Structural probing of Zn(II), Cd(II) and Hg(II) binding to human ubiquitin†

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A structural investigation performed on adducts of human ubiquitin with group-12 metal ions reveals common preferential anchoring sites, the most populated one being His68; at higher metal ion concentration a second and a third site, close to the N-terminus of the protein, become populated and promote a polymorphic transition from orthorhombic to cubic form; Glu16 and Glu18, involved in the latter metal binding, undergo a remarkable displacement from their position in native ubiquitin; the aggregate stereochemistry appears to be driven by the clustering of deshielded backbone hydrogen-bond patches, and metal ions foster this process.

Ubiquitin (Ub) is a small protein of 76 residues (MW 8565 Da) folded in a compact globular structure in which a mixed parallel/anti-parallel β-sheet packs against an α-helix generating a hydrophobic core. Not found in bacteria, this protein is ubiquitous in eukaryotes and has highly conserved sequences, the human and the yeast proteins differing by only three residues.[1] The remarkable degree of sequence conservation underscores its important physiological role. Ub becomes attached to lysine residues of proteins to be degraded and targets them to the proteasome, the complex molecular machinery where the ATP-dependent process of degradation takes place.[2] Besides protein degradation, Ub is known to activate cell signals in several pathways: tolerance to DNA damage, inflammatory response, protein trafficking, and ribosomal protein synthesis.[3] The presence of Ub-positive protein aggregates is a biomarker of neurodegeneration,[4] but the molecular mechanism underlying their accumulation is unknown. Protein aggregation is believed to be favored by metal ions, such as Cu(II) and Zn(II), whose levels are increased in brains of patients with Parkinson's and Alzheimer's diseases, the two most common neurodegenerative disorders.[5] Ub has been widely used as model for stability, folding, and structural studies,[6] and carefully characterized both in solution[7] and the solid state.[8] However, despite the plethora of structural investigations, only few studies concern the interaction with metal ions. Recently, binding of Cu(II) to Ub has been shown to destabilize the protein, while other metal ions, including Zn(II) and Cd(II), have no effect on the unfolding temperature.[9] In the present study, adduct formation between human ubiquitin (hUb) and group-12 metal ions Zn(II), Cd(II) and Hg(II) has been investigated by X-ray crystallography. While Zn(II) is an essential element involved in several important cellular functions,[10] Cd(II) and Hg(II) are toxic metal ions released into the environment by human activities, which may bind to adventitious sites, thus compromising protein functions.[11] The characterization of metal-binding sites and the patterns of metal ion-induced crystallization of hUb can provide structural insights into the early aggregation mechanism of folded systems.

Crystals of hUb were grown in the presence of different concentrations of Zn(II), Cd(II) or Hg(II) acetate.‡ Zn-hUb adducts crystallize in orthorhombic or cubic form depending upon the metal/protein molar ratio used (8 : 1 and 70 : 1, respectively). Crystallization of a Cd-hUb adduct takes place only at high metal : protein molar ratio (70 : 1), while crystals of Hg-hUb were obtained only at very low Hg(II)/protein molar ratio (1 : 1). Details on data collection and structure refinement are reported in Tables 1SI and 2SI of ESI.†

The structure of orthorhombic Hg-hUb was solved at 1.8 Å resolution. The asymmetric unit contains one protein molecule and one Hg(II) ion. Inspection of the electron density maps shows other regions which could host metal ions with low occupancy factors (*i.e.* the fraction of sites occupied by metal ions) and high thermal factors, these ill-defined metal sites were not considered. His68 binds Hg(II) through NE2 of the imidazole ring. The metal ion completes its linear coordination geometry by binding NE2 of Gln31* of a symmetry-related molecule (Fig. 1(A) and Table 3SI of ESI†). This site has an occupancy factor of 0.77 and the bond distances for Hg1–NE2 His68 and Hg1–NE2 Gln31* are 2.2 and 2.4 Å, respectively.

