New crystal form of human ubiquitin in the presence of magnesium

Ana Camara-Artigas,^a Marina Plaza-Garrido,^a Sergio Martinez-Rodriguez^b and Julio Bacarizo^{a*}

^aDepartment of Chemistry and Physics, University of Almeria, Agrifood Campus of International Excellence (ceiA3), Carretera de Sacramento (s/n), 04120 Almeria, Spain, and ^bDepartment of Physical Chemistry, University of Granada, Avda. de Fuentenueva s/n, 18071 Granada, Spain. *Correspondence e-mail: juliobacarizo@hotmail.com

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Ubiquitin is a small globular protein that has a considerable number of lysine residues on its surface. This results in a high surface entropy that precludes the formation of crystal-packing interactions. To date, only a few structures of the native form of ubiquitin have been solved, and most of the crystals that led to these structures were obtained in the presence of different divalent metal cations. In this work, a new crystallographic structure of human ubiquitin solved from crystals grown in the presence of magnesium is presented. The crystals belonged to a triclinic space group, with unit-cell parameters $a = 29.96$, $b = 30.18$, $c = 41.41$ Å, $\alpha = 88.52$, $\beta = 79.12$, $\gamma = 67.37^\circ$. The crystal lattice is composed of stacked layers of human ubiquitin molecules with a large hydrophobic interface and a smaller polar interface in which the magnesium ion lies at the junction between adjacent layers in the crystal. The metal ion appears in a hexa-aquo coordination, which is key to facilitating the crystallization of the protein.

1. Introduction

Ubiquitin (Ub) is a small protein of 76 amino acids (molecular weight 8565 Da), the polypeptide sequence of which is highly conserved among different organisms. The ubiquitin–proteasome system (UPS) is involved in the destruction of proteins for the maintenance of protein homeostasis in cells, where Ub acts by tagging and targeting proteins to the large proteolytic chamber of the proteasome, causing their elimination. Ubiquitination also occurs in a wide range of other cellular processes, including translation, transcription, cytokine, protein kinase and DNA-damage signalling, intracellular trafficking and most forms of protein degradation. Imbalances in the ubiquitin system can lead to disease, as many proteins involved in the assembly, binding or disassembly of ubiquitin conjugates are mutated in human disorders (Glickman & Ciechanover, 2002).

As of October 2015, 34 crystallographic structures of ubiquitin had been deposited in the Protein Data Bank (PDB; Berman *et al.*, 2000). Most of the structures are of modified ubiquitin constructs and only 14 structures are of monomeric wild-type human ubiquitin (HUb). Interestingly, ten of these structures were obtained in the presence of divalent metal cations such as Cd^{2+} , Hg^{2+} , Pt^{2+} or Zn^{2+} (Falini *et al.*, 2008; Arnesano *et al.*, 2011; Ma *et al.*, 2015). These metals are mainly located at the crystal contacts and may facilitate the growth of the crystals.

The likelihood of obtaining HUb crystals has been reported to be dependent to the presence of seven lysine residues located on the protein surface (9.2% of the residues of the

Table 1

Macromolecule-production information.

Source organism	<i>Homo sapiens</i>
DNA source	Addgene plasmid 12647
Forward primer	5'-CACCATATGCATCACCATCACCAGATCTTCGTCGAAGACGTTAAC-3'
Reverse primer	5'-AAGCTTTCAACCACCTCTTAGTCTTAAGACAAGATGTAAGGTCGAC-3'
Cloning vector	pET-101 Directional TOPO plasmid (Invitrogen)
Expression vector	pET-101D
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	HHHHHHQIFVKLTGKTTITLEVEPSDTIENVKAK-IQDKEGIPPDQQLIFAGKQLEDGRITLSDYNI-QKESTLHLVLRG

protein). These residues increase the surface entropy and preclude packing interactions. Loll *et al.* (2014) have explored the effect of these lysine residues by mutating them to serine. Crystallization experiments conducted with the serine mutants revealed that some of them improved the crystallization process, while other reduced the number of crystallization hits. Interestingly, one of these lysine residues (Lys29) is present in the crystal contacts of several ubiquitin crystals obtained in the presence of various metal cations.

Here, we describe a new crystal form of monomeric wild-type HUb in the triclinic space group *P*1. We have analysed the crystal contacts and, especially, the Mg^{2+} ion coordinated by six water molecules located in a symmetry-related position that facilitates crystallization through improving crystal contacts and packing. Finally, we compared our structure with previous crystal structures of HUb obtained in the presence of metal cations.

