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Crystal structures of Lys-63-linked tri- and di-ubiquitin reveal a highly extended chain architecture

Stephen D. Weeks^{1,3}, Kimberly C. Grasty¹, Lisa Hernandez-Cuebas^{1,2}, and Patrick J. Loll^{1,*}

¹ Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19102

² Graduate Program in Biochemistry, Drexel University College of Medicine, Philadelphia, PA 19102

Abstract

The covalent attachment of different types of poly-ubiquitin chains signal different outcomes for the proteins so targeted. For example, a protein modified with Lys-48-linked poly-ubiquitin chains is targeted for proteasomal degradation, whereas Lys-63-linked chains encode non-degradative signals. The structural features that enable these different types of chains to encode different signals have not yet been fully elucidated. We report here the X-ray crystal structures of Lys-63-linked tri- and di-ubiquitin at resolutions of 2.3 and 1.9 Å, respectively. The tri- and di-ubiquitin species adopt essentially identical structures. In both instances the ubiquitin chain assumes a highly extended conformation with a left-handed helical twist; the helical chain contains four ubiquitin monomers per turn and has a repeat length of approximately 110 Å. Interestingly, Lys-48 ubiquitin chains also adopt a left-handed helical structure with a similar repeat length. However, the Lys-63 architecture is much more open than that of Lys-48 chains and exposes much more of the ubiquitin surface for potential recognition events. These new crystal structures are consistent with the results of solution studies of Lys-63 chain conformation, and reveal the structural basis for differential recognition of Lys-63 versus Lys-48 chains.

Keywords

ubiquitin; crystallography; proteasome; protein degradation; molecular recognition

Introduction

The covalent modification of proteins by ubiquitin is a critical mechanism for propagating signals in cells 1. Ubiquitination of different target proteins is heterogeneous; both the number of ubiquitin molecules that are attached and their topology are variable. Thus, ubiquitin can be added as single molecules 2 or as poly-ubiquitin chains 3, and the chains can take different forms, depending on the structural details of chain assembly. Within poly-ubiquitin chains, individual ubiquitin monomers are linked by isopeptide bonds between one molecule's C-terminus and a lysine side chain of the adjacent monomer. Ubiquitin contains seven lysines, and isopeptide-linked chains can be formed using any of these residues. Chains containing all seven possible linkage types have been found in living cells 4-5. Different ubiquitin chain types encode different biological signals, allowing this single protein to mediate many diverse functions. For example, chains built using Lys-48 linkages

*Corresponding author: Patrick J. Loll, Dept. of Biochemistry and Molecular Biology, Drexel University College of Medicine, 245 N. 15th St. Philadelphia, PA 19102 USA, pat.loll@drexel.edu, (215) 762-7706 (phone), (215) 762-4452 (fax).

³Current address: Laboratory for Biocrystallography, University of Leuven, Faculty of Pharmaceutical Sciences, Leuven, Belgium.

(K48 chains) signal degradation of almost any protein to which they are attached 6. In contrast, K63-linked chains participate in a variety of non-proteasomal signaling pathways, including the initiation of DNA damage responses and the formation of aggresomes 7–9.

Because different ubiquitin chain types are associated with different signaling pathways, cellular machinery must be able to recognize and distinguish chains containing different isopeptide linkages. The molecular basis underlying this selective recognition is of great interest. The principal epitope on monomeric ubiquitin that is recognized by ubiquitin-binding domains is the so-called Ile-44 patch, a surface cluster of hydrophobic side chains that includes Leu-8, Ile-44, and Val-70 10. Since this patch is found on all ubiquitin chains, its presence alone is not sufficient to distinguish chain types. Hence, if this patch, or indeed any region of the ubiquitin surface, is to contribute to specificity of recognition, it must be differentially accessible in different chain types, implying conformational differences between chains with different linkages. This notion is supported by structural information that is emerging for different types of poly-ubiquitin chain, which points to significant conformational differences between K48- and K63-linked chains.

The structures of K48 chains are the most thoroughly characterized. K48-linked di-ubiquitin forms a tight dimer in which the Ile-44 patch of each monomer is buried at the dimer interface. This is known as the closed conformation, and is the predominant form in solution at physiological pH 11. This same structural motif is seen in K48-linked tetra-ubiquitin; at neutral pH (where presumably the most physiologically relevant form is found), two di-ubiquitin dimers stack atop one another to produce a compact globular assembly 12. This topology exposes short hydrophobic stripes along the length of the chain, which has been suggested to represent a unique motif for K48 chain recognition.

