



Novel physicochemical properties of polyubiquitin chains

PhD Thesis
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Preface

This thesis is submitted for the degree of Doctor of Philosophy at Kyoto University. The research described herein was conducted under the supervision of Professor Masahiro Shirakawa in the Department of Molecular Engineering, Graduate School of Engineering, Kyoto University between 2010 and 2014.

I declare that except where specific reference is made to the work of others, the contents of this thesis are original. Neither this, nor any substantially similar thesis has been or is being submitted for any other degree or other qualification at any other university.

This thesis is my own work and contains nothing that is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements.

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Abstract

Ubiquitylation is one of the most well studied post-translational modifications. Several ubiquitin molecules and/or polymeric chains are covalently conjugated to substrate proteins. Conjugated ubiquitin molecules/chains are then specifically recognized by downstream proteins in cells. Ubiquitylation is related with no less extensive cellular processes such as cell cycle progression than phosphorylation; however, ubiquitin is physically much larger when compared with other non-protein modifiers such as methyl and phosphate groups. This suggests that (poly-)ubiquitylation may exert some physical influence such as increasing the molecular weight/volume and molecular anisotropy of substrate proteins. More importantly, although ubiquitin is an extremely soluble and stable protein, it is found in inclusion bodies associated with neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. To gain insight into such contradictory behaviors, it is necessary to compare the physicochemical properties of ubiquitin with those of its polymeric chains and those of ubiquitylated proteins.

First of all, a method to enzymatically synthesize polyubiquitin chains on a large-scale and to separate them by the chain length has been established. By using the method, a large amount of and highly pure ubiquitin chains with a particular chain-length are obtained, which has been confirmed by the crystallization of K48-linked tetra-, hexa- and octa-ubiquitin. Next, the thermodynamic investigations of isolated ubiquitin chains revealed that their folding stability decreases with increasing chain length, resulting in the formation of amyloid-like fibrils. Furthermore, when expressed in cells, polyubiquitin chains covalently linked to EGFP also form intracellular aggregates depending on chain length. They are then selectively degraded by macroautophagy. Finally, the fluorescence analysis indicated that a substrate protein is also destabilized thermodynamically due to ubiquitylation similarly to the folding instability of polyubiquitin chains. In addition, the NMR relaxation experiments detected the ubiquitylation-induced structural fluctuations of the protein, which might lead to the folding destabilization.

Thus, this thesis has revealed the novel physicochemical properties of ubiquitin, which has been thought of as a protein signal. The physical and chemical instabilities of polyubiquitin chains and ubiquitylated proteins, which result in aggregate formation, may be important evidences to take into account the pathological hallmarks of human sporadic proteinopathies (*i.e.*, abnormal ubiquitin-positive inclusions observed in the diseases).

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Chapter 1. General Introduction

1-1. Ubiquitin

Ubiquitin is a small eukaryotic protein of 76 amino acids (8.6 kDa) that folds into a compact β -grasp structure with a flexible C-terminal tail (Figure 1-1) (Vijay-Kumar et al., 1987a). Although researchers working in the field of protein chemistry widely use it as a model protein (Ibarra-Molero et al., 1999; Lindorff-Larsen et al., 2005), this protein has unique physicochemical and biological properties. First of all, the tertiary structure of ubiquitin is one of the most rigid among eukaryotic intracellular proteins. Ubiquitin is much more tolerant to extreme pH changes, high pressure and high temperature than most cellular proteins. Due to its high evolutionary sequence conservation: only three conservative amino acid differences between human and yeast ubiquitin (Wilkinson et al., 1986), such a rigid structure is also highly conserved among eukaryotes (Vijay-Kumar et al., 1987b). Recently, it has been reported that a certain kind of *Archaea* (*C. subterraneum*) possesses a ubiquitin-like protein modifier and its system (Nunoura et al., 2011). In addition, ubiquitin exists in any cells and organizations as its name suggests and it is quite unique that the intracellular concentration of ubiquitin is extremely high: $\sim 85 \mu\text{M}$ (Kaiser et al., 2011).

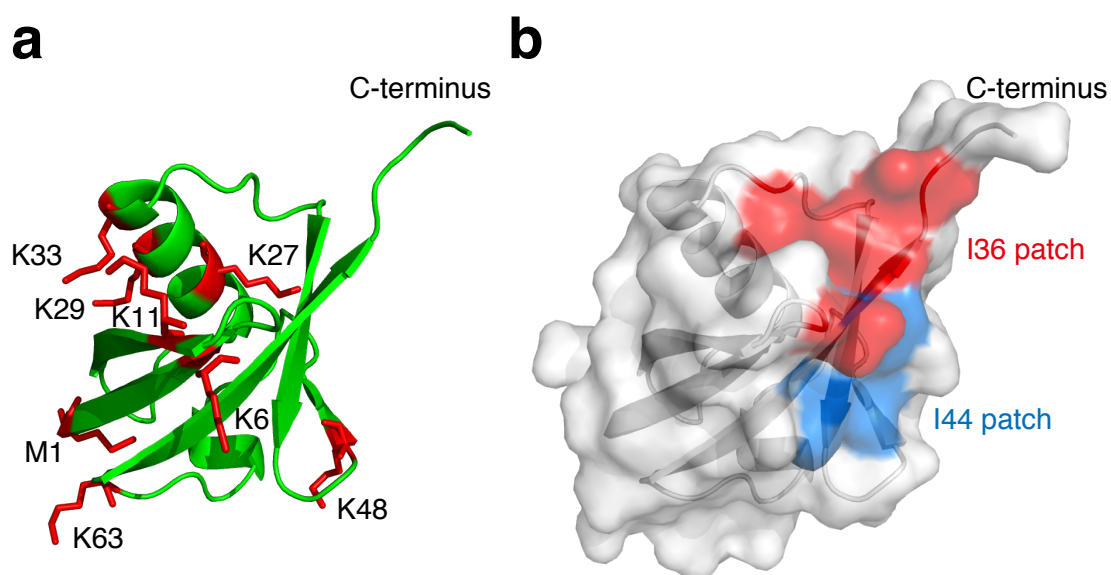


Figure 1-1. **Structure of ubiquitin.** **a**, The carboxyl group of the C-terminus of a certain ubiquitin molecule can covalently bind to the amino group of a lysine residue or the N-terminus on a substrate protein or the amino group of seven lysine residues or M1 residue on another ubiquitin molecule. **b**, Two hydrophobic patches on ubiquitin. The structures shown are based on the crystal structure of ubiquitin. PDB ID, 1UBQ (Vijay-Kumar et al., 1987a). The two structures (**a** and **b**) have the same orientation.

