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Purification, crystallization and preliminary crystallographic studies of Lys48-linked polyubiquitin chains

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Post-translational modification of proteins by covalent attachment of ubiquitin regulates diverse cellular events. A Lys48-linked polyubiquitin chain is formed *via* an isopeptide bond between Lys48 and the C-terminal Gly76 of different ubiquitin molecules. The chain is attached to a lysine residue of a substrate protein, which leads to proteolytic degradation of the protein by the 26S proteasome. In order to reveal the chain-length-dependent higher order structures of polyubiquitin chains, Lys48-linked polyubiquitin chains were synthesized enzymatically on a large scale and the chains were separated according to chain length by cation-exchange column chromatography. Subsequently, crystallization screening was performed using the hanging-drop vapour-diffusion method, from which crystals of tetraubiquitin, hexaubiquitin and octaubiquitin chains were obtained. The crystals of the tetraubiquitin and hexaubiquitin chains diffracted to 1.6 and 1.8 Å resolution, respectively. The tetraubiquitin crystals belonged to space group $C222_1$, with unit-cell parameters a = 58.795, b = 76.966, c = 135.145 Å. The hexaubiquitin crystals belonged to space group $P2_1$, with unit-cell parameters a = 51.248, b = 102.668, c = 51.161 Å. Structural analysis by molecular replacement is in progress.

1. Introduction

Ubiquitin is a highly conserved eukaryotic protein composed of 76 amino acids and regulates diverse biological processes such as stress responses, cell-cycle progression, gene transcription and DNA repair through covalent attachment to target proteins (Weissman, 2001; Pickart & Fushman, 2004). The attachment of monomeric ubiquitin or a polymeric ubiquitin chain is catalyzed by an enzymatic cascade involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin–protein ligase (E3), in which an isopeptide bond is formed between the C-terminal Gly76 of ubiquitin and a lysine residue of the target protein. Polyubiquitin chains are formed in the same manner. The C-terminal carboxyl group of one ubiquitin forms an isopeptide bond to the N-terminus or one of seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) of another ubiquitin molecule (Pickart & Fushman, 2004; Kirisako *et al.*, 2006). Various ubiquitin-binding proteins recognize differences in length and/or linkage-specific structural properties of these polyubiquitin chains and are involved in diverse ubiquitin-mediated signalling pathways.

Of these polyubiquitins, Lys48-linked polyubiquitin chains are mainly involved in ATP-dependent proteasomal degradation. The cellular function and structural properties of the Lys48-linked polyubiquitin chain have been extensively studied, as have those of the Lys63-linked polyubiquitin chain, which mediates various cellular signals that do not rely on proteasomal protein degradation. In contrast to the extended but flexible conformation adopted by the Lys63-linked polyubiquitin chain (Datta *et al.*, 2009; Komander *et al.*, 2009; Tenno *et al.*, 2004), the Lys48-linked chain forms a compact quaternary structure in crystals. To date, three distinct crystal structures of the Lys48-linked tetraubiquitin have been reported, two of which contain ubiquitin units that are arranged in such a way that a

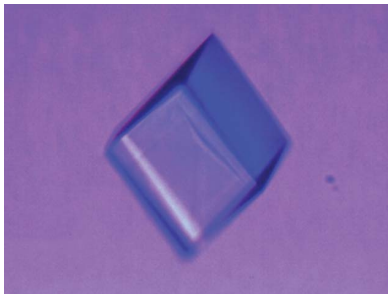

Table 1
Primers used for PCR synthesis of the mUBA gene.

Restriction sites are shown in bold.

Forward 1	GG**CATATG**TCCAGCTCGCCGCTGTC	(*Nde*I)
Reverse 1	**ACTAGT**TTCCGAATAAGGTCTTCATCCAAGC	(*Spe*I)
Forward 2	**GCTAGC**TTATGTTGCTGCTGGGG	(*Nhe*I)
Reverse 2	**AGATCT**TGGCTAGAGCTGTAGGATTCTG	(*Bgl*II)
Forward 3	**GGATCC**ACCAGAGAAATCCATCCC	(*Bam*HI)
Reverse 3	**GGATCC**AGTCAACCCATAGGTCTG	(*Bam*HI)
Forward 4	**AGATCT**CAAGACCGAGCTGCTGTG	(*Bgl*II)
Reverse 4	GG**CTCGAG**TTATCAGCGAATGGTATATCGGA-CATAAGGG	(*Nde*I)