2. Materials and methods

2.1. Macromolecule production

Addgene plasmid 12647 was used as a template for the HUb gene. PCR amplification was carried out using recombinant *Pfu* DNA Polymerase (Thermo Scientific). The primers used

(IDT) were HumUb5 (5'-CACCATATGCATCACCATCACCATCACCAGATCTTCGTCGAAGACGTTAAC-3') and HumUb3 (5'-AAGCTTTCAACCACCTCTTAGTCTTAAGACAAGATGTAAGGTCGAC-3'). The obtained PCR fragment was purified from an agarose gel using a gel-extraction kit (Takara) and cloned using a pET-101 Directional TOPO plasmid (Invitrogen). Once the fragment had been cloned, its sequence was confirmed by sequencing. The resulting construction allows the production of recombinant HUb fused at the N-terminus to a polyhistidine tag (His₆ tag; Table 1).

Escherichia coli BL21 (DE3) cells were transformed with the pET-101D HUb plasmid and grown in solid LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. A single colony was transferred into 10 ml LB medium with ampicillin at the concentration given above and incubated overnight at 37°C. 500 ml LB supplemented with ampicillin was then inoculated with 5 ml of the overnight culture. After 3–4 h of incubation at 37°C with vigorous shaking, the OD₆₀₀ of the resulting culture was 0.8–1.0. To induce overexpression of the HUb gene, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture was kept at 34°C for a further 12 h. The cells were collected by centrifugation (4500g, 4°C, 20 min) and subsequently resuspended in 50 mM sodium phosphate pH 8.0, 300 mM NaCl (column buffer; CB). The cells were lysed *via* sonication in five cycles of 40 s using a Dr Hielscher UP200S Ultraschallprozessor sonicator (working at 60% amplitude and a cycle time of 0.5 s) and were then centrifuged (14 000g, 4°C, 30 min). The resulting supernatant was applied to 5 ml Ni-NTA agarose (Qiagen) previously equilibrated with 50 ml CB. The column was successively washed with 50 ml CB, 50 ml CB supplemented with 20 mM imidazole and 50 ml CB supplemented with 50 mM imidazole. Finally, the protein was eluted with 30 ml CB supplemented with 250 mM imidazole. The protein was dialyzed against CB to remove imidazole and further purified using a HiLoad 16/60 Superdex 75 size-exclusion column (GE Healthcare) using 20 mM Tris-HCl pH 8 as the mobile phase. Pure protein was concentrated to 10–15 mg ml⁻¹ using 3 kDa cutoff concentrators (Amicon Ultra, Millipore) and stored at –80°C.

Figure 1

HUb crystal of 0.10 × 0.02 × 0.02 mm in size in a sitting-drop vapour-experiment setup before the X-ray experiment and mounted in a Molecular Dimensions LithoLoop. Crystals were cooled in a chemically identical solution to the crystallization solution supplemented with 10%(v/v) PEG 300 for cryoprotection.

Table 2
Crystallization.

Method	Vapour diffusion, sitting drop
Plate type	MRC crystallization plate, SWISSCI MRC 2 Well (Jena Bioscience)
Temperature (K)	288
Protein concentration (mg ml ⁻¹)	19
Buffer composition of protein solution	20 mM Tris pH 8.0
Composition of reservoir solution	0.2 M magnesium chloride hexahydrate, 30% (w/v) PEG 4000, 0.1 M Tris pH 8.5
Volume and ratio of drop (μl)	3 (1.5 + 1.5)
Volume of reservoir (μl)	100

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	ID30A1, ESRF
Wavelength (Å)	0.97
Temperature (K)	100
Detector	Pilatus 2M, Dectris
Crystal-to-detector distance (mm)	142.96
Rotation range per image (°)	0.25
Total rotation range (°)	168
Exposure time per image (s)	0.07
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	29.96, 30.18, 41.41
α , β , γ (°)	88.52, 79.12, 67.37
Mosaicity (°)	0.26
Resolution range (Å)	27.82–1.32 (1.34–1.32)
Total No. of reflections	48369 (2300)
No. of unique reflections	28713 (2783)
Completeness (%)	93 (89.5)
Multiplicity	1.7
$\langle I/\sigma(I) \rangle$	5.8 (2.2)
$R_{\text{merge}}^{\dagger}$	0.064 (0.385)
$R_{\text{r.i.m.}}^{\ddagger}$	0.100 (2.649)
$R_{\text{p.i.m.}}$	0.064
Overall <i>B</i> factor from Wilson plot (Å ²)	11.750
CC _{1/2}	0.991 (0.646)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted average intensity for all *i* observations of reflection *hkl*. $\ddagger$ Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where *N* is the data multiplicity.