Ubiquitin exhibits its biological functions when it is covalently conjugated to intracellular proteins in a specific manner. The covalent conjugation of ubiquitin has been initially identified as an ATP-dependent proteolysis signal (Ciechanover et al., 1978). Ubiquitylation (the covalent modification of a protein with ubiquitin) is a successive enzymatic reaction by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligase enzymes (E3) (Figure 1-2) (Deshaies and Joazeiro, 2009; Schulman and Harper, 2009). Ubiquitin is first activated through the formation of an E1-ubiquitin thioester in an ATP-dependent manner and then transferred to an E2 enzyme via a thioester linkage. Finally, E3 enzymes mediate the formation of an isopeptide bond between a lysine residue on a substrate protein and the C-terminal tail of ubiquitin, or the formation of a peptide bond between the N-terminus of a substrate protein and the C-terminus of ubiquitin (N-terminal ubiquitylation) (Ciechanover and Ben-Saadon, 2004a). Conjugated ubiquitin molecules are recognized by downstream proteins containing a ubiquitin-binding domain (UBD) (Dikic et al., 2009) and can be removed from the substrate protein by deubiquitinating enzymes (DUBs) (Komander et al., 2009a), which counterbalance the action of the E1-E2-E3 machinery in cells (Komander and Rape, 2012). The intracellular balance between conjugation and recycling is highly regulated, and the amount of free ubiquitin molecules and unanchored chains in cells is tightly controlled (Kimura et al., 2009).

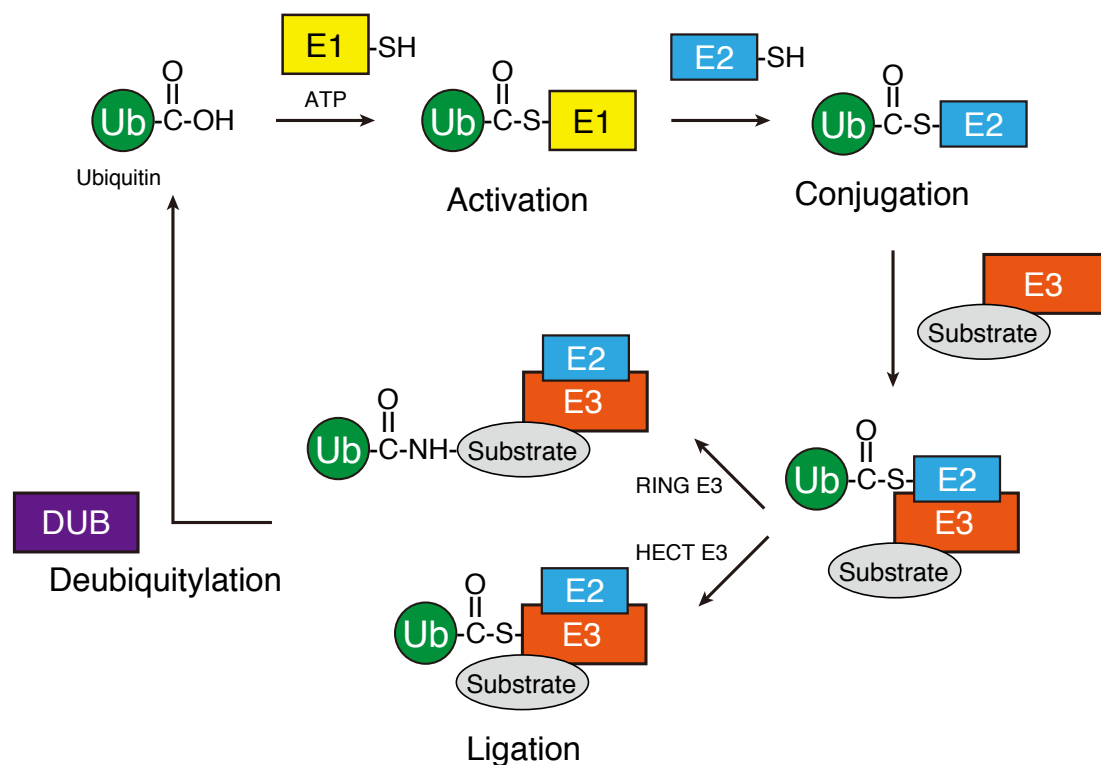


Figure 1-2. **Schematic diagram of a ubiquitylation reaction.** Free ubiquitin is activated in an ATP-dependent manner via a thioester bond between ubiquitin-activating enzyme (E1) and the carboxyl terminus of ubiquitin. The activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2), resulting in the formation of E2-ubiquitin complex through a thioester bond. Then, the ubiquitin-charged E2 associates with a specific ubiquitin ligase (E3). The E3 ligase site-specifically conjugates ubiquitin with its substrate protein in an E3-type dependent manner. RING-type E3 ligases directly transfer ubiquitin from the E2 enzyme to the substrate protein; in contrast, in the case of HECT-type E3 ligases, ubiquitin is transferred to the active-site cysteine of the E3 ligase, followed by conjugation between ubiquitin and a substrate protein (Deshaies and Joazeiro, 2009). In cells, a deubiquitinating enzymes (DUB) ubiquitin from modified proteins and/or disassemble unanchored ubiquitin chains (Komander et al., 2009a).