While crystal structures have recently become available for K63-linked ubiquitin dimers bound to antibody Fab fragments 13 and to the de-ubiquitinating enzyme AMSH 14, no crystal structure has yet been published for a free K63-linked poly-ubiquitin chain. However, the relative positions of K48 and K63 on the surface of the ubiquitin molecule suggest that K63 chains cannot adopt the same compact folding pattern as K48 chains. This idea is supported by solution NMR studies of K63 di-ubiquitin, in which analysis of chemical shift perturbations indicated little or no direct contact between the two monomers 15,16. The K48 dimer behaves very differently, with its intimate dimer interface giving rise to significant differences in chemical shifts between monomer and dimer 11. The lack of contacts between the two subunits of the K63 dimer suggests the dimer adopts an open, extended structure. This finding is supported by small angle X-ray scattering (SAXS) analysis of the dimer 15; SAXS studies also suggest that the K63 tetramer adopts a similarly open conformation.

We present here the X-ray crystal structures of K63-linked tri- and di-ubiquitin. Both species adopt essentially identical extended structures in which the isopeptide linkage is the only interaction between adjacent monomers in the chain. The K63 chain topology that we observe is radically different from that of K48 chains, and suggests structural mechanisms by which potential binding partners could distinguish between the two chain types.

Materials and Methods

Protein preparation

K63-linked di- and tri-ubiquitin chains were prepared using the protocol of Pickart and Raasi 17. Briefly, recombinant hexahistidine-tagged human E1 and the yeast E2 complex (Mms2/Ubc13) were produced in *E. coli* and purified by immobilized metal ion chromatography (IMAC). Ubiquitin dimers were assembled by the E1/E2-catalyzed linkage

of K63R and D77 ubiquitin mutants. Following synthesis the reaction was stopped by the addition of 5 mM 2-mercaptoethanol and the terminal D77 residue removed from the ubiquitin moiety using recombinant hexahistidine-tagged Yuh1. The dimer chains were purified first by IMAC to remove the E1, E2 and YUH1 enzymes, and then via cation exchange chromatography at pH 5.2 to separate the dimers from unreacted monomers. Trimers were prepared by using the E1 and E2 enzymes to add an additional D77 ubiquitin molecule to the proximal end of the di-ubiquitin species, followed by Yuh1 deprotection and chromatographic purification. K63-linked poly-ubiquitin was obtained by incubation of the E1 and E2 enzymes with wild type ubiquitin. The identities of the di- and tri-ubiquitin species were verified by ESI-mass spectrometry, carried out by the Proteomics Facility of the Wistar Institute (Philadelphia, PA, USA). Intact protein masses were obtained by direct infusion of purified ubiquitin chains in an LTQ Orbitrap mass spectrometer operating at 100K resolution (Thermo Scientific, Waltham, MA, USA). Experimental masses are in excellent agreement with values calculated for the mono-sodium species (calculated/experimental masses (Da): di-ubiquitin, 17126/17130; tri-ubiquitin, 25670/25671).

Crystallization and data collection

Crystals were grown by the microbatch under oil method 18, using Al's Oil (Hampton Research, Aliso Viejo, CA). Equal (1 μ l) volumes of protein and precipitant solutions were mixed and incubated at 291K; crystals appeared within 24 hours. The di-ubiquitin solution contained 8 mg/ml protein in 20 mM Tris pH 7.5, and the precipitant solution contained 0.2 M cadmium sulphate, 0.1 M imidazole-Cl pH 6.5, and 5% w/v PEG 8000 (final pH = 5.2). The tri-ubiquitin solution contained 8.5 mg/ml protein in 20 mM Tris pH 7.5, and the precipitant solution contained 0.2 M zinc acetate, 0.1 M imidazole-Cl pH 6.5, and 6% w/v PEG 8000 (final pH = 5.2). Metals were required for crystal growth; both the di- and tri-ubiquitin species could be crystallized using either zinc or cadmium. Cadmium gave larger crystals for di-ubiquitin, while zinc produced larger crystals for tri-ubiquitin. Microcrystals of K63-linked poly-ubiquitin were obtained using a precipitant solution of 0.2 M zinc acetate, 0.1 M imidazole-Cl pH 6.5, and 6% w/v PEG 8000 (final pH = 5.2). Crystals were cryoprotected using a freshly prepared precipitant solution with the same concentrations given above, but containing in addition 25% w/v glycerol. Diffraction data were collected at NSLS beamline X6A, and integrated and scaled using XDS 19. Data collection statistics are given in Table I.