Under these conditions HUB is stable for several months. Different stages of the protein purification and the final purity of recombinant HUB were evaluated by SDS–PAGE.

2.2. Crystallization

Crystals of HUB were obtained by the vapour-diffusion technique using a sitting-drop setup at 288 K with Crystal Screen and Crystal Screen 2 from Hampton Research (Fig. 1). 3 μl droplets were prepared by mixing 1.5 μl protein solution (concentrated as described above to 19 mg ml⁻¹ in 20 mM Tris pH 8.0) and 1.5 μl reservoir solution. The mixture was vapour-equilibrated against 100 μl reservoir solution. Crystals were obtained after three months in 0.2 M magnesium chloride, 30% (w/v) PEG 4000, 0.1 M Tris pH 8.5 (Table 2).

2.3. Data collection, processing, structure solution and refinement

A 1.32 Å resolution data set was collected at a wavelength of 0.965 Å on the ID30A1 beamline (Svensson *et al.*, 2015)

Table 4
Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	27.82–1.32 (1.37–1.32)
Completeness (%)	93 (89.5)
σ Cutoff	2.2
No. of reflections, working set	27251 (2645)
No. of reflections, test set	1458 (138)
Final R_{cryst}	0.178 (0.2215)
Final R_{free}	0.201 (0.2568)
Cruickshank DPI	0.111
No. of non-H atoms	
Proteins	1170
Ion	1
Ligand	0
Water	140
Total	1311
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.217
Average <i>B</i> factors (Å ²)	
Protein	15.1
Ion	14.7
Water	25.80
Ramachandran plot	
Most favoured (%)	100
Allowed (%)	0

at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. The diffraction images were processed and scaled using the *autoPROC* package (Vonnrhein *et al.*, 2011) and *AIMLESS* (Evans & Murshudov, 2013). Data-collection and processing statistics are shown in Table 3.

The solution of the structure was performed using the *PHENIX* suite (Adams *et al.*, 2010). Phasing was performed by molecular replacement using *Phaser* (Bunkóczi *et al.*, 2013) with the coordinates of the crystallographic structure of the monomeric mutant of ubiquitin K33S (PDB entry 4pih; Loll *et al.*, 2014). Manual model building was performed using *Coot* (Emsley *et al.*, 2010). Refinement was performed using *phenix.refine* in *PHENIX* (Afonine *et al.*, 2012). The final cycles of refinement were performed using anisotropic *B* factors for all atoms except waters. Overall structure comparison and analysis were performed with *LSQKAB* (Kabsch, 1976), *CONTACT* and *AREAIMOL* from the *CCP4* suite (Winn *et al.*, 2010) and *MolProbity* (Chen *et al.*, 2010). Structure-refinement statistics are shown in Table 4.

2.4. Dynamic light scattering

Dynamic light-scattering (DLS) experiments were performed at 288 K in a Zetasizer Nano instrument (Malvern Instruments, UK) equipped with a 10 mW helium–neon laser ($k = 632.8$ nm) and a thermoelectric temperature controller. These experiments were analysed with the *Zetasizer* software (Malvern Instruments) as described elsewhere (Bacarizo *et al.*, 2014).

3. Results and discussion

We have obtained the high-resolution structure of HUB (1.32 Å) in a new crystal form using magnesium chloride in the

precipitant solution. The asymmetric unit is composed of two monomers of HUB related by a twofold rotational axis. Comparison of the two chains in the asymmetric unit gives an overall r.m.s.d. for C^α atoms of 0.31 Å. A DLS experiment performed with HUB (19 mg ml⁻¹) at pH 8.5 (100 mM Tris buffer) show a hydrodynamic radius (R_h) of 2.0 ± 0.2 nm. Considering a globular protein, this R_h value results in an estimated molecular mass of 9.2 kDa, suggesting that HUB is a monomer in solution at the same temperature and pH as that at which the crystals were grown.

The protein secondary-structure motifs were analysed using *PROMOTIF* in the *PDBsum* web interface (Hutchinson & Thornton, 1996; de Beer *et al.*, 2014). HUB residues Gln2–Gly74 were modelled in the difference electron-density maps. Additionally, two histidine residues belonging to the His₆-tagged region were also modelled. The overall structure of

HUB includes an α -helix (three-and-a-half turns; residues Ile23–Glu34), six β -turns (Thr7–Gly10, Glu18–Asp21, Ile44–Gly47, Phe45–Lys48, Glu51–Arg54 and Gln62–Ser65), two short pieces of 3_{10} -helix at residues Pro38–Gln40 and Ser57–Tyr59, a parallel β -sheet formed by two strands at residues His1–Thr7 and Thr66–Leu71, and an antiparallel β -sheet formed by three strands at residues Thr12–Val17, Gln41–Phe45 and Lys48–Gln49.