1-2. Ubiquitylation

Ubiquitin is attached to target proteins either as a single moiety (monoubiquitylation) or as several independent ubiquitin molecules (multi-monoubiquitylation) (Figure 1-3). Multiple ubiquitin moieties also can be covalently linked via their N-terminus (M1) or any of the seven lysine (K6, K11, K27, K29, K33, K48 and K63) residues on a given ubiquitin and the C-terminus of the next ubiquitin molecule, thereby forming polymeric ubiquitin chains (Komander and Rape, 2012). Although most ubiquitin chains in cells are homo-typic chains (possessing a single type of linkage), some chains have mixed topology if different linkages are formed at successive positions of the chain: hetero-typic chains possessing more than one linkage type (Figure 1-3) (Komander and Rape, 2012). Hetero-typic ubiquitin chains include branched ubiquitin chains (Kulathu and Komander, 2012). Furthermore, some heterologous ubiquitin modifications have also been recently observed: a modification by small ubiquitin-like modifier (SUMO) mixed with ubiquitylation (Tatham et al., 2008) and post-translational modifications of ubiquitin itself (acetylated and phosphorylated ubiquitin) (Koyano et al., 2014; Ohtake et al., 2014). Although these novel types of ubiquitylation have been reported, most populations of polyubiquitin chains in cells consist of K48-linked and K63-linked polyubiquitin chains: the population of the two linkage types in intracellular polyubiquitin chains is more than 80% (Kaiser et al., 2011).

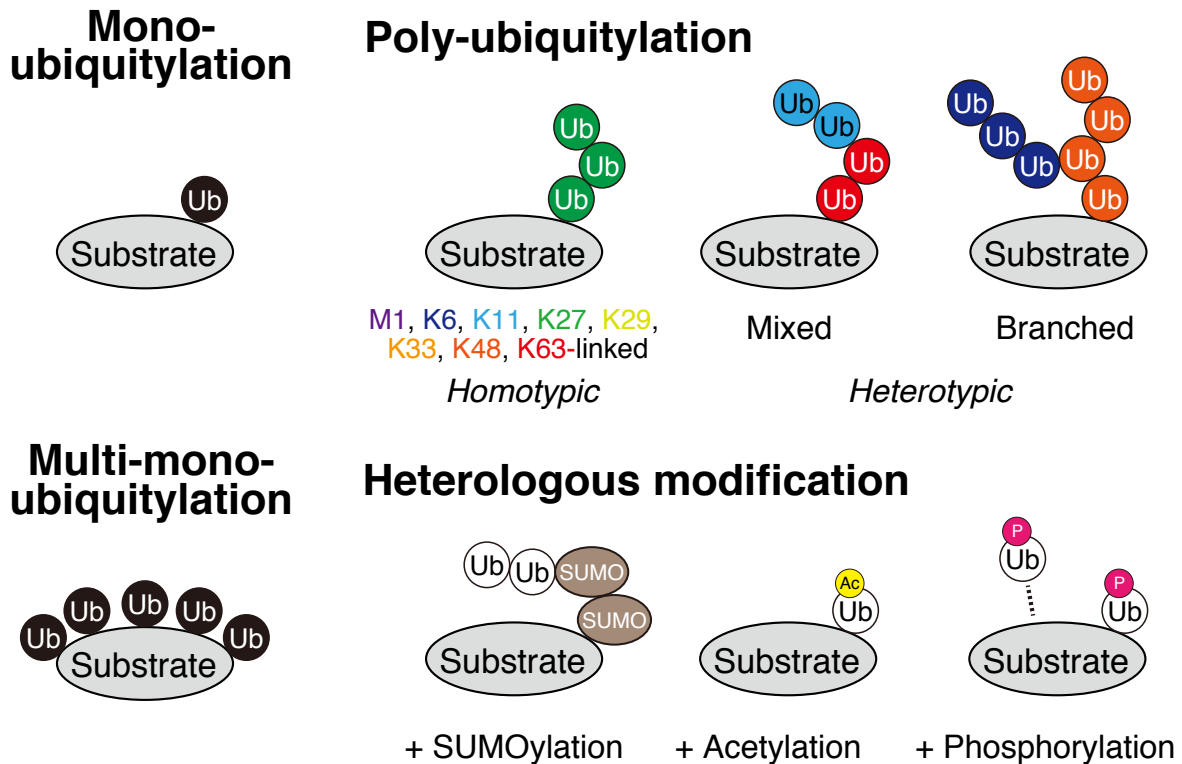


Figure 1-3. **Different modification types by ubiquitin.** Substrate proteins can be modified by mono-, multi-mono- or polyubiquitin. Ubiquitin chains are colored according to linkage-type. Poly-ubiquitylation includes modifications by a single linkage-type (homotypic) ubiquitin chain or a ubiquitin chain with more than one linkage type (heterotypic). Heterotypic chains are either non-branched or branched. Some heterologous modifications with ubiquitin have been found: a ubiquitylation mixed with conjugation of small ubiquitin-like modifier (SUMO) (Tatham et al., 2008), and acetylation (Ohtake et al., 2014) and phosphorylation of ubiquitin. In particular, phosphorylated ubiquitin seems to function as an allosteric activator for a substrate protein (Koyano et al., 2014).