hydrophobic patch composed of Leu8, Ile44 and Val70 is concealed at the interface between two spatially proximate units (Cook *et al.*, 1994; Phillips *et al.*, 2001; Eddins *et al.*, 2007). NMR studies also showed that the Lys48-linked chain has substantial interactions between ubiquitin units, whereas the Lys63-linked chain has few stable interunit contacts (Varadan *et al.*, 2002; Tenno *et al.*, 2004). Nevertheless, crystallographic and NMR analyses suggest that Lys48-linked polyubiquitin chains can form different quaternary structures depending on pH and crystal packing and that they possess conformational flexibility (Cook *et al.*, 1994; Phillips *et al.*, 2001; Eddins *et al.*, 2007). In the three reported crystal structures of Lys48-linked tetraubiquitin, the Lys48–Gly76 linkage between the second and third subunits was only observed in a crystal grown at neutral pH in which Lys48 of the second ubiquitin was replaced by an aminoethylcysteine possessing an S atom at the γ position instead of the C^{γ} methylene of lysine (Cook *et al.*, 1994; Phillips *et al.*, 2001; Eddins *et al.*, 2007).

Polyubiquitin chains with a wide range of lengths, often longer than a tetramer, have been identified *in vivo*. A systematic approach is required to fully elucidate the molecular mechanism underlying ubiquitin-mediated signalling pathways as well as the structural and dynamic properties of polyubiquitin chains of various lengths. To further analyze the structure of ubiquitin chains, we have established a protocol for efficient enzymatic synthesis of Lys48-linked polyubiquitin chains with native isopeptide bonds and separation of the chains based on their lengths. Here, we describe the purification and crystallization of Lys48-linked tetraubiquitin, hexaubiquitin and octaubiquitin. Preliminary X-ray analysis of tetraubiquitin and hexaubiquitin is also reported.

2. Materials and methods

2.1. Cloning, overproduction and purification of the ubiquitin-activating E1 enzyme

An E1 enzyme from mouse (mUBA1) was overproduced in insect cells. mUBA1 cDNA was cloned from the mouse kidney-derived cDNA library. The coding region of full-length mUBA1 was not efficiently amplified by PCR. We reconstructed the mUBA1 cDNA by enzymatically ligating the PCR fragments amplified using four pairs of primers (Table 1). The PCR-synthesized mUBA1 cDNA fragment was subcloned into pFastBacI baculovirus transfer vector (Invitrogen) together with a polyhistidine tag. The reconstructed mUBA1 cDNA inserted into the transfer vector was verified by sequencing. A primary baculovirus stock was produced according to the manufacturer's instructions. The baculovirus was transfected into Sf9 cells. The cells were cultured in serum-free insect-cell medium (SF900-II) for 3 d and were harvested by centrifugation at 1000*g* for 10 min. The cells were resuspended in lysis buffer (50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 1 m*M* DTT, 1% Triton X-100, 10 m*M* NaF, 10 m*M* disodium hydrogen phosphate and 10 m*M* sodium pyrophosphate) and lysed by sonication. After centrifugation at 48 384*g* for 20 min, the supernatant was mixed with 10 ml Ni–NTA–agarose resin (Qiagen) equilibrated with buffer *A* (1× PBS pH 7.4 containing 300 m*M* NaCl and 10 m*M* β-mercaptoethanol). After extensive washing with buffer *A* containing 20–60 m*M* imidazole–HCl, the protein was eluted by increasing the concentration of imidazole–HCl from 80 to 300 m*M* in buffer *A*. The protein solution was dialyzed against 5 l 50 m*M* Tris–HCl buffer pH 8.0 containing 500 m*M* NaCl and 1 m*M* DTT. A preparation from a 300 ml culture yielded approximately 8 mg mUBA1.