Structure determination

For both crystal forms, the location of the ubiquitin monomers was determined by molecular replacement, using a truncated model from which the K63 side chain and residues 74–76 were removed. EPMR 20 and Phaser 21 gave essentially identical solutions. Refinement was carried out using phenix.refine 22, and the structures were completed using alternating cycles of refinement and rebuilding in Coot 23. As independent checks on the structure, the locations of metal ions were determined from anomalous difference maps calculated using phases derived from the initial molecular replacement models. These metal positions were used to calculate SAD phases in OASIS, after which the maps were improved by solvent flattening in DM. For both the di- and tri-ubiquitin structures, the SAD maps were in good agreement with the structures obtained by refinement of the molecular replacement solutions. Refinement statistics are given in Table II. Coordinates and structure factors for the di- and tri-ubiquitin structures have been deposited with the Protein Data Bank (accession numbers 3H7P and 3H7S, respectively). While this manuscript was in preparation, an independent structure for K63-linked di-ubiquitin was published 24, and appears very similar to the di-ubiquitin structure reported here.

Results and Discussion

Composition of the crystal asymmetric unit

Both the di- and tri-ubiquitin structures crystallize in the same spacegroup, with essentially identical unit cell parameters (Table I). Calculation of Matthews coefficients and solvent content revealed that, in principle, the asymmetric unit of this crystal could accommodate either two or three molecules of ubiquitin, giving V_m values of 2.9 and 1.9 Å³/Da and solvent contents of 57% and 35%, respectively 25·26. Hence, at the outset of the structure determination it was formally possible that the two crystal forms might differ, with the di-ubiquitin crystals containing two ubiquitin molecules per asymmetric unit, and the tri-ubiquitin crystals three. However, once the data were analyzed it became clear that the two different crystal forms are essentially identical and that both contain two ubiquitin molecules per asymmetric unit. This is not the result of degradation of the tri-ubiquitin chains, as SDS-PAGE analysis of dissolved crystals verified that the crystals contain the expected species (Figure 1).

These results imply that the tri-ubiquitin crystals contain positional disorder. As described below, the ubiquitin molecules pack head-to-tail in infinite chains. The two molecules in the asymmetric unit make up two links in one such chain, and pack head-to-tail at either end with adjacent pairs of molecules. This head-to-tail packing means that translational disorder along the direction of the chain is easily accommodated by the crystal lattice. We denote the two ubiquitin monomers in the asymmetric unit as A and B, and an adjacent pair of monomers along the extended ubiquitin chain as A' and B'. We have modeled the three subunits of the ubiquitin trimer as occupying positions A-B-A' in 50% of the crystal, and positions B-A'-B' in the remaining 50% of the crystal. This simple disorder model leads to a structurally reasonable result, as explained below. Consistent with this model for packing disorder, we have also been able to crystallize heterogeneous preparations of K63-linked poly-ubiquitin chains that consist mostly of tetramers and larger species, and which contain very little dimer. Unfortunately, only microcrystals have been obtained thus far for the poly-ubiquitin, and we have been unable to measure their diffraction (data not shown). However, these poly-ubiquitin crystals form under the same conditions used to crystallize the di- and tri-ubiquitin species, suggesting that they may adopt the same lattice packing and exhibit the same type of translational disorder along the direction of the poly-ubiquitin chain.

As a consequence of the synthetic strategy used, both the di- and tri-ubiquitin chains contain the K63R mutant as their most distal molecule. In the di-ubiquitin structure we have modeled the K63R molecule in the A position of the chain, placing the isopeptide linkage between the C-terminus of molecule A and the K63 side chain of molecule B. In the tri-ubiquitin structure, the translational disorder requires that both the wild type and mutant proteins be present in both the A and B positions, with relative occupancies of 2:1 wild type:mutant.