1-3. Recognition of Ubiquitin

Most proteins containing UBDs interact with the two solvent-exposed hydrophobic patches centered on Ile36 in the loops between α -helix and β -strand 3, or on Ile44 in the β -strand 5 of ubiquitin (Figure 1-1). Some of the ubiquitin binding proteins are able to recognize the topology of these hydrophobic patches in polyubiquitin chains and/or the length of chains (Dikic et al., 2009; Kulathu and Komander, 2012; Winget and Mayor, 2010). The topology of ubiquitin chains depends on the chain-linkage: differently linking of ubiquitin chains results in a distinct conformation of the polymer. Five ubiquitin chain types (M1-, K6-, K11-, K48 and K63-linked ubiquitin chains) have been structurally characterized so far by using X-ray crystallography, small angle X-ray scattering (SAXS) and nuclear

magnetic resonance (NMR) spectroscopy (Figure 1-4). M1-linked and K63-linked diubiquitin form extended structures (Komander et al., 2009b), whereas the other linkage diubiquitin form compact structures by the interactions between two hydrophobic patches, the Ile36 patch and the Ile48 patch (Bremm et al., 2010; Cook et al., 1992; Virdee et al., 2010) (Figure 1-4). Importantly, the combination of solvent-exposed hydrophobic patches in polyubiquitin chains is different dependently on the chain linkage (Figure 1-4), which suggests that each linkage-type chain is specifically recognized by down-stream proteins containing UBDs (Komander and Rape, 2012; Kulathu and Komander, 2012) (Figure 1-5). In addition, a recent work suggests that ubiquitin chains have not only linkage-specific structures, but also linkage-dependent dynamics in solution (Ye et al., 2012).

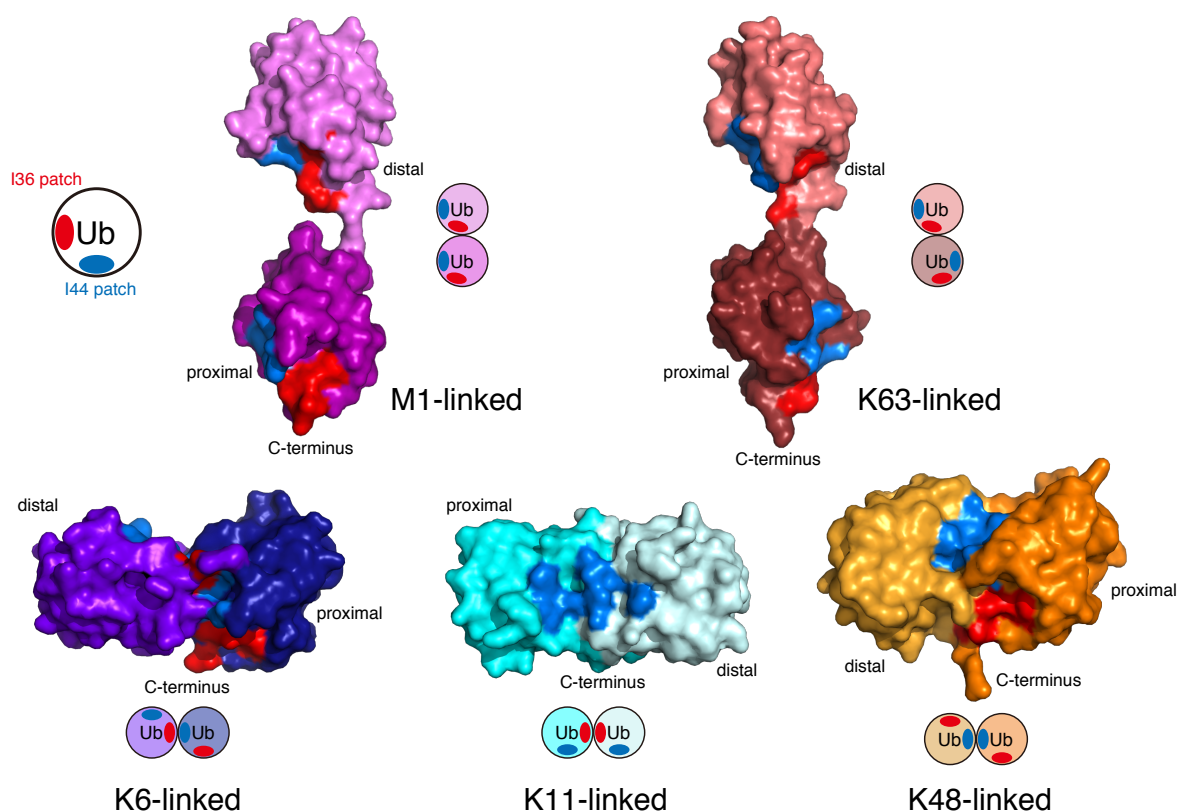


Figure 1-4. **Distinct conformations of polyubiquitin chains.** Crystal structures of M1-, K6-, K11-, K48- and K63-linked diubiquitin. Respective PDB IDs, 2W9N (Komander et al., 2009b), 2XK5 (Virdee et al., 2010), 3NOB (Matsumoto et al., 2010), 1AAR (Cook et al., 1992) and 2JF5 (Komander et al., 2009b). Their schematic diagrams are shown below each structure. The red and blue regions show hydrophobic patches centered on Ile36 and Ile44 in ubiquitin, respectively.