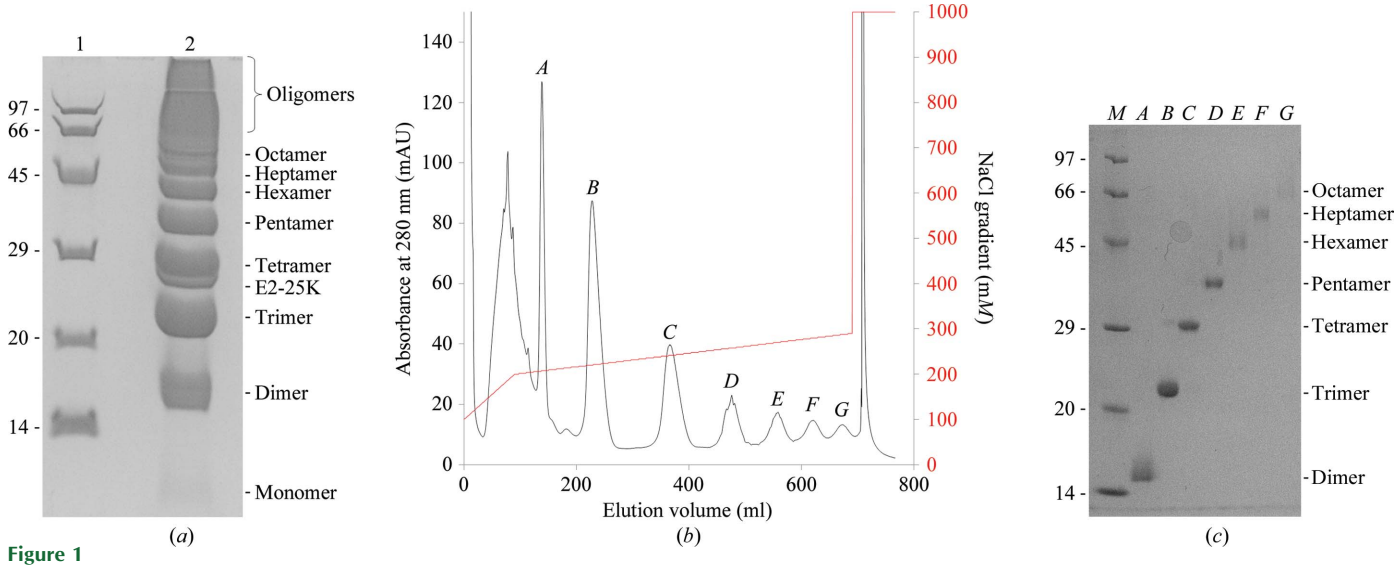

Figure 1
Preparation of polyubiquitin chains of different lengths. (*a*) Synthesis of Lys48-linked polyubiquitin chains using E1 and E2 enzymes *in vitro*. The reaction mixture was analyzed on a 17.5% SDS–PAGE gel. Lane 1, molecular-mass markers (kDa); lane 2, enzymatically synthesized polyubiquitin chains. (*b*) Chromatogram of the polyubiquitin chains. Lys48-linked polyubiquitin chains were separated based on length: *A*, dimer; *B*, trimer; *C*, tetramer; *D*, pentamer; *E*, hexamer; *F*, heptamer; *G*, octamer. A red line shows NaCl gradients (100–200 m*M* NaCl in six column volumes and 200–290 m*M* NaCl in 40 column volumes). Nucleotides included in the enzymatic reaction were eluted in the bulk peak at the start of the second gradient. (*c*) Analysis of the fractions indicated in (*b*) on a 15% SDS–PAGE gel. Lane *M*, molecular-mass markers (kDa); lanes *A*–*G*, fractions *A*–*G* in (*b*).

2.2. Purification of polyubiquitin chains

Ubiquitin was overproduced in *Escherichia coli* and purified as previously reported (Piotrowski *et al.*, 1997). A Lys48-link-specific E2 enzyme, E2-25K, was overproduced in *E. coli* and purified as described previously (Piotrowski *et al.*, 1997). Lys48-linked polyubiquitin chains were enzymatically synthesized *in vitro* as described previously (Chen & Pickart, 1990). Each polyubiquitin chain was purified using cation-exchange column chromatography with Source 15S (GE Healthcare). The polyubiquitin chain was applied onto a Source 15S column equilibrated with 50 m*M* sodium acetate buffer pH 4.5 containing 1 m*M* DTT. The bound ubiquitin chains were eluted by increasing the salt concentration using two sequential linear gradients: a steeper gradient (100–200 m*M* NaCl in six column volumes) and a shallow gradient (200–290 m*M* NaCl in 40 column volumes). Protein solutions were concentrated using 3000 molecular-weight cutoff polyethersulfone filters (Sartorius Stedim). The protein concentration was determined from the absorbance at 280 nm.

2.3. Crystallization

Crystallizations were carried out using the hanging-drop vapour-diffusion method at 293 K. Lys48-linked tetraubiquitin and hexaubiquitin chains were each screened in various conditions from commercially available kits. For each condition, 1 µl protein solution was mixed with 1 µl reservoir solution and the mixture was equilibrated against 500 µl reservoir solution.

2.4. X-ray diffraction experiments

Initial X-ray diffraction experiments were performed in-house using an R-AXIS IV++ imaging-plate detector system installed on a MicroMax-007 rotating-anode X-ray generator (Rigaku) operating at 40 kV and 20 mA with an Osmic mirror system. X-ray diffraction data sets were collected at a wavelength of 1.000 Å on beamline BL5A at the Photon Factory (Tsukuba, Japan). Crystals were flash-cooled at 100 K in cryoprotectant containing 20% glycerol.