The crystal lattice is arranged with stacked layers of HUB molecules; the interfaces were analysed using the *PISA* server (Krissinel, 2011). The largest interface (surface area of 704 Å²) is composed by chain *A* (or *B*; the interface interactions are the same) and a symmetry-related molecule (Fig. 2*a*): the contacts are established by 11 residues in chain *A* and ten residues in a symmetry-related molecule (or by ten residues in chain *B* and 11 residues of the symmetry-related

Figure 2

Crystal packing of the HUB crystals in the presence of $Mg(H_2O)_6^{2+}$. (a) The large and small interfaces and the position of a magnesium cation in a symmetry-related position are shown. (b) The crystal lattice is composed of stacked layers of HUB molecules. Chains *A* and *B* are in blue and yellow, respectively, the outline of the triclinic unit cell is shown in green, and the magnesium cations are shown as red spheres, emphasizing their position between the layers in the lattice. (c) Orthogonal view of the lattice packing.

molecule). There are only two hydrogen bonds (Arg74 O–Gln49 NE2, 2.94 Å; Gly75 O–Gln49 N, 3.12 Å) and 45 non-bonded contacts. These interactions are mainly hydrophobic and are established between lateral groups of chains *A* (or *B*) and partially buried residues from a symmetry-related chain (Val70, Leu8, Ile44, Leu73 and Leu71). The smaller interface area (comprising 442 Å²) shows a more polar character than the larger interface and includes four hydrogen bonds (Ala46*A* N–Ala46*B* O, 2.76 Å; Glu64*A* O–His68*B* NE2, 2.88 Å; Lys6*A* NZ–Asn60*B* O, 2.91 Å; Lys6*A* NZ–Gln62*B* OE1, 2.59 Å) and 54 nonbonded contacts. The histidine residues belonging to the His₆-tagged region are placed neither on these major interfaces nor near the magnesium and do not interfere in the packing of the crystal.

The packing of this triclinic structure is similar to that observed in the K33S HUB mutant, in which a Ca²⁺ ion appears at the contact of the crystal instead an Mg²⁺ ion (see

Supplementary Table S1). In our structure the Mg²⁺ cation is coordinated by six water molecules [Mg(H₂O)₆]²⁺, which are bound to residues belonging to different molecules in the asymmetric unit. The crystal contact mediated by the Mg(H₂O)₆²⁺ complex is not found in either of the major packing interfaces. Instead, this metal ion lies at the junction between adjacent layers of the crystal, participating in important crystal contacts (Fig. 2). Magnesium ions tend to coordinate water molecules rather than other residues or anions in solution. The usual coordination number of Mg²⁺ in crystal structures is six, forming hexa-aquo magnesium ions, Mg(H₂O)₆²⁺ (Bock *et al.*, 1994). Thus, the use of MgCl₂ as an additive in the crystallization condition provided six ordered water molecules that could establish interactions with residues in the protein surface, facilitating the packing of the crystal (Fig. 3*a*). In this way, the correct packing of the HUB molecules requires the stabilization of some flexible residues in

Figure 3

Crystal packing of HUB in the presence of (a) Mg²⁺ (PDB entry 5dk8), (b) Ca²⁺ (PDB entry 4pih) and (c, d) Zn²⁺ (PDB entries 4xol and 4xok, respectively). Symmetry-related HUB molecules are represented in blue, yellow and green. H₂O, Mg²⁺, Ca²⁺ and Zn²⁺ are represented as spheres in red, green, white and grey, respectively. The hydrogen-bond network established by an Mg(H₂O)₆²⁺ coordination complex and various ions is shown with dashed black lines. Structures of HUB in the presence of zinc (c, d) are composed of three symmetry-related molecules and those in the presence of Ca²⁺ and Mg²⁺ of two (a, b). (a) Lys29 in chain *A* is hydrogen-bonded to two water molecules (W2 and W3) of the Mg(H₂O)₆²⁺ complex and to a bulk-solvent water molecule (W7); Lys29 in chain *B* is hydrogen-bonded to waters 1 and 6 of the Mg(H₂O)₆²⁺ complex and to residues Glu18*A* and Asn25*B*. The related ions are found in a symmetry position coordinating the residues in a crystal contact; the residues involved in these interactions are Glu18*A/B/C*, Asp21*A/B/C*, Asn25*A/B* and Lys29*A/B*.

critical positions to allow the formation of crystal contacts with their neighbouring molecules, as in the case of Lys29, which is involved in metal-site interactions. Loll *et al.* (2014) reported that mutation of this residue to serine reduces the number of crystallization hits. These authors reported that Lys29 is not buried and interacts with the backbone carbonyl O atom of Glu16, as well as with the side chain of Asp21, and attributed the low crystallization hit rate to destabilizing effects upon mutation to serine as a consequence of a loss of intramolecular interactions.

We have compared the coordination of the Mg^{2+} ion in our structure with others in which metal ions are present. The monomeric wild-type HUB has also been crystallized in the presence of Cd^{2+} (PDB entry 3eec; Falini *et al.*, 2008), Hg^{2+} (PDB entry 3efu; Falini *et al.*, 2008), Pt^{2+} (PDB entry 3n32; Arnesano *et al.*, 2011) or Zn^{2+} (PDB entries 3ehv, 3n30, 4k7s, 4k7u, 4k7w, 4xok and 4xol; Falini *et al.*, 2008; Arnesano *et al.*, 2011; Ma *et al.*, 2015). This is the first crystal structure of the monomeric wild-type HUB crystallized in the presence of magnesium. The Mg^{2+} ion is placed in an equivalent position to that found in the structures obtained in the cubic space group $P4_332$ in the presence of Cd^{2+} (PDB entry 3eec) and Zn^{2+} (PDB entry 3n30). It is worth mentioning that both structures are at low resolution (3 Å), hampering accurate modelling of the solvent. In fact, the Cd^{2+} and Zn^{2+} ions in these structures do not fulfill the typical chemical coordination found for these cations (Harding *et al.*, 2010). A new structure of Zn^{2+} -HUB in the cubic space group $P4_332$ has recently been reported (PDB entry 4xol; 2.9 Å resolution), in which the zinc ions have been modelled taking into account Zn^{2+} chemical coordination (Ma *et al.*, 2015; Fig. 3c).

We have used *CONTACT* to account for the residues interacting with the different metal ions. The same residues that are implicated in the coordination of the Zn^{2+} and Cd^{2+} ions are also implicated in the coordination of the hexa-aquo coordinated Mg^{2+} ion present in our structure: Glu18A, Asp21A, Lys29A, Asn25A and the corresponding residues of the symmetry-related chain *B*. The main difference is that in the Mg^{2+} -HUB structure the two ubiquitin molecules in the crystal contact are related by a twofold symmetry axis (Fig. 3a), while in the cubic space-group structures the ubiquitin molecules are related by a threefold symmetry axis (Figs. 3c and 3d). The triclinic structure of the Ca^{2+} -HUB K33S mutant shows a similar coordination in which residues Glu18 and Asp21 belonging to chains *A* and *B* coordinate the metal cation directly and some water molecules mediate the interaction with symmetry-related Asp21 and Asn25 residues (Fig. 3b). In this case, the interaction with Lys29 is weaker (>4 Å; Supplementary Table S1). These differences in coordination at the metal site containing calcium or magnesium result in different orientations of the HUB molecules in the stacked layers that compose each triclinic crystal.

Additional metal sites are present in the Zn^{2+} -HUB, Cd^{2+} -HUB and Ca^{2+} -HUB structures that also favour crystal contacts, but their counterparts are not occupied in the Mg^{2+} -HUB structure reported in this work. All the cubic space-group structures show the same crystal contact as is present in

the triclinic structures (Fig. 3). Besides, some of the structures of Zn^{2+} -HUB obtained in the orthorhombic space group $P2_12_12_1$ also show an equivalent crystal contact mediated by Zn^{2+} (Fig. 3d, Supplementary Table S2). This experimental evidence indicates that this metal-binding site is key in the formation of the HUB crystals.

In conclusion, the hexa-aquo coordination around the magnesium ion allows the improvement of a set of packing interactions previously observed in other metal-bound HUB structures. Lys29 also participates at this location, and its side chain has been modelled with average side-chain *B* factors of 25 and 16.5 Å² for chains *A* and *B*, respectively (overall average *B* values: chain *A*, 17.4 Å²; chain *B*, 15.8 Å²), which indicates a reduced mobility. Large and flexible side chains are usually excluded from crystal contacts because of the unfavourable loss of entropy produced in the residue-locking process to produce packing interactions (Derewenda & Vekilov, 2006). The formation of new and strong interactions around the metal site results in an enthalpy release that may compensate for the loss of entropy. Thus, this high-resolution crystal form provides information about the role of magnesium in the binding and crystallization of HUB through water-mediated hydrogen bonds, allowing a better understanding of how reducing the flexibility of some residues facilitates packing interactions in crystals.

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