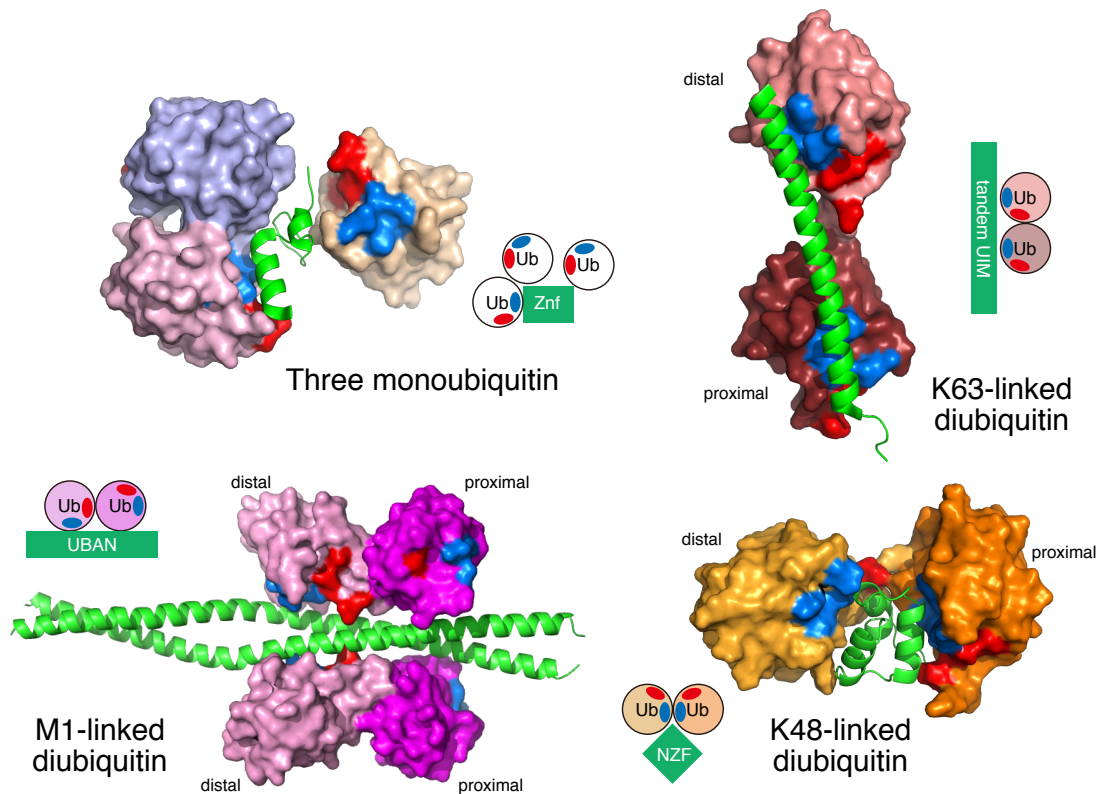


Figure 1-5. **Diverse recognitions of ubiquitylation by ubiquitin-binding proteins.** Crystal structures of the complex of monoubiquitin, M1-linked, K48-linked and K63-linked diubiquitin with specific ubiquitin-binding proteins (PDB IDs: 3OJ3, 2ZVN, 1ZO6 and 3A1Q). The schematic diagrams show that each ubiquitin-binding protein recognizes the orientation and combination of the two hydrophobic patches. As the same as Figure 1-4, the red and blue regions indicate hydrophobic patches centered on Ile36 and Ile44 in ubiquitin, respectively.

Specific recognition of monoubiquitylation and polyubiquitylation by down-stream proteins results in diversity of physiological roles of ubiquitin in cellular events (Figure 1-5). Monoubiquitylation regulates the cellular system such as endocytosis and DNA repair (Bienko et al., 2005; Hoeller et al., 2006). The biological functions of K48- and K63-linked ubiquitin chains have been most extensively characterized in various types of ubiquitin chains. K48-linked polyubiquitylation predominantly targets substrate proteins for ATP-dependent proteasomal degradation (Chau et al., 1989). K63-linked polyubiquitylation regulates non-proteolytic protein function, subcellular localization and protein-protein interactions (Dikic et al., 2009; Komander and Rape, 2012); it has also been reported to relate with proteolytic system, lysosomal degradation (Mukhopadhyay and Riezman, 2007). Recently, the cellular functions of M1-linked polyubiquitylation have been revealed: it plays

an essential role in NF- κ B activation related with inflammatory, anti-apoptosis and immune processes (Tokunaga et al., 2009). The roles of other atypical types of ubiquitin chain have not been studied in great detail, but data on their specific functions are just starting to emerge (Geisler et al., 2010; Wickliffe et al., 2011), suggesting that ubiquitylation can act as a code to store and transmit information by means of specific recognition by downstream ubiquitin-binding proteins of polyubiquitin chains and/or substrate proteins (Kulathu and Komander, 2012).

Polyubiquitin chains not only with various linkage types, but also with a wide range of lengths, often longer than a tetramer, have been identified *in vivo*; however, the length of ubiquitin chain in cells has not been precisely determined. Longer chains achieve more sufficient binding affinity by ubiquitin-binding proteins because of higher local concentration of ubiquitin molecules. Actually, the previous *in vitro* analysis for the ubiquitin chain length indicates that polyubiquitin signals with more than four units of ubiquitin are efficient for the targeting signal (Thrower et al., 2000). Moreover, the length-dependent inter-unit interactions have been observed in the crystal structures of K48-linked tetraubiquitin (Cook et al., 1994; Eddins et al., 2007). Thus, it is possible that ubiquitin-binding proteins recognize the difference of chain-length and that such a length-dependent recognition contribute to the complexity of ubiquitin intracellular functions. However, the importance of ubiquitin chain-length for cellular signaling has not been precisely characterized yet.

1-4. Ubiquitin-like Modifiers

Eukaryotes possess not only ubiquitin, but also ubiquitin-like modifiers (Ubls) including SUMO, ISG15, Atg12, and NEDD8 to exert post-translational modification of target proteins (Kerscher et al., 2006). Although the amino acid sequence similarity between each Ubl and ubiquitin is not high, all Ubls possess ubiquitin-like β -grasp structures (Hochstrasser, 2000). In addition, in a similar manner to ubiquitylation, Ubl is covalently conjugated to a lysine residue of the substrate protein via a carboxyl group of the C-terminus residue in the Ubl, a glycine, by E1-E2-E3 enzymatic cascades (Hochstrasser, 2000). Similarly to polyubiquitin chains, poly-SUMO chains have been observed both *in vitro* and *in vivo* (Fu et al., 2005; Pichler et al., 2002; Tatham et al., 2001) and poly-Nedd8 chains also have been detected *in vitro* (Ohki et al., 2009); however, their physiological functions remain unclear.