Overall structure of the K63-linked dimer/trimer

Lys-63 is on the opposite face of the ubiquitin molecule from the carboxy terminus, allowing the two molecules in the asymmetric unit to assemble in an extended head-to-tail fashion, with the C-terminus of the A molecule pointing toward the K63 side chain of the B molecule. Using the standard nomenclature for ubiquitin chains, in the A-B pair A is the distal molecule, and B the proximal. The C-terminus of the B molecule points toward the K63 side chain of a symmetry-related A' molecule (i.e., B is distal to A'). In this way, alternating A and B molecules form an elongated chain that runs through the crystal, explaining how both tri-ubiquitin and di-ubiquitin can be accommodated in the same crystal lattice (Figure 1).

The structures in the di- and tri-ubiquitin crystals are essentially identical; superposition of the ubiquitin dimers that constitute the asymmetric units in the two crystal forms yields an rms difference in C α positions of 0.23 Å. Another, independent structure of K63-linked di-ubiquitin has recently appeared 24, and is also essentially identical to our dimer and trimer structures, with rms differences in C α positions of 0.28 and 0.24 Å, respectively. Because of the similarity of the dimer and trimer structures, the descriptions that follow are equally applicable to the two crystal forms, unless otherwise noted.

The chain of linked ubiquitin molecules is highly extended, with no monomer in the chain making close contacts with any adjacent monomers in the same chain. Except for the covalently linked residues that form the links in the chain—the C-terminal Gly-76 of the distal monomer and the K63 side chain of the proximal monomer—no residue of any ubiquitin monomer approaches to within 4.2 Å of another monomer. Each of the monomers that make up the chain is rotated about the long axis of the extended ubiquitin chain with respect to its proximal and distal neighbors. The rotation of the B molecule relative to the A molecule is 107°, and the rotation of A' with respect to B is 73° (crystallographic symmetry requires that these two angles sum to 180°). This imparts a helical character to the chain, with a repeat of four monomers per turn (Figure 2).

There are only small, localized differences between the two ubiquitin monomers in the asymmetric unit; superposition of the A and B monomers yields an RMS difference of 0.86 Å for C α positions. Most of the differences are localized to the C-termini and to two surface loops that flank the C-termini, spanning residues 7–11 and 30–39. Both of these loops show considerable structural variation in solution structures 27,28 and are involved in crystal contacts. Neither of the loops is within contact distance of the adjoining monomer in the chain; hence, it seems likely that the differences seen between the two monomers in the crystal structure reflect innate mobility and lattice packing effects, and are not imposed by the K63 chain linkage.

Metal binding

Divalent cations are required for crystal growth. We used anomalous difference maps to unambiguously define the locations of numerous ordered metal ions in the di- and tri-ubiquitin structures. Seven metal sites are found in both of the two structures, including three pairs of equivalent sites in which both ubiquitin molecules in the asymmetric unit bind metals at the same positions. These sites include Met-1/Glu-16 (both molecules A and B), Glu-18/Asp-21 (both molecules), Glu 24/Asp 52 (both molecules), and Glu-64/His-68 (molecule A only). Similar metal binding patterns have been previously observed for Cu(II), Cd(II), and/or Zn(II) in monomeric ubiquitin and ubiquitin-like proteins 29 suggesting that metal binding may be a general property of ubiquitin and related proteins, and not merely an artifact of crystallization. This result is intriguing, given the recent observation that cadmium induces the formation of aggresomes in a poly-ubiquitin-dependent manner 30.

The isopeptide linkage

In the di-ubiquitin structure, the isopeptide linkage is modeled at the A–B junction (i.e., connecting the C-terminus of molecule A with the K63 side chain of molecule B). However, while it is possible to build a sterically reasonable connection, the electron density for the linkage is too weak to allow the connection to be built with confidence. In the tri-ubiquitin structure, the packing disorder requires that the isopeptide linkage be present at both the A–B and B–A' junctions in the chain at an occupancy of 0.67. However, the electron density at both positions is also weak, and does not support building the connection. Since we are confident that the isopeptide links are present in both the di- and tri-ubiquitin crystals (Figure 1), it seems likely that the poor quality of the electron density reflects disorder in the

linkage region. The C-terminus of ubiquitin forms an isolated strand that extends away from the body of the protein. As described earlier, adjacent molecules in the extended poly-ubiquitin chain do not pack against each other, and so the only packing interactions that are available to stabilize the conformation of this extended C-terminus are supplied by neighbors in the crystal lattice. None of these neighboring molecules, however, packs very tightly against the C-terminus of either the A or B molecule, and so it is likely that the atoms participating in the A–B and B–A' linkages are free to sample multiple conformations. In addition to this motional disorder, the tri-ubiquitin crystals also contain static disorder, as discussed above. It is not necessary to invoke a similar translational disorder for the di-ubiquitin crystals, but it is possible that they contain some degree of such disorder, which would also contribute to the lack of definition in the maps at the linkage sites.