Orthorombic crystals of Zn-hUb (1.8 Å resolution) contain, in the asymmetric unit, three hUb molecules (A, B and C) and one Zn(II) ion, which interacts with two protein molecules (A and B) by coordination to NE2 of His68 residues (Fig. 1(B)). This site has an occupancy factor of 0.50 and the bond distances for Zn–NE2 His68A and Zn–NE2 His68B are 2.5 and 2.4 Å, respectively. The Zn(II) ion interacts weakly also with two water molecules. His68 of the third protein molecule (C) in the asymmetric unit does not appear to have metal ions in its proximity.

The crystal structure of Cd-hUb has been solved at 3.0 Å resolution in a cubic form. In the asymmetric unit there are two protein molecules (A and B), each one interacting with

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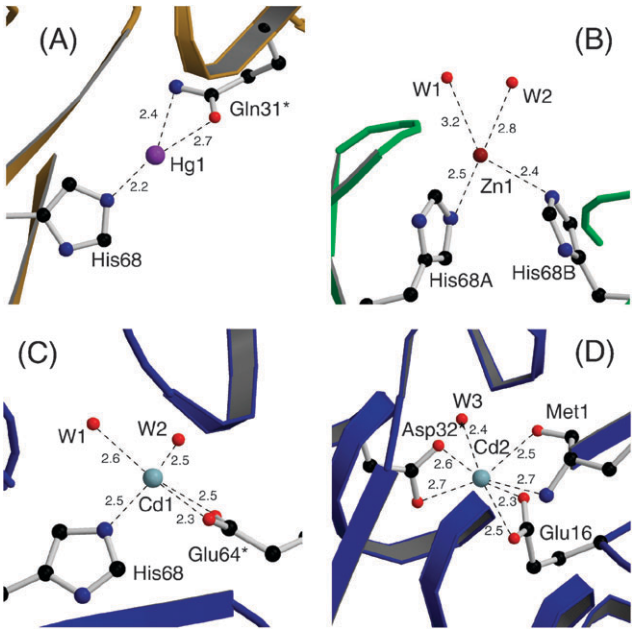

Fig. 1 hUb metal binding sites Me1 (A, B, C) and Me2 (D). (A) Structure of the Hg-hUb adduct; (B) structure of the orthorhombic Zn-hUb adduct; (C, D) structure of the cubic Cd-hUb adduct. The distances are reported in Å.

several Cd(II) ions. The metal binding sites are the same for the two protein molecules, but the occupancy factors are different (Table 4SI, ESI†). One cadmium ion (Cd1) is anchored to NE2 of His68 and completes its quasi-tetrahedral geometry by binding the carboxylate group of Glu64* of a symmetry-related protein molecule and two water molecules (Fig. 1(C)). A second Cd(II) ion (Cd2) is anchored to the carboxylate group of Glu16, which undergoes a remarkable displacement from its position in native hUb (PDB code: 1UBQ,[8] Fig. 2(A)). Cd2 also binds the NH_2 and CO of Met1, the carboxylate group of Asp32* of a symmetry-related molecule, and a water molecule (Fig. 1(D)). Binding of Cd2 appears to weaken the hydrogen bond between CO of Met1 and NH of Val17, the distance between these two groups

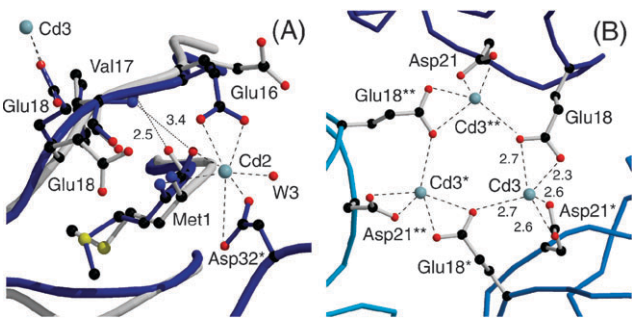

Fig. 2 (A) Superposition of the structures of native hUb (white) and Cd-hUb (blue) in the region of the binding sites Cd2 and (only partially) Cd3. The interactions between the metal ions (Cd2 and Cd3) and donor atoms are indicated by dashed lines. The Met1 CO···Val17 NH interactions are indicated by dotted lines. (B) Cd3 metal binding sites in Cd-hUb. The three symmetry-related hUb molecules (coloured in different blue tones) are linked by a bond network involving three Cd2 and three Cd3 ions (light blue spheres).

