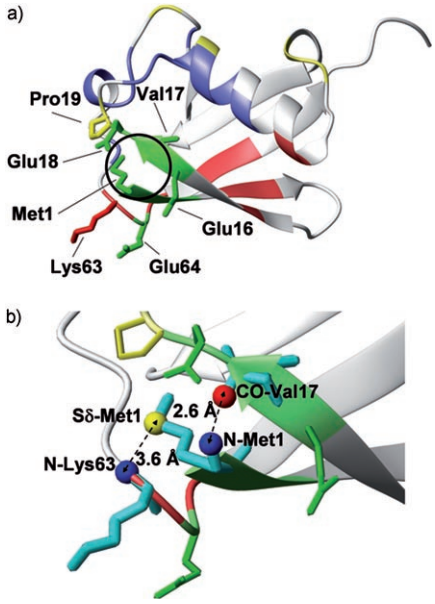

Figure 2. Mapping the effects of the binding of paramagnetic Cu^{II} ions on the NMR signals of Ub (structure: PDB ID 1UBQ). a) Full view with the putative location of the Cu^{II} ion inscribed in a circle. b) Enlarged bottom view with two key hydrogen-bonding interactions involving Met1 indicated with dotted lines. Color code: green: Ub signals broadened beyond detection in ^{1}H,^{15}N HSQC NMR spectra, red: negative pseudocontact shifts, blue: positive pseudocontact shifts, yellow: nondetectable proline groups and solvent-exchanging residues.

experience small but measurable pseudocontact shifts (PCS) arising from dipolar coupling with the electron (see Figure S6 in the Supporting Information). The size and the sign of PCS depend on the distance between the metal and the nucleus examined (^{1}H in the present case), and the anisotropy and orientation of the magnetic susceptibility tensor χ.[14] The z axis of the χ tensor is nearly orthogonal to the ideal plane formed by the Cu^{II} donor atoms in a tetragonal environment and causes a positive shift on the nuclei close to the z axis (mapped in blue in Figure 2) and a negative shift on the nuclei close to the coordination plane (mapped in red in Figure 2).

Besides ^{1}H,^{15}N experiments, ^{1}H,^{13}C HSQC and ^{13}C,^{13}C CACO[15] were recorded with substoichiometric Cu^{II}/Ub ratios to better define the location of the Cu^{II} ion. It was found that the Cα and the CO of Met1, Val17, and Glu18, together with the side chains (Cγ and Cδ) of Glu16 and Glu18, are the most affected ^{13}C resonances (see Figures S4 and S5 in the Supporting Information). Therefore, both the ^{15}N and ^{13}C NMR data clearly confine the Cu^{II} ion in a well-defined region at the N terminus of the protein (indicated by a circle in Figure 2). All NMR spectral changes induced by Cu^{II} disappeared upon addition of stoichiometric amount of ascorbic acid and reduction of Cu^{II} to Cu^{I}. Therefore, under "normal" conditions, in which intracellular copper is mainly Cu^{I}, Ub does not bind copper. The situation, however, could be rather different under disease conditions. It has already been proposed that under certain adverse circumstances Cu^{II} can interact with intracellular proteins such as α-synuclein.[16]

Far-UV CD measurements indicate very little overall change in Ub secondary structure, and the small decrease with time of the CD intensity can be a consequence of Cu^{II}-induced protein aggregation rather than protein unfolding (see Figure S7 in the Supporting Information). Indeed, light-scattering measurements of Ub recorded in the presence of 1 mol equiv of Cu^{II} show the appearance of high molecular weight species (> 100 Å hydrodynamic radius), which can be filtered off leaving the signal of monomeric Ub (36 ± 1 Å hydrodynamic radius).

Addition of Cu^{II} above 1:1 ratio produces spectral changes in the region around His68 (i.e. residues 66–68). Despite the affinity of Cu^{II} for the imidazole nitrogen of histidine, the lack of a preorganized set of donor atoms near His68 renders the binding of Cu^{II} to this site less effective than its binding to the N-terminal region of Ub. It is worth mentioning that in the case of the Cu^{II}–iminodiacetic acid complex (Cu^{II}-IDA), in which three coordination sites of Cu^{II} were firmly occupied by the IDA ligand and the protein could provide only one easily accessible donor atom, Cu^{II} coordination occurred to His68 on the Ub surface.[17]

In summary, we have located the primary binding of Cu^{II} ions to a specific site at the N terminus of Ub, involving the Met1 nitrogen and three oxygen donor ligands in a tetragonal geometry. Potential oxygen donor ligands are Met1 and Val17 (carbonyl groups), and Glu16 and Glu18 (carboxylate groups). At physiological pH the N-terminal group ($pK_a \approx 9$) of uncomplexed Ub is completely protonated and Cu^{II} has to compete strongly with protons for coordination to this site (see the Supporting Information). Since the residues close to the N terminus are involved in the formation of a β strand, Cu^{II} binding may destabilize the protein starting from its N-terminal region. Furthermore, in native Ub, the first residue, Met1, is involved in two key hydrogen bonds:[18] one occurring between the N-terminal group and the CO of Val17 and the other between the side chain sulfur atom of Met1 and the amide NH of Lys63 (Figure 2). Therefore Cu^{II} binding to Ub might hamper the proteins' turnover and other in vivo signaling events regulated by Lys63-linked polyubiquitination.[10,19]

The Cu^{II} affinity of the primary site of Ub appears to be competitive with that of amyloidogenic proteins involved in prion, Alzheimer's, and Parkinson's diseases. In all cases the affinity is in the submicromolar range,[16] a fact that may have important biological implications.

Experimental Section

Unlabeled and isotopically labeled Ubiquitin (Ub) was purchased from VLI Research Inc. and used without further purification. DSC runs were recorded as reported elsewhere.[20] ESI mass spectra were recorded as described in the Supporting Information. Ammonium acetate (5 mM) was added before injection in order to obtain a good volatilization in mildly acidic conditions (pH 6.5). CD spectra were recorded on a Jasco J-810 spectropolarimeter as described in the Supporting Information. UV/Vis spectra were recorded by using a Hewlett-Packard 8452A spectrophotometer equipped with a circulation thermostat. The affinity constant of Ub for Cu^{II} was determined from spectrophotometric titrations as reported in the Supporting Information. All EPR spectra were recorded at 150 K on a Bruker Elexsys E500 CW-EPR spectrometer operating at 9.3–9.5 GHz. All NMR experiments were performed on a 0.5 mM $^{13}C,^{15}N$-enriched sample of Ub in ammonium formiate buffer (pH 6.5) at 25 °C. The titration of Ub with Cu^{II} was followed by $^{1}H,^{15}N$ and $^{1}H,^{13}C$ HSQC and $^{13}C,^{13}C$ CACO[15] experiments (see Table S1 in the Supporting Information).

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[1] C. Soto, *Nat. Rev. Neurosci.* **2003**, *4*, 49 – 60.
[2] F. Chiti, C. M. Dobson, *Annu. Rev. Biochem.* **2006**, *75*, 333 – 366.
[3] D. R. Brown, K. Qin, J. W. Herms, A. Madlung, J. Manson, R. Strome, P. E. Fraser, T. A. Kruck, A. Von Bohlen, W. Schulz-Schaeffer, A. Giese, D. Westaway, H. A. Kretzschmar, *Nature* **1997**, *390*, 684 – 687.
[4] C. S. Atwood, R. D. Moir, X. Huang, R. C. Scarpa, N. M. Bacarra, D. M. Romano, M. A. Hartshorn, R. E. Tanzi, A. I. Bush, *J. Biol. Chem.* **1998**, *273*, 12817 – 12826.
[5] S. R. Paik, H. J. Shin, J. H. Lee, C. S. Chang, J. Kim, *Biochem. J.* **1999**, *340*, 821 – 828.
[6] C. J. Morgan, M. Gelfand, C. Atreya, A. D. Miranker, *J. Mol. Biol.* **2001**, *309*, 339 – 345.
[7] A. J. McClellan, S. Tam, D. Kaganovich, J. Frydman, *Nat. Cell Biol.* **2005**, *7*, 736 – 741.
[8] L. M. Billings, S. Oddo, K. N. Green, J. L. McGaugh, F. M. LaFerla, *Neuron* **2005**, *45*, 675 – 688.
[9] A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **1998**, *67*, 425 – 479.
[10] C. M. Pickart, D. Fushman, *Curr. Opin. Chem. Biol.* **2004**, *8*, 610 – 616.
[11] R. Varadan, M. Assfalg, A. Haririnia, S. Raasi, C. Pickart, D. Fushman, *J. Biol. Chem.* **2003**, *279*, 7055 – 7063.
[12] M. Amici, K. Forti, C. Nobili, G. Lupidi, M. Angeletti, E. Fioretti, A. M. Eleuteri, *J. Biol. Inorg. Chem.* **2002**, *7*, 750 – 756.
[13] A. Ciechanover, P. Brundin, *Neuron* **2003**, *40*, 427 – 446.
[14] F. Arnesano, L. Banci, M. Piccioli, *Q. Rev. Biophys.* **2005**, *38*, 167 – 219.
[15] W. Bermel, I. Bertini, L. Duma, L. Emsley, I. C. Felli, R. Pierattelli, P. R. Vasos, *Angew. Chem.* **2005**, *117*, 3149 – 3152; *Angew. Chem. Int. Ed.* **2005**, *44*, 3089 – 3092.
[16] R. M. Rasia, C. W. Bertoncini, D. Marsh, W. Hoyer, D. Cherny, M. Zweckstetter, C. Griesinger, T. M. Jovin, C. O. Fernández, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 4294 – 4299.
[17] M. Nomura, T. Kobayashi, T. Kohno, K. Fujiwara, T. Tenno, M. Shirakawa, I. Ishizaki, K. Yamamoto, T. Matsuyama, M. Mishima, C. Kojima, *FEBS Lett.* **2004**, *566*, 157 – 161.
[18] S. Vijay-Kumar, C. E. Bugg, W. J. Cook, *J. Mol. Biol.* **1987**, *194*, 531 – 544.
[19] M. J. Eddins, C. M. Carlile, K. M. Gomez, C. M. Pickart, C. Wolberger, *Nat. Struct. Mol. Biol.* **2006**, *13*, 915 – 920.
[20] M. Sciacca, D. Milardi, M. Pappalardo, C. LaRosa, D. Grasso, *J. Therm. Anal. Calorim.* **2006**, *86*, 311 – 314.