In these UbIs, physiological functions of SUMO have been most extensively studied. Many substrate proteins of SUMO in mammals are related with gene expression, transcriptional activators and repressors (Johnson, 2004). Furthermore, it has been reported that sumoylation (the covalent modification of proteins with SUMO) regulates high-order chromatin structure and chromosome segregation (Johnson, 2004). Moreover, it has been revealed that Atg12 is specifically conjugated to Atg5 and then Atg12-Atg5 conjugates drive the formation of autophagic vesicles (Kerscher et al., 2006; Ohsumi, 2001). Table 1-1 summarized physiological functions of UbIs. Although the manner of modification and structure of UbIs are quite similar to those of ubiquitin, they contribute to distinct biological processes, respectively.

Table 1-1. **Ubl family and its physiological functions** (Kerscher et al., 2006). E1, E2 enzymes and E3 ligases in each ubiquitin-like modifier are shown.

Modifier	E1 enzyme	E2 enzymes	E3 ligases	Functions
Ubiquitin	Uba1	Ubc1-8, -10, -11, Ubc13-Mms2	Many (RING and HECT families)	Substrate degradation, localization, interactions (Komander and Rape, 2012)
Smt3/ SUMO1-4	Uba2-Aos1	Ubc9	Siz1, Siz2, Mms21	Substrate localization, transcriptional regulations, chromosome organization (Johnson, 2004)
Atg12	Atg7	Atg10	–	Macroautophagy (Ohsumi, 2001)
ISG15	UbeL1	UbcH8	Herc5, Efp	Translation, glycolysis, stress response (Giannakopoulos et al., 2005)
Rub1/ NEDD8	Uba3-Ula1	Ubc12	Dcn1	Regulation of SCF complexes, transcriptional regulation of p53 (Xirodimas et al., 2004)
Atg8	Atg7	Atg3	–	Macroautophagy (Ohsumi, 2001)
Urm1	Uba4	–	–	Oxidative-stress response (Goehring et al., 2003)
UFM1	Uba5	Ufc1	–	Function unknown (Komatsu et al., 2004)
Fat10	–	–	–	Ubiquitin-independent substrate degradation (Hipp et al., 2005)
FUBI/ MNSFβ	–	–	–	Functions in T cell activation (Nakamura and Tanigawa, 2003)
Hub1	–	–	–	Pre-mRNA splicing (Wilkinson et al., 2004)

1-5. Ubiquitin-positive Inclusions

In spite of exceptional structural rigidity and high solubility *in vitro*, ubiquitin has been identified as a major component of neurofibrillary tangles (protein inclusion bodies) in various intractable diseases including cancer and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Gallo and Anderton, 1989; Lennox et al., 1989; Lennox et al., 1988; Lowe et al., 1989; Lowe et al., 1988; Mori et al., 1987; Ross and Poirier, 2004). Table 1-2 summarizes the abnormal cellular inclusions containing ubiquitin in neurological disorders.

In general, ubiquitylated proteins do not continue to accumulate in healthy cells because ubiquitylated substrates to be eliminated are supposed to be rapidly degraded by ubiquitin-proteasome system (Schrader et al., 2009). Even if ubiquitylated proteins form aggregates in cells, the aggregates are immediately and selectively degraded by macroautophagy (hereafter referred to as autophagy), in which isolation membranes engulf cytoplasmic constituents and the resulting autophagosomes fuse with lysosomes, leading to degradation of their constituents (Chapter 1-6). Therefore, the deposits of ubiquitylated proteins in cells are thought to be the result of failure to eliminate ubiquitylated substrates by the 26S proteasome or autophagy. It has been suggested that some stress factors such as reactive oxygen species and neurotoxic compounds may induce formation of such ubiquitin-positive inclusion bodies (Alves-Rodrigues et al., 1998); however, their formation mechanism and function have remained unclear ever since ubiquitin was first identified as a component of paired helical filaments in Alzheimer's disease more than 25 years ago (Mori et al., 1987). In addition, the molecular mechanism by which the aggregates are specifically degraded by autophagy is largely unknown.

Table 1-2. **Cell inclusions containing ubiquitin in diseases** (Alves-Rodrigues et al., 1998). Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CBD, corticobasal ganglionic degeneration; CJD, Creutzfeldt-Jacob disease; COFS, cerebro-oculo-facio-skeletal syndrome; DLBD, diffuse Lewy bodies disease; MND, motor neuron disease; PD, Parkinson's disease; TSE, transmittable spongiform encephalopathies.

Inclusions	Diseases	Protein components
Cytoplasmic		
Ballooned neurons	Pick's disease, CBD, AD, CJD, ALS, COFS and seizures	Neurofilaments
Dystrophic neurites	AD, PD, DLBD, MND	Neurofilaments and proteasomes
Granulovascular bodies	AD, normal aging	Neurofilaments and tau
Lewy bodies	PD, DLBD	Neurofilaments, α -crystallin, 26S proteasome
Lewy body-like inclusions	ALS	Neurofilaments
Pick bodies	Pick's disease	PHF with tau Neurons
Rosenthal fibers	Alexander's disease	α -crystallin and intermediate filaments
Endosomal/lysosomal		
Lysosome-related structures	TSE, AD	Prion proteins and HSP70
Bunina bodies	ALS	Vimentin and HSP70
Nuclear		
Intranuclear inclusion	Machado-Joseph disease, Huntington's disease	Ataxin 3, huntingtin Neurons

1-6. Autophagic Protein Degradation

Autophagy is an evolutionarily conserved lysosomal degradation pathway where cytoplasmic cargo is sequestered inside double-membrane vesicles, known as autophagosomes. This system is essential for turnover and recycling of intracellular constituents, and it plays an important role in cellular survival, starvation responses, differentiation and development (Levine and Kroemer, 2008). The autophagic pathway occurs at a low level, however, it is immediately upregulated when cells need intracellular nutrients and energy: starvation or bioenergetic demands (Levine and Kroemer, 2008). Thus, autophagy is an essential cellular event and inactivation of autophagy leads to an accumulation of both cytoplasmic protein inclusions and excess deformed organelles, which causes cancer, liver injury, diabetes, heart disease, and neurodegeneration (Mizushima et al., 2008).

