Structure of a new crystal form of tetraubiquitin

Cynthia L. Phillips, Julia Thrower, Cecile M. Pickart and Christopher P. Hill
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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 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, Ub₂ (Cook et al., 1992), and tetraubiquitin, Ub₄-1 (Cook et al., 1994). The ubiquitin moiety retains its compact globular conformation in both Ub₂ and Ub₄-1 structures, although the flexible linkage...
(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, bound ubiquitin moieties pack much more closely in Ub2 than in Ub4-1 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 (Ub4-2), 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, tetramer conformations are possible in this crystal structure, both of which are different from the previously determined Ub4-2 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.

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 mM ammonium acetate pH 4.5, 0.001 mM EDTA, 5 mM NaCl, 1 mM 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 (NH₄)₂SO₄, 1.05 M Li₂SO₄. The protein solution contained 0.44 mM Ub4 and 1 mM 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 Ub4-2 and refer to the previously published tetramer structure (Cook et al., 1994) as Ub4-1.

2.2. Data collection and processing

The Ub4-2 crystals belong to space group I4,22, 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-pllate 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.

3. Results and discussion

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

The two ubiquitin moieties in the Ub4-2 asymmetric unit are very similar to each other (RMSD = 0.87 Å on the first 73 Ca atoms). They are also very similar to all previously reported ubiquitin structures [Ub...
respectively. The Ub2 structure, including the buried hydrophobic interface, superimposes on the second (yellow) ubiquitin moiety on the left with the red first ubiquitin moiety on the right. In (a), the extended Ub2-2ex 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 Ub2-2ex tetramer is shown in gray. Note that in the Ub2 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 Ub2 and Ub1 structures. For both Ub2 and Ub2-2, 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 Ub2 asymmetric unit is extended, the two ubiquitins of Ub2 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 Ub2-2 structure is rotated by 180° relative to the position of the second ubiquitin moiety in the Ub2 structure, revealing the dramatic inherent flexibility of this linkage (Fig. 1). Likewise, the conformation of linkages between adjacent ubiquitin moieties in Ub2-1, which are related by a 21 screw axis, are quite different from that seen here for Ub2-2. Thus, the Ub2, Ub2-1 and Ub2-2 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 Ub2 crystal structure is not detectably populated in solution (Lam et al., 1997).

Figure 2
The Ub2-2 conformations. These ribbon diagrams show the connectivity between ubiquitin moieties in the two tetramer models: (a) Ub2-2cl, (b) Ub2-2ex. 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 101–276), 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 Ub2-2cl 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 Ub2-2ex 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 Ub2-2ex tetramer is shown in gray. Note that in the Ub2 crystal the red and yellow ubiquitin moieties are crystallographically equivalent to the green and blue ubiquitin moieties.

Figure 3
Stereoview of buried hydrophobic residues in the Ub2-2cl 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 Ub2 structure, including the buried hydrophobic interface, superimposes on the second (yellow) and third (green) moieties of the Ub2-2cl structure.

Table 1
Data-collection statistics.

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† Rmerge = Σhk − (hk)Σhk.

3.2. Structure of tetrabiubiquitin in Ub2-2 crystals

Although the Ub2-2 crystals are formed of tetrabiubiquitin, 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 tetrabiubiquitin 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 Cα of the last visible residue, Leu173, in the second ubiquitin moiety. There are two possible pairs of symmetry-related dimers in Ub2-2 crystals that meet these criteria, resulting in two possible tetramer conformations: Ub2-2cl (closed) and Ub2-2ex (extended) (Figs. 2a and 2b). The Leu173 Cα–Lys248 Cα distance in Ub2-2cl is 10.3 Å and is 14.0 Å for Ub2-2ex. The disordered Arg174, Gly175 and Gly176 residues and Lys248 side chain can be reasonably built into either model. Refinement of the tetrameric Ub2-2cl and Ub2-2ex structures in the lower symmetry space group P41 did not reveal any additional electron density at the linkage between the second and third ubiquitin moieties. Therefore, the data do
imposed upon the Ub₂ dimer). Thus, the Ub₂ conformation can allow a longer polyubiquitin chain to make a tight 180° turn that reverses the chain direction. Because the compact Ub₂-2/cl structure is nearly a closed circular tetramer, it seems that steric constraints prohibit formation of a long polyubiquitin chain comprised exclusively of successive Ub₂-2/cl units. Thus, this conformation will be limited to short chains or local segments of longer polyubiquitin chains. One possibility is that the Ub₂ 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 Ub₂-2/cl model.

The Ub₂-2/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 Ub₂-2/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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References