Table 2
Data-collection statistics for tetraubiquitin and hexaubiquitin crystals.

	Tetraubiquitin	Hexaubiquitin
Wavelength (Å)	1.0000	1.0000
Space group	$C222_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 58.795, b = 76.966, c = 135.145, $\alpha = \beta = \gamma$ = 90	a = 51.248, b = 102.668, c = 51.161, $\alpha = \gamma$ = 90, β = 113.419
Resolution range (Å)	50.0–1.60 (1.66–1.60)	50.0–1.80 (1.86–1.80)
No. of unique reflections	40198 (3566)	44166 (3935)
Total No. of reflections	261653	146701
Completeness (%)	98.5 (88.9)	97.2 (87.0)
R_{merge} (%)	7.5 (46.1)	7.9 (36.0)
Mean $I/\sigma(I)$	12.5 (2.8)	11.9 (2.1)
Redundancy	6.5 (5.6)	3.4 (2.5)

3. Results and discussion

3.1. Large-scale preparation of polyubiquitin chains of different lengths

Lys48-linked polyubiquitin chains were produced through *in vitro* enzymatic reaction using recombinant UBA1 (E1) and the Lys48-specific E2 enzyme E2-25K (E2). For ubiquitination reactions, 1.2 m*M* ubiquitin was mixed with 1 µ*M* UBA1 and 4 µ*M* E2-25K in 50 m*M* Tris–HCl pH 8.0 buffer containing 5 m*M* $MgCl_2$, 4 m*M* ATP, 1 m*M* DTT and 20 m*M* phosphocreatine. After adding creatine phosphokinase and inorganic pyrophosphatase to a final concentration of 0.6 U ml^{-1} each, the reaction mixture was incubated at 310 K. As shown in Fig. 1(*a*), the formation of Lys48-linked polyubiquitin chains from dimers was observed.

The mixture of enzymatically synthesized Lys48-linked polyubiquitins was separated into chains of distinct lengths using cation exchange (Fig. 1*b*). The pH of the reaction mixture was adjusted to

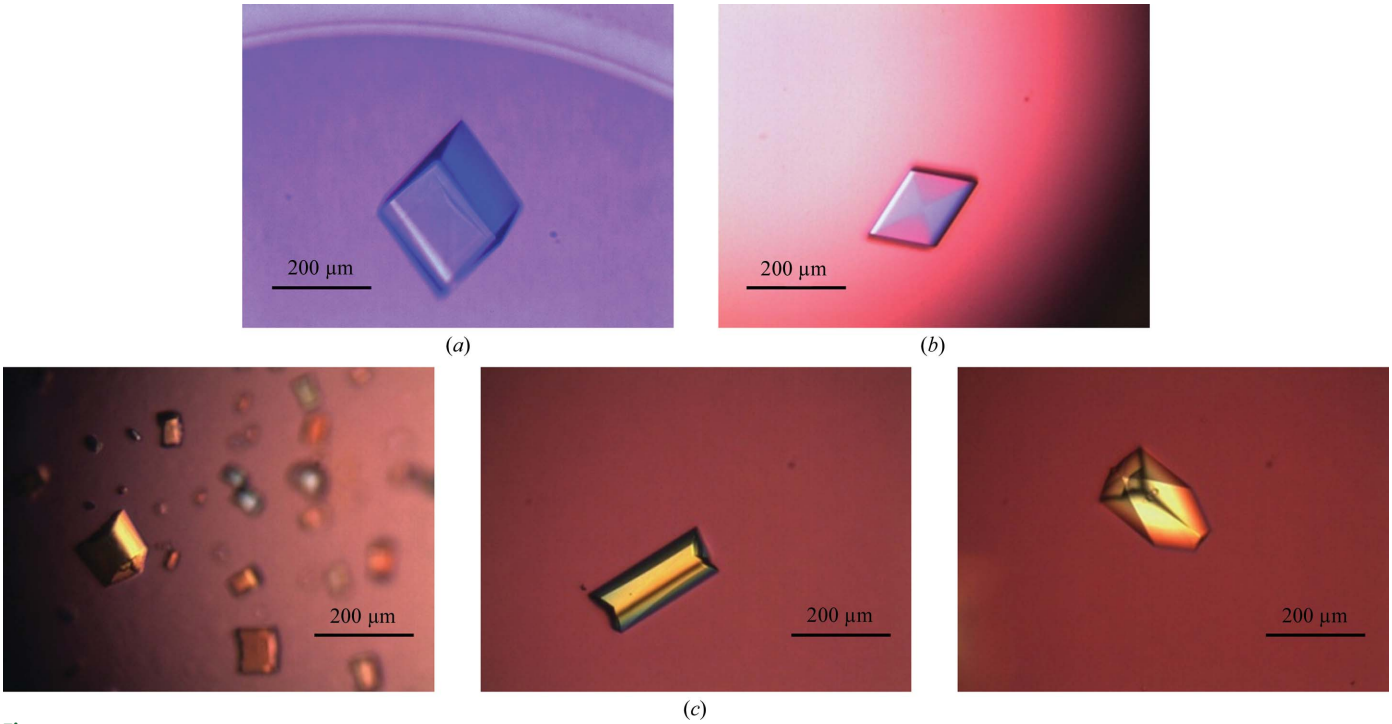

Figure 2
Crystals of polyubiquitins. (*a*) A crystal of tetraubiquitin. (*b*) A crystal of hexaubiquitin. (*c*) Crystals of octaubiquitin.

pH 4.5 in order to enhance the binding of ubiquitin chains to the ion-exchange column. Polyubiquitin chains longer than octamers were not efficiently obtained because they aggregated during the pH-adjustment step.

This protocol can be applied to the preparation of polyubiquitins with other linkages and enables us to further investigate the structural and functional properties of polyubiquitin chains in the context of the type of linkage and length.

3.2. Crystallization of Lys48-linked tetraubiquitin, hexaubiquitin and octaubiquitin

The purified Lys48-linked tetraubiquitin, hexaubiquitin and octaubiquitin concentrations were adjusted to 2.5, 6.0 or 12.5 mg ml^{-1} with 3000 molecular-weight cutoff PES filters (Sartorius Stedim) and were subjected to crystallization screening. Crystals of tetraubiquitin were grown in drops containing 2.5 mg ml^{-1} protein, 1.8 *M* ammonium sulfate, 100 m*M* citrate pH 5.4 and 4% PEG 400. Single crystals with typical dimensions of 200 × 150 × 150 µm appeared within a week at 293 K (Fig. 2*a*). Crystals of hexaubiquitin were obtained from a crystallization solution containing 12.5 mg ml^{-1} protein, 50 m*M* MES pH 6.6, 38% PEG 300 and 200 m*M* calcium acetate. Single crystals with typical dimensions of 150 × 100 × 50 µm appeared within a week at 293 K (Fig. 2*b*). The crystals of Lys48-linked tetraubiquitin and hexaubiquitin diffracted to 2.4 and 2.6 Å resolution, respectively, on an in-house X-ray source. Lys48-linked octaubiquitin was crystallized from 6 mg ml^{-1} protein solution containing 100 m*M* Tris–HCl pH 7.5, 200 m*M* ammonium sulfate and 20% PEG 4000; it was also crystallized from 3 mg ml^{-1} protein solution containing 100 m*M* Tris–HCl pH 7.5 and 8.0, 200 m*M* ammonium sulfate and 15% PEG 8000. Crystals with an oval shape appeared within a week at 293 K (Fig. 2*c*). These crystals diffracted to 8 Å resolution.

3.3. X-ray diffraction data collection of Lys48-linked tetraubiquitin and Lys48-linked hexaubiquitin

X-ray diffraction data sets were collected using a single crystal each of Lys48-linked tetraubiquitin and Lys48-linked hexaubiquitin. The crystals of Lys48-linked tetraubiquitin and Lys48-linked hexaubiquitin diffracted to 1.6 and 1.8 Å resolution, respectively, using synchrotron radiation at the Photon Factory, Tsukuba, Japan. The diffraction data sets were processed using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The crystal of Lys48-linked tetraubiquitin belonged to space group $C222_1$, with unit-cell parameters a = 58.795, b = 76.966, c = 135.145 Å. The intensity data set contained 261 653 reflections, covering 98.5% of the theoretical reflections; these reflections were obtained with an overall $R_{\rm merge}$ of 7.5% at 1.6 Å resolution. The crystal of Lys48-linked hexaubiquitin belonged to space group $P2_1$, with unit-cell parameters a = 51.248, b = 102.668, c = 51.161 Å, β = 113.419°. The intensity data set contained 146 701 reflections, covering 97.2% of the theoretical reflections; these reflections were obtained with an overall $R_{\rm merge}$ of 7.9% at 1.8 Å resolution. Crystallographic data-collection statistics are summarized in Table 2. Further structural determination is in progress and the details will be reported elsewhere.

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