increasing from 2.5 Å in the native form to 3.4 Å in the adduct (Fig. 2(A)); however, the local fold of hUb does not change significantly, due to the persistence of the hydrogen bond between NH_2 of Met1 and CO of Val17. Donor atoms for the third Cd(II) ion (Cd3) are the carboxylate oxygens of Glu18 and of Asp21* and Glu18* of a symmetry-related molecule (Fig. 2(B)). Similarly to Glu16, also Glu18 undergoes a remarkable displacement from its position in native hUb (Fig. 2(A)). Cd2 and Cd3 bridge, in pairs, three protein molecules of different asymmetric units (Fig. 3(A)), thus playing a crucial role in the molecular packing. Other Cd(II) ions are localized on the protein surface and bound to carboxylate groups, however their occupancy factors are generally low.

Cubic crystals of Zn-hUb adduct were also obtained in the presence of a high concentration of Zn(II). Unfortunately, their low quality prevented an accurate determination of the crystallographic structure which, however, appeared to be similar to that of cubic Cd-hUb.

In general, the observed metal ion–protein donor atom distances are slightly larger than the average for inorganic compounds. This is not unexpected since the donor atoms are linked to the protein frame and thus are more subject to spatial constraints.

The three structures (superimposed in Fig. 1SI, ESI†) allow a hierarchy among protein metallation sites to be established. At low metal/protein ratio the surface exposed His68 appears

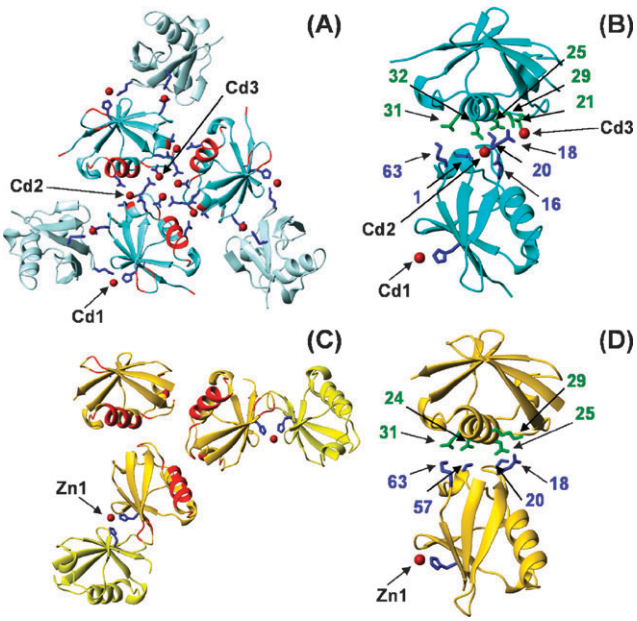

Fig. 3 Packing of molecules in cubic Cd-hUb (A, B) and orthorhombic Zn-hUb (C, D) crystals. hUb molecules are coloured in cyan and yellow, respectively. In (A) and (C) hUb molecules forming one trimer are shown in more intense colour; Zn(II) or Cd(II) ions are shown as red spheres, while the Zn(II) or Cd(II) ligands are shown as blue sticks. Regions containing poorly wrapped backbone hydrogen bonds (*dehydrons*) are shown in red. Conserved contacts between two hUb molecules within the trimer are shown in detail in (B) and (D). Residues at the interface are shown as green and blue sticks and pointed out by arrows. The metal binding sites are indicated for one hUb molecule.