The autophagic degradation has been considered to be a non-selective bulk degradation system of the cell. On the other hand, it has been recently reported that there are more specific types of autophagic degradation: selective degradation of intracellular aggregated proteins (aggrephagy), damaged mitochondria (mitophagy), and invading bacterial cells (xenophagy) (Johansen and Lamark, 2011). Autophagic adaptors, p62 and NBR1, have been characterized as substrates for such selective autophagic degradation (Bjørkøy et al., 2005; Kirkin et al., 2009a) and they play important roles in each pathway (Kirkin et al., 2009b). Under the conditions of each processes, namely aggrephagy, mitophagy and xenophagy, different proteins are ubiquitylated by distinct E3 ligases such as Chip, Parkin, and LRSAM1 (Johansen and Lamark, 2011), implying that polyubiquitin chains with distinct topology give rise to different types of selective autophagy. Actually it has been proposed that K63-linked ubiquitylation functions specifically for selective autophagy (Mukhopadhyay and Riezman, 2007). Nevertheless, in any types of these selective autophagy, ubiquitylation triggers a common transduction signal: the assembly of core Atg proteins and adaptor proteins, p62 and NBR1. In addition, insoluble polyubiquitin chains accumulating in autophagy-deficient tissues showed no ubiquitin chain linkage specificity (Riley et al., 2010). Thus, the relationship between selective autophagy and ubiquitylation remains unclear.

Chapter 2. Synthesis of Long Polyubiquitin Chains

2-1. Abstract

Polyubiquitin chains with various lengths have been identified in cells. Recently, it has been proposed that longer chains would be more significant in biological processes and that polyubiquitin chains with different lengths have distinct structural properties. Some methodologies to synthesize long polyubiquitin chains have been established so far; however, it is necessary to use some kinds of ubiquitin mutants in the methods, and their quantity and purity of products should be improved for structural studies using X-ray crystallography and/or NMR spectroscopy. In this study, a novel method to synthesize native long polyubiquitin chains on a large scale has been established. By using the customized cation exchange chromatography, K48-linked polyubiquitin chains synthesized enzymatically are separated according to chain length. In particular, the purified K48-linked tetra-, hexa- and octa-ubiquitin chains are crystalized using hanging-drop vapor-diffusion method, which suggests that the purity of the sample prepared by this novel method is appropriate for protein crystallization. Furthermore, the crystals of tetra- and hexa-ubiquitin chains diffracted to 1.6 Å and 1.8 Å, respectively.

2-2. Introduction

Ubiquitin is a highly conserved protein that can be covalently conjugated to many cellular proteins as a monomer and/or polymeric chain. A variety of polyubiquitin chains have been identified in cells: more than eight kinds of linkage types are found and ubiquitin chains with various lengths have been detected. Although the biological and structural characters driven by the chain-linkage difference have been well characterized, the role of chain-length in biological events and tertiary structures of polyubiquitin remains unclear. It has been recently suggested that longer chains would play more significant roles in the ubiquitin-proteasome degradation system (Thrower et al., 2000). In addition, several length-dependent structural properties of polyubiquitin chains have been reported. For example, the inter-unit interactions in K48-linked diubiquitin are different from those of K48-linked tetraubiquitin (Cook et al., 1994; Eddins et al., 2007; Phillips et al., 2001). The two ubiquitin in the K48-linked diubiquitin units interact with each other via the Ile44 hydrophobic interface (Cook et al., 1992); however, not only interactions between Ile44 hydrophobic patches, but also interactions between Ile36 ones have been observed in the K48-linked tetraubiquitin crystal structure (Figure 2-1). In addition, the chain-length specific structural and dynamical properties of K63-linked tetraubiquitin have also been observed (Datta et al., 2009).

To reveal these length-dependent properties of polyubiquitin chains, several methodologies to synthesize the chains with different chain-lengths have been constructed so far (Chen and Pickart, 1990; Pickart and Rose, 1985; Piotrowski et al., 1997). Using these methods, it is possible to synthesize long polyubiquitin chains and to obtain the chains with a certain chain-length, however, their methods need to use some kinds of ubiquitin mutants (mutants at a particular lysine residue and the C-terminal residue) and their protocols have many experimental steps. More importantly, their quantity and purity of products should be improved for structural studies using X-ray crystallography and/or NMR spectroscopy because their structural studies need a large amount of pure samples. Therefore, in this study, a novel length-dependent separation method for native ubiquitin chains has been established. By using this method, it is easier to synthesize polyubiquitin chains with a certain chain-length on a large-scale and with high purity.

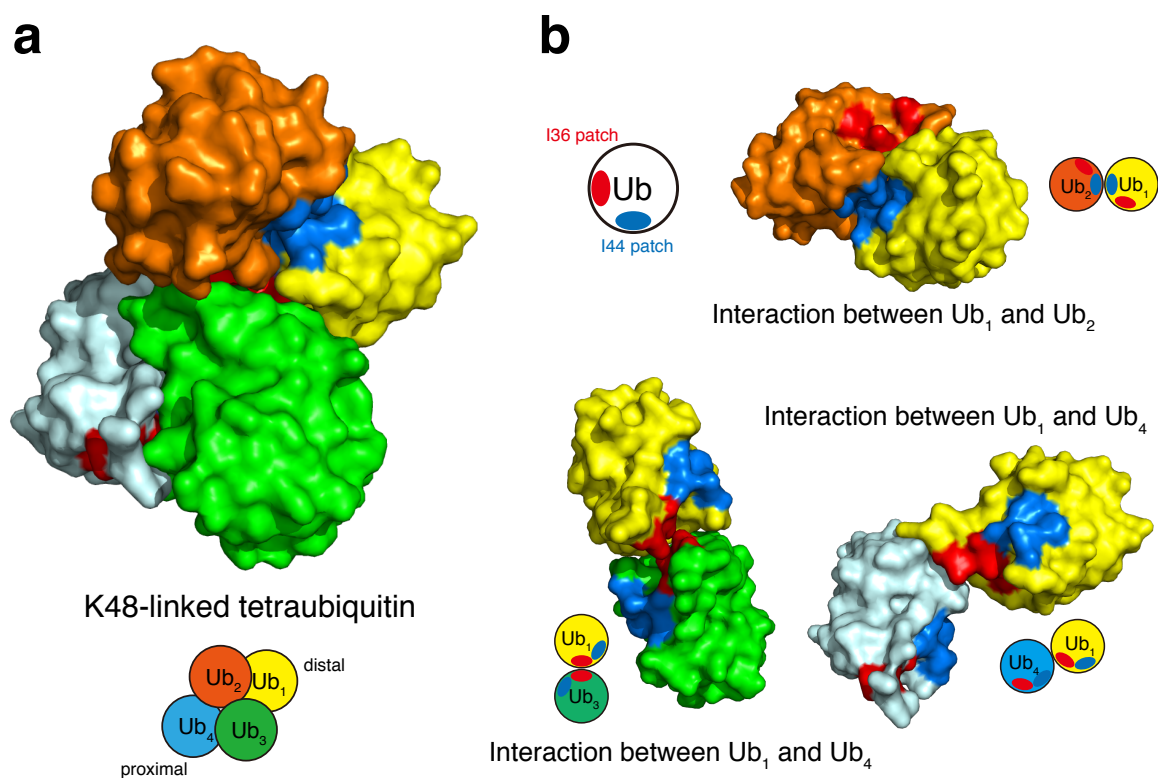


Figure 2-1. Conformation of K48-linked tetraubiquitin and its intra-molecular interactions. **a**, The crystal structure of K48-tetraubiquitin. PDB ID, 2O6V (Eddins et al., 2007). Its schematic diagram is shown below. The C-terminal unit is tagged as number 1 and colored in yellow. The conformation of K48-linked tetraubiquitin is a dimer of diubiquitin: it consists of one diubiquitin (Ub₁:Ub₂) and another diubiquitin (Ub₃:Ub₄). **b**, Intra-molecular interactions in K48-linked tetraubiquitin. Both conformations of the diubiquitin (Ub₁:Ub₂ and Ub₃:Ub₄) are similar to that of diubiquitin reported previously as PDB ID, 1AAR (Cook et al., 1992). The ubiquitin units in each structure of the diubiquitin interact with each other via the interface containing the Ile44 hydrophobic patch (upper), which is quite similar to the crystal structure of K48-linked diubiquitin (Figure 1-4). In contrast, the Ub₁ unit interacts with the Ub₃ unit via the interface containing Ile36 hydrophobic patch (lower left). The red region and blue one indicate hydrophobic patches centered on Ile36 and Ile44 in ubiquitin, respectively.

2-3. Large-scale Preparation of Ubiquitin-activating Enzymes

In some methods established previously (Chen and Pickart, 1990; Pickart and Rose, 1985; Piotrowski et al., 1997), ubiquitin-activating (E1) enzymes have been purified from mammalian reticulocytes, erythrocytes or liver extracts. For stable and large-scale preparation, it is necessary to construct a method to overexpress recombinant E1 enzymes. Herein, mouse E1 enzymes (mUBA1) have been overproduced in insect cells (SF9 cells). The cDNA of mUBA1 was cloned from the mouse kidney-derived cDNA library. The DNA fragments of the mUBA1 gene with specific sequences recognized by restriction enzymes were amplified using four pairs of primers by polymerase chain reaction (PCR) (Table 2-1). The PCR-synthesized mUBA1 DNA fragments were sub-cloned into pFastBacI baculovirus transfer vector (Invitrogen) with a hexa-histidine tag.

Table 2-1. **Primers used for PCR synthesis of the mUBA gene.** Restriction sites are underlined.

Primer	Sequence	Site
Forward 1	GG <u>CATATG</u> TCCAGCTCGCCGCTGTC	<i>NdeI</i>
Reverse 1	<u>ACTAGTTT</u> CCGAATAAGGTCTTCATCCAAGC	<i>SpeI</i>
Forward 2	<u>GCTAGCTT</u> ATGTTGCTGCTGGGG	<i>NheI</i>
Reverse 2	<u>AGATCTTG</u> GCTAGAGCTGTAGGATTCTG	<i>BglII</i>
Forward 3	<u>GGATCC</u> ACCAGAGAAATCCATCCC	<i>BamHI</i>
Reverse 3	<u>GGATCC</u> AGTCAACCCATAGGTCTG	<i>BamHI</i>
Forward 4	<u>AGATCT</u> CAAGACCGAGCTGCTGTG	<i>BglII</i>
Reverse 4	GG <u>CTCGAG</u> TTATCAGCGAATGGTATATCGGACATAAGGG	<i>NdeI</i>

The baculovirus coding mUBA1 was transfected into Sf9 cells. Transfected cells were cultured in serum-free insect cell media (SF900-II) for three days and were harvested by centrifugation at 1,000 g for 10 minutes. The cells were resuspended and sonicated with the lysis buffer containing 50 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM DTT, 1% tritonX-100, 10 mM NaF, 10 mM disodium hydrogen phosphate and 10 mM sodium pyrophosphate. After centrifