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Structure of a new crystal form of tetraubiquitin

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Polyubiquitin chains, in which the C-terminus and a lysine side chain of successive ubiquitin molecules are linked by an isopeptide bond, function to target substrate proteins for degradation by the 26S proteasome. Chains of at least four ubiquitin moieties appear to be required for efficient recognition by the 26S proteasome, although the conformations of the polyubiquitin chains recognized by the proteasome or by other enzymes involved in ubiquitin metabolism are currently unknown. A new crystal form of tetraubiquitin, which has two possible chain connectivities that are indistinguishable in the crystal, is reported. In one possible connectivity, the tetraubiquitin chain is extended and packs closely against the antiparallel neighbor chain in the crystal to conceal a hydrophobic surface implicated in 26S proteasome recognition. In the second possibility, the tetraubiquitin forms a closed compact structure, in which that same hydrophobic surface is buried. Both of these conformations are quite unlike the structure of tetraubiquitin that was previously determined in a different crystal form [Cook *et al.* (1994), *J. Mol. Biol.* **236**, 601–609]. The new structure suggests that polyubiquitin chains may possess a substantially greater degree of conformational flexibility than has previously been appreciated.

1. Introduction

Ubiquitin is a compact 76 amino-acid protein that is found both as the monomer and also as covalent adducts to a wide variety of proteins, including other ubiquitin molecules. A principal role of ubiquitin is to target proteins for degradation by the 26S proteasome, a process that accounts for most turnover of abnormal and short-lived proteins in the cytosol and nucleus of eukaryotic cells (Ciechanover *et al.*, 1984; Finley *et al.*, 1984). Efficient recognition by the 26S proteasome appears to require that a substrate protein is conjugated to a chain of at least four ubiquitin moieties (Piotrowski *et al.*, 1997; Deveraux *et al.*, 1994; Thrower *et al.*, 2000), in which the principal ubiquitin–ubiquitin linkage is an isopeptide bond between the C-terminus of one ubiquitin and the ε-amino group of Lys48 in the next ubiquitin (Finley *et al.*, 1994; Spence *et al.*, 1995; Chau *et al.*, 1989; Gregori *et al.*, 1990). For reviews, see Pickart (1997), Dubiel & Gordon (1999), Hochstrasser (1996) and Hershko & Ciechanover (1998).

Several proteins are involved in the synthesis and disassembly of polyubiquitin chains. The C-terminus of monomeric ubiquitin is activated by the ubiquitin-activating (E1) enzyme and transferred to a ubiquitin-conjugating (E2) enzyme. The E2 enzyme, usually in conjunction with its cognate ubiquitin ligase (E3 enzyme), effects attachment of the ubiquitin C-terminus to a lysine side chain on the substrate protein (or another ubiquitin) by an isopeptide bond. In many cases, the E2–E3 complex may also catalyze the conjugation of additional ubiquitins through Lys48, producing a polyubiquitin chain which then targets the substrate for degradation by the 26S proteasome. In some cases, the mechanism of chain assembly may be more complex. For example, an elongation factor, E4, has been shown to allow extension of chains that would otherwise not extend beyond three ubiquitin moieties *in vitro* (Koegl *et al.*, 1999). E4 does not appear to interact directly with the E1, E2 or E3 proteins and it is proposed to function by altering the conformation or linkage of the polyubiquitin chain. Another level of enzymatic regulation is provided by families of deubiquitinating enzymes, which liberate monomeric ubiquitin from a variety of C-terminal adducts.

There is considerable interest in understanding how polyubiquitin chains are recognized by the 26S proteasome and other proteins. Crystal structures of two polyubiquitin chains have been reported previously: diubiquitin, Ub_2 (Cook *et al.*, 1992), and tetraubiquitin, Ub_4-1 (Cook *et al.*, 1994). The ubiquitin moiety retains its compact globular conformation in both Ub_2 and Ub_4-1 structures, although the flexible linkage

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(C-terminal four residues and Lys48 side chain) allows the ubiquitin moieties to adopt dramatically different relative orientations. Superposition of the first ubiquitin in each of these structures gives a relative orientation for the second ubiquitin that differs by about 120°. Consequently, linked ubiquitin moieties pack much more closely in Ub_2 than in Ub_4-1 and the two structures present radically different surfaces to potentially interacting proteins.

The existence of polyubiquitin chains linked through different lysine residues of ubiquitin, some which have been implicated in processes other than targeting to proteasomes, suggests that there must be mechanisms to allow the differentiation of various polyubiquitin chains from one another (*e.g.* Spence *et al.*, 1995; for a review, see Pickart, 1997). One potential mechanism is conformationally based. Potential conjugating lysine residues can be quite distant from one another. Therefore, chains assembled through certain lysines might present different surfaces of the ubiquitin molecule for recognition. As only limited structural data are yet available for polyubiquitin chains, the validity of this model remains to be tested.

In an effort to co-crystallize tetraubiquitin with an interacting peptide derived from a subunit of the 26S proteasome, we instead crystallized tetraubiquitin alone in a new conformation (Ub_4-2), which we report here at 2.7 Å resolution. Owing to disorder and associated absent electron density for the linkage between the second and the third ubiquitin moieties, two tetramer conformations are possible in this crystal structure, both of which are different from the previously determined Ub_4-1 structure. The new structure confirms the dramatic flexibility of the connection between successive ubiquitin moieties and reveals that hydrophobic residues known to be important for binding to the 26S proteasome can be buried in a polyubiquitin chain.

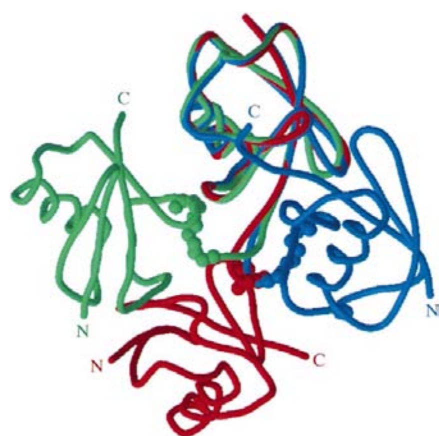

Figure 1
The flexible linkages between ubiquitin moieties. Superposition of the first ubiquitin moiety of each of the three polyubiquitin crystal structures reveals each of the second ubiquitin moieties to be in a different position. The Ub_4-2 dimer is shown in red, the Ub_4-1 dimer in green and the Ub_2 dimer in blue. In each case, the Gly76–Lys148 linkage is shown in CPK representation. The N- and C-termini of the second ubiquitin moiety in each structure are labeled. Figures were created using *MOLSCRIPT* (Kraulis, 1991).

2. Materials and methods

2.1. Crystallization

Tetraubiquitin was synthesized as described (Piotrowski *et al.*, 1997) and concentrated to 15 mg ml^{-1} in 0.5 m*M* ammonium acetate pH 4.5, 0.001 m*M* EDTA, 5 m*M* NaCl, 1 m*M* dithiothreitol. Crystals were grown at 293 K by vapor diffusion in 10 µl hanging drops comprised of equal volumes of the protein solution and a reservoir solution of 0.1 *M* sodium citrate pH 5.0, 0.4 *M* $(NH_4)_2SO_4$, 1.05 *M* Li_2SO_4. The protein solution contained 0.44 m*M* Ub_4 and 1 m*M* of a 36-residue synthetic peptide derived from the S5a polyubiquitin-binding protein of the human proteasome 19S regulatory complex (residues Met217–Gln252 of S5a; Young *et al.*, 1998). Unfortunately, analysis of washed crystals by SDS–PAGE revealed that crystallization had excluded the peptide and that crystals were composed exclusively of tetrameric ubiquitin (data not shown). Furthermore, no evidence for bound peptide was observed during the structure determination. Crystalline needles grew to full size (0.025 × 0.025 × 0.3 mm) in 4 d. We call this crystal form Ub_4-2 and refer to the previously published tetramer structure (Cook *et al.*, 1994) as Ub_4-1.

2.2. Data collection and processing

The Ub_4-2 crystals belong to space group $I4_122$, with half a tetraubiquitin chain in the asymmetric unit and a solvent content of 52% (Matthews, 1968). Prior to data collection at 90 K, the crystal was cryoprotected by brief immersion in reservoir solution brought to 20% glycerol, suspended in a rayon loop and cooled by plunging into liquid nitrogen. Data were collected at a wavelength of 1.08 Å on a MAR18 imaging-plate detector at beamline 7-1 of the Stanford Synchrotron Radiation Laboratory. Data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1995). Data-processing statistics are shown in Table 1.

2.3. Structure determination and refinement

Crystallographic calculations employed programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The scaled diffraction intensities were converted to structure factors using the program *TRUNCATE* (French & Wilson, 1978) and the Ub_4-2 structure was determined by molecular replacement using the program *AMoRe* (Navaza, 1994). Residues 1–72 of the first ubiquitin in the Ub_4-1 structure (Cook *et al.*, 1994; PDB entry 1tbe) were used as the search model. Two clear solutions fit in the asymmetric unit with a correlation coefficient of 58.4% and an *R* factor of 39.0% against data in the resolution range 3.6–8.0 Å.

The model was refined using the program *X-PLOR* (Brünger, 1996) by rigid-body, simulated-annealing, positional and *B*-factor protocols. Rounds of automated refinement were interspersed with manual rebuilding into $2F_o - F_c$ and $F_o - F_c$ omit maps using the program *O* (Jones *et al.*, 1991). A bulk-solvent correction was applied in the final rounds of refinement and map calculation. The refined model of Ub_4-2 has a free *R* factor of 29.2%, a working *R* factor of 22.4% and good geometry (Table 2). The crystallographic model includes two ubiquitin moieties, numbered 1–76 and 101–176, that are joined by an isopeptide bond between the carboxyl terminus of Gly76 and the side chain of Lys148. All of the residues in each ubiquitin moiety are well defined in electron-density maps, with the exception of the last three residues of the second ubiquitin moiety (Arg174, Gly175 and Gly176), which completely lack defined density and are not included in the final model. Although the asymmetric unit contains only two ubiquitin moieties, analysis by SDS–PAGE confirmed that the crystals are comprised of tetraubiquitin; the tetramer must therefore be formed of two adjacent asymmetric units. In discussion of possible tetraubiquitin conformations, we number residues of the third and fourth ubiquitin moieties 201–276 and 301–373, respectively.

3. Results and discussion

3.1. Structure of the two ubiquitin moieties in the asymmetric unit

The two ubiquitin moieties in the Ub_4-2 asymmetric unit are very similar to each other (RMSD = 0.87 Å on the first 73 C^{α} atoms). They are also very similar to all previously reported ubiquitin structures [Ub

(PDB code 1ubi), Vijay-Kumar *et al.*, 1987; Ub_2 (PDB code 1aar), Cook *et al.*, 1992; Ub_4-1 (PDB code 1tbe), Cook *et al.*, 1994] with RMSDs that range from 0.72 to 1.18 Å for the first 73 C^{α} atoms in each ubiquitin. The only non-covalent contacts seen between the two ubiquitin moieties of the asymmetric unit of Ub_4-2 are one hydrogen bond between Gln40 $N^{\varepsilon 2}$ and Ala146 O (3.0 Å) and one van der Waals contact between the side chains of Arg74 and Asn160 (3.4 Å between Arg74 C^{δ} and Asn160 C^{β}). These limited interactions indicate that this dimer conformation will not be maintained in solution in the absence of other contacts.

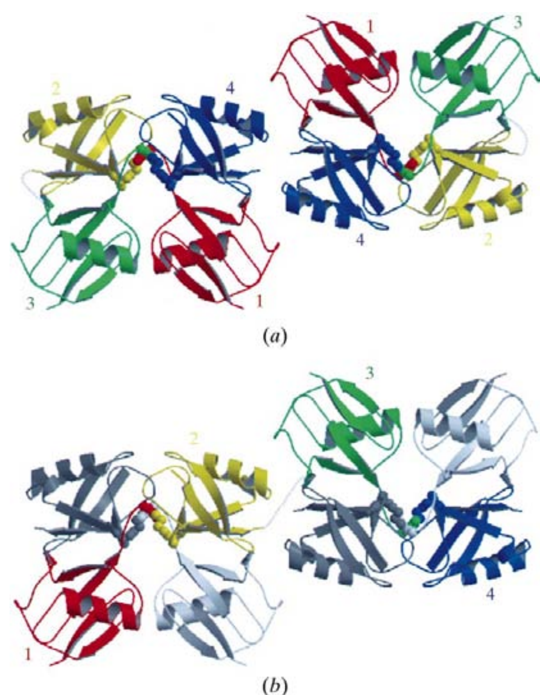

Figure 2
The Ub_4-2 conformations. These ribbon diagrams show the connectivity between ubiquitin moieties in the two tetramer models: (*a*) Ub_4-2/cl, (*b*) Ub_4-2/ex. Each ubiquitin moiety is shown in a different color, the first ubiquitin in the chain is colored red (moiety 1: residues 1–76), the second ubiquitin yellow (moiety 2: residues 101–173), the third ubiquitin green (moiety 3: residues 201–276) and the fourth ubiquitin blue (moiety 4: residues 301–373). The ordered Gly76–Lys178 and Gly276–Lys348 linkages are shown in CPK representation. Disordered connections are shown with dotted lines. (*a*) shows two adjacent Ub_4-2/cl tetramers that could be joined to form an octamer to link the blue fourth ubiquitin moiety on the left with the red first ubiquitin moiety on the right. In (*b*), the extended Ub_4-2/ex arrangement of ubiquitin moieties repeats infinitely throughout the crystal, with the blue fourth ubiquitin moiety connecting to the first ubiquitin moiety in the next tetramer (not shown). The antiparallel Ub_4-2/ex tetramer is shown in gray. Note that in the Ub_4-2 crystal the red and yellow ubiquitin moieties are crystallographically equivalent to the green and blue ubiquitin moieties.

The conformation of the Gly76–Lys48 linkage seen here is very different from those seen in the Ub_2 and Ub_4-1 structures. For both Ub_2 and Ub_4-2, the two ubiquitin moieties in the asymmetric unit (Cook *et al.*, 1992; PDB code 1aar) are related to each other by a non-crystallographic twofold axis. However, the different orientation of these twofold axes gives rise to dramatically different structures, such that whereas the Ub_4-2 asymmetric unit is extended, the two ubiquitins of Ub_2 pack very closely together to bury hydrophobic surfaces and form a number of hydrogen-bonding interactions. When the first ubiquitin moieties are superimposed, the second ubiquitin moiety in the Ub_4-2 structure is rotated by 180° relative to the position of the second ubiquitin moiety in the Ub_2 structure, revealing the dramatic inherent flexibility of this linkage (Fig. 1). Likewise, the conformation of linkages between adjacent ubiquitin moieties in Ub_4-1, which are related by a 2_1 screw axis, are quite different from that seen here for Ub_4-2. Thus, the Ub_2, Ub_4-1 and Ub_4-2 structures reveal a total of three very different relative orientations between adjacent ubiquitin moieties (Fig. 1). A wide range of other relative orientations will also be accessible in solution. However, previous NMR studies indicated that the conformation seen in the Ub_2 crystal structure is not detectably populated in solution (Lam *et al.*, 1997).

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.70–2.80 Å).

Space group	$I4_122_4$
Unit-cell parameters (Å)	
a	97.02
c	88.97
Resolution (Å)	2.70–19.92
No. of observations	27007
No. of unique reflections	5846
Mosaicity (°) (refined value)	1.2
Completeness of data (%)	96.3 (96.6)
$I/\sigma(I)$	6.0 (4.3)
R_{sym}† (%)	13.6 (38.3)

† $R_{sym} = \sum|I_i - \langle I\rangle|/\sum I_i$.

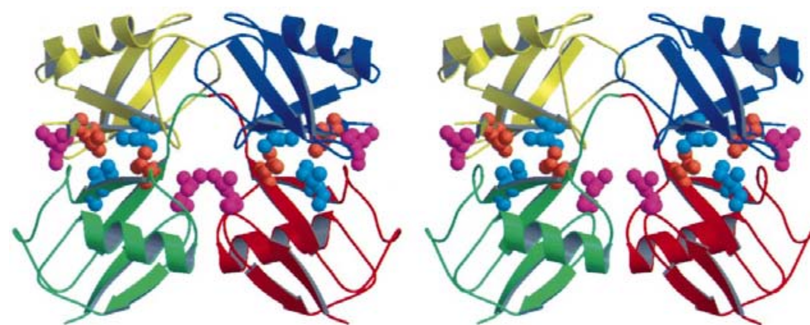

Figure 3
Stereoview of buried hydrophobic residues in the Ub_4-2/cl structure. Ubiquitin moieties are colored as in Fig. 2(*a*). Leu8, Ile44 and Val70 in each ubiquitin moiety are shown in CPK format and colored pink, orange and cyan, respectively. The Ub_2 structure, including the buried hydrophobic interface, superimposes on the second (yellow) and third (green) moieties of the Ub-2/cl structure.

3.2. Structure of tetraubiquitin in Ub_4-2 crystals

Although the Ub_4-2 crystals are formed of tetraubiquitin, the asymmetric unit contains only two crystallographically distinct ubiquitin moieties, with no electron density apparent for the disordered covalent linkage between the ubiquitin dimers of adjacent asymmetric units. We have, therefore, inferred the possible tetraubiquitin conformations in these crystals on the basis of stereochemical and spatial considerations. To make an isopeptide linkage, the third ubiquitin in the tetramer is constrained to have Lys248 C (the lysine side chain is not well ordered) within ~16 Å of the C^{α} of the last visible residue, Leu173, in the second ubiquitin moiety. There are two possible pairs of symmetry-related dimers in Ub_4-2 crystals that meet these criteria, resulting in two possible tetramer conformations: Ub_4-2/cl (closed) and Ub_4-2/ex (extended) (Figs. 2*a* and 2*b*). The Leu173 C^{α}–Lys248 C^{α} distance in Ub_4-2/cl is 10.3 Å and is 14.0 Å for Ub_4-2/ex. The disordered Arg174, Gly175 and Gly176 residues and Lys248 side chain can be reasonably built into either model. Refinement of the tetrameric Ub_4-2/cl and Ub_4-2/ex structures in the lower symmetry space group $I4_1$ did not reveal any additional electron density at the linkage between the second and third ubiquitin moieties. Therefore, the data do

Table 2
Refinement statistics.

All data were used without rejection based on the estimated standard deviations. A bulk-solvent correction was applied for the final map and *R*-factor calculations. Stereochemical criteria were defined using *PROCHECK* (Laskowski *et al.*, 1993).

R_{cryst} (%)†	22.4
R_{free} (%)‡	29.2
RMS deviations from ideal	
Bond length (Å)	0.008
Bond angle (°)	1.4
Dihedral angle (°)	26.0
Improper angle (°)	1.22
Average *B* factor, protein ($Å^2$)	41
Ramachandran plot statistics	
Residues in most favored regions (%)	92.3
Residues in additionally allowed regions (%)	6.9
Residues in generously allowed regions (%)	0.8
Residues in disallowed regions (%)	0.0

† $R_{cryst} = \sum(||F_{obs}| - |F_{calc}||)/\sum|F_{obs}|$, crystallographic *R* factor. ‡ R_{free} is the *R* factor for a selected subset (10%) of the reflections which were not included in prior refinement calculations.

not distinguish between these two very different possible tetramer structures. It is even possible that both tetraubiquitin conformations are present in different domains of the crystal.

3.3. The extended tetraubiquitin model: Ub_4-2/ex

The extended tetraubiquitin model, Ub_4-2/ex, forms a chain from alternating conformations of ubiquitin–ubiquitin linkages. One of the linkages has the conformation that is well defined in electron-density maps and is described above. The other linkage positions the disordered residues Leu73, Arg74, Gly75 and Gly76 in an extended conformation such that this tetramer conformation has almost no non-covalent inter-ubiquitin contacts and therefore will not be stable alone in solution. Although antiparallel pairs of Ub_4-2/ex chains pack together to bury a hydrophobic surface in the crystal, no evidence for dimerization or higher order aggregates of tetraubiquitin in solution has been observed by gel-filtration chromatography (C. M. Pickart, unpublished observations).

3.4. The closed tetraubiquitin model: Ub_4-2/cl

The two ubiquitin dimers that form the Ub_4-2/cl model are related to each other by an exact crystallographic twofold axis that results in numerous intimate contacts. The relationship between the second and third ubiquitin moieties of this conformation is the same as that seen earlier in the Ub_2 structure (RMSD = 1.15 Å on C^{α} atoms 101–173 and 201–273 of Ub_4-2/cl superimposed upon the Ub_2 dimer). Thus, the Ub_2 conformation can allow a longer polyubiquitin chain to make a tight 180° turn that reverses the chain direction. Because the compact Ub_4-2/cl structure is nearly a closed circular tetramer, it seems that steric constraints prohibit formation of a long polyubiquitin chain comprised exclusively of successive Ub_4-2/cl units. Thus, this conformation will be limited to short chains or local segments of longer polyubiquitin chains. One possibility is that the Ub_2 turn could link two segments of a longer polyubiquitin chain that packs against itself in the same way as for two adjacent tetraubiquitins of the Ub_4-2/ex model.

The Ub_4-2/cl conformation buries a large hydrophobic surface (Fig. 3) that includes the Leu8, Ile44 and Val70 residues of each ubiquitin moiety. Mutation of these residues results in lowered rates of degradation by the 26S proteasome *in vitro* (Beal *et al.*, 1998) and lowered affinity between tetraubiquitin and S5a, a subunit of the regulatory complex of the 26S proteasome (Beal *et al.*, 1996). Because these observations have been interpreted to imply a direct interaction between these residues and components of the 26S proteasome, the physiological relevance of the Ub_4-2/cl structure is questionable.

The most important conclusion from this study is that Lys48-linked polyubiquitin chains are inherently flexible and whereas the ubiquitin moieties themselves behave as rigid units, the connecting residues are able to adopt very different conformations. The non-covalent interactions observed between ubiquitin moieties in the various crystal structures, including the ones that we describe here, are probably relatively weak. Therefore, the different crystallographic conformations appear to be defined primarily by lattice interactions. The principal lesson from this study is that knowledge of relevant polyubiquitin conformations will probably require co-crystallization with an appropriate binding partner.

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