to be the dominant anchoring site. By increasing the metal ion concentration, the carboxylate side chains of Glu16 and Glu18, located near the N-terminus of hUb, can also load a metal ion. Binding to His68 and to the N-terminus has been already observed, respectively, in a Ub variant crystallized in the presence of Cd(II) ions[12] and in a Ub-like protein crystallized in the presence of Zn(II) ions.[13] His68 and Met1 are also the sites of copper ion binding. Specifically, Met1 was found to be the primary site of attack by Cu(II) ion, which attains a tetragonal geometry by coordinating the nitrogen of Met1 and three oxygen donor ligands.[9] In contrast, when three copper coordination positions are held by a tridentate ligand (such as iminodiacetate, IDA), the imidazole nitrogen of His68 becomes the preferential site of attack.[14]

Hg-hUb and Zn-hUb show preferential coordination to His68, thus resembling the hUb-Cu(IDA) system. It is most likely that, in the solid state, an adjacent hUb molecule plays a similar role like the IDA ligand in solution experiments, that is to provide additional donor atoms to the metal ion. Binding of Cu(II) to Met1 of hUb was shown to cause a destabilization of the protein, such a destabilization is not observed in the case of Cd(II) in agreement with previous calorimetric determinations.[9] This could be a consequence of the different coordination mode of Cu(II) with respect to Cd(II). While Cu(II) is bound to a single hUb molecule, Cd(II) binds to Met1 and Glu16 of one hUb molecule and completes its coordination shell by binding to Asp32* of a symmetry-related molecule and to water molecules of crystallization. Moreover, the coordination geometry of Cd(II) is generally tetrahedral or octahedral, but not tetragonal as for Cu(II). It is also worth noting that, notwithstanding the several attempts, all trials to crystallize hUb in the presence of copper were unsuccessful, even when using very small amounts of Cu(II). This could be a consequence of Cu(II) destabilizing the protein structure and hampering crystallization.

The analysis of molecular packing shows that, in cubic crystals of Cd-hUb, helix α1 (residues 23–31) of one hUb molecule packs against the N-terminal region of another molecule and comes in contact with residues 18–20 (located in the loop between β2 and α1) and with the side chain of Lys63. Each of the two hUb molecules, in turn, establishes complementary contacts with a third molecule, thus giving rise to a symmetric trimer stabilized by three Cd2 and three Cd3 ions (Fig. 3(A)).

Interestingly, a similar trimeric arrangement of hUb molecules is also found in orthorhombic crystals of Zn-hUb, where Me2 and Me3 ions are missing, thus suggesting that such an intermolecular interaction involving helix α1 of one hUb and the N-terminal region of another hUb does not necessarily require support from metal ions (Fig. 3(C)).

Instead, a series of electrostatic intermolecular interactions (H-bonds and salt bridges) are established (Glu18/Lys29, Ser20/Asn25, Ser57/Glu24 and Lys63/Gln31; Fig. 3(B) and (D)). Helix α1 of native hUb contains a large number of poorly wrapped backbone hydrogen bonds,[15] called dehydrons, which are generally correlated to the aggregation propensity of a protein.[16] Mapping on the Zn-hUb and Cd-hUb structures reveals that dehydrons are clustered in the core of the trimer (Fig. 3(A) and (C)). Therefore, such "structural defects" in isolated hUb molecules may be partially offset in the crystals by intermolecular interactions at the trimeric core. From this analysis it is also inferred that bridging metal ions may enhance the aggregation propensity of surface regions of hUb, thus shifting the equilibrium towards the formation of oligomeric species.

In conclusion, we have shown that hUb can bind group 12 metal ions at different sites, His68, Glu16/Met1 and Glu18 being, in the given order, the preferred anchoring residues. Metal binding to the second and third site causes a polymorphic transition and stabilizes a trimeric arrangement of hUb molecules characterized by the clustering of dehydrons.

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Notes and references

‡ Crystallization and structure determination. The crystallization of the metal ion-hUb adducts was carried out by the hanging drop method at 293 K. The structures were solved by molecular replacement, using the structure of native ubiquitin (1UBQ) as probe. The refinement was performed with CNS[17] and the models were rebuilt with the graphic program XtalView.[18] Further details are reported in the ESI.†

The coordinates for the Zn-hUb, Cd-hUb and Hg-hUb adducts have been deposited in the Protein Data Bank under the accessions 3EHV, 3EEC and 3EFU, respectively.

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