Correlation of the X-ray structure with solution studies

NMR solution studies performed at a neutral pH reveal that the ubiquitin monomers making up a K63-linked dimer fail to interact, except for the residues forming the linkage 15·16, in excellent agreement with the extended structure seen in the crystals. Comparison of rotational diffusion tensors obtained from the NMR experiments also show that adjacent molecules in the K63-linked ubiquitin chain are rotated with respect to one another, as is seen in the crystal structure. The sense of this rotation cannot be inferred from the NMR data alone, as both right-handed and left-handed helical arrangements are consistent with the solution data; in contrast, the crystal structure clearly shows that the helical twist of the chain is left-handed. The magnitude of the rotation estimated from the NMR data is somewhat lower than the values seen in the crystal structure (ca. 35–45°, as compared to 73–107°); however, as discussed below, the K63 linkage is likely to be highly flexible and capable of assuming a wide range of conformations. Such a flexible “beads on a string” model is supported by small angle X-ray scattering experiments showing that, while elongated, K63-linked tetra-ubiquitin does not behave like a fully extended rigid rod 15.

K63-linked vs. K48-linked chains

A comparison of the K63-linked di- and tri-ubiquitin structures with the crystal and solution structures of K48-linked tetra-ubiquitin reveals both marked differences and interesting similarities in conformation. The K48-linked structure is compact, and consists of stacked ubiquitin dimers (see Figure 2, lower panel). Dimer formation buries much of the Ile-44 recognition patch, exposing only a narrow hydrophobic stripe made up of the two Leu-8 side chains, which has been proposed to represent a potential recognition motif 12. In contrast, the extended K63-linked chain fully exposes most of the ubiquitin surface, including the entire Ile-44 patch. In K48-linked chains, successive dimers are rotated 90° about the chain axis with respect to one another, and hence the putative Leu-8 recognition motif forms a repeating structure consisting of a left-handed helical array of hydrophobic stripes with eight molecules (four dimers) per turn and a repeat length of approximately 100 Å. In the K63-linked chain, successive monomers are also rotated roughly 90° about the chain axis with respect to each other, and also form left-handed helical arrays. The repeat length is approximately 110 Å, similar to that of the K48-linked chain, but the K63 chain is much less dense, with only 4 monomers per turn. Thus, both types of chains present multiple copies of hydrophobic recognition surfaces that might facilitate specific interactions; both form left-handed helical arrays; and the repeat lengths for the two chain types are quite similar. However, the two chain types differ in the nature of the recognition surface that is presented and the flexibility of the chain architecture. Of the residues making up the Ile-44 recognition patch, only Leu-8 is exposed in the K48-linked chains, whereas the entire patch is accessible in the K63-linked chains. In the K48-linked chains, successive ubiquitin monomers make intimate contacts with one another, leading to a closed and relatively stiff chain architecture being favored under physiological conditions 31. In contrast, the isopeptide links in the K63-

linked chains are the only contacts between adjacent monomers. These links are highly flexible, and hence the K63 chains are more open than the K48 chains. We also predict that K63 chains will exhibit more conformational heterogeneity and be more readily deformable. This is consistent with the two crystal structures currently available for K63-linked ubiquitin dimers in complex with other proteins. In the structure of the complex of K63 di-ubiquitin with the de-ubiquitinating enzyme AMSH, the dimer adopts an extended structure in which the two monomers are related by an approximately 90° left-handed helical twist 14, very similar to the structure seen in our crystals. In contrast, in the structure of K63 di-ubiquitin bound to an antibody fragment, the dimer structure is distorted and the linkage between the two monomers is bent into a V-shape 13. In both instances, recognition of K63 di-ubiquitin involves interactions with both ubiquitin monomers in the dimer, including portions of the ubiquitin monomers that would not be accessible in a K48 ubiquitin chain. Thus, the extended architecture of the K63-linked ubiquitin chain displays two critical features that can be exploited for specific recognition of this chain type: An open structure that provides access to the entire surface of each monomer in the chain, and an inherently flexible linkage that allows the relative orientation of any two adjacent monomers to vary significantly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Verification of the ubiquitin species contained within our crystals. Lane 1, purified tri-ubiquitin; Lane 2, purified di-ubiquitin; Lanes 3 and 4, molecular weight markers (molecular weights are shown in the space between the two gels); Lane 5, a different sample of purified di-ubiquitin that was used for crystallization experiments; Lane 6, washed and dissolved di-ubiquitin crystals; Lane 7, washed and dissolved tri-ubiquitin crystals. Crystals were washed repeatedly with protein-free mother liquor, transferred to sample buffer and run on a 12–20% SDS-PAGE gradient gel, which was fixed and stained with Coomassie Brilliant Blue. The formation of SDS-resistant higher molecular weight species is likely due to residual PEG in the dissolved crystals. (b) Structure of the di-ubiquitin chain found in the asymmetric unit of both the di- and tri-ubiquitin crystals. The distal molecule is colored cyan and the proximal molecule yellow. The side chains of Lys-63 (on the proximal molecule) and Arg-63 (on the distal molecule) are shown in ball-and-stick representation. (c) Positional disorder in the tri-ubiquitin structure. At top is shown a portion of one of the extended ubiquitin chains running throughout the crystal; the distal and proximal ends of the chain are marked. Three adjacent asymmetric units are shown (A'-B', A-B, and A'-B'), separated by dotted lines. Below is a schematic representation of the packing of the tri-ubiquitin species. The distal-most subunit of the trimer will alternately occupy the A and B positions in the asymmetric unit. Figures 1 and 2 were prepared using MacPyMol (<http://www.pymol.org>).

Figure 2.

Stereo views of K63- and K48-linked ubiquitin chains. *Upper panel:* The extended K63-linked ubiquitin chain that runs through the di- and tri-ubiquitin crystals. Shown are surface representations for six monomers (three adjacent asymmetric units). Molecules occupying the distal (A) position are colored blue and molecules in the proximal (B) position are colored yellow. The proximal end of the chain is at the bottom of the figure, and the distal end at the top. On each molecule, the hydrophobic patch comprising residues Leu-8, Ile-44, and Val-70 is colored red. *Lower panel:* A tetramer of K48-linked ubiquitin molecules. Starting from the distal end and moving toward the proximal end of the chain, the monomers are colored cyan, magenta, blue, and yellow, respectively. The hydrophobic patch on each monomer is colored red, as in the upper panel.

Table I

Data Collection Statistics

Data set	Di-ubiquitin	Tri-ubiquitin
Wavelength (Å)	0.980	0.980
Space group	$P4_332$	$P4_332$
Cell dimensions (Å)	a = b = c = 105.53	a = b = c = 105.32
Resolution range (Å) ^a	25-1.90 (1.95-1.90)	25-2.3 (2.36-2.30)
No. observations	303,537 (9245)	208,084 (15,039)
No. unique reflections	16,374 (1179)	9,348 (669)
Completeness (%)	99.8 (99.4)	99.8 (100.0)
Multiplicity	18.5 (7.8)	22.2 (22.5)
Mean I/σ(I)	23.9 (3.0)	28.2 (4.9)
R_{merge}	0.067 (0.631)	0.074 (0.701)
R_{meas}^b	0.069 (0.675)	0.076 (0.722)

^a Values in parentheses refer to the highest resolution shell

^b The redundancy-independent residual on intensities R_{meas} is defined according to Diederichs & Karplus³⁴.

Table II

Refinement statistics

<i>Refinement</i>	Di-ubiquitin	Tri-ubiquitin
Resolution range (Å)	24.2–1.90	24.8–2.30
Number of reflections used	16,373	9,345
Number of protein atoms	1201	1194
Number of solvent atoms		
water	107	47
Cd ²⁺ ions	7	---
Zn ²⁺ ions	---	11
Mean B values (Å ²)		
protein	45.2	58.4
water	51.2	59.9
metal ions	45.2	110.4
RMS deviations from ideal geometry		
Bond distances (Å)	0.010	0.005
Bond angles (deg)	1.16	0.82
R _{cryst} /R _{free}	0.193/0.236	0.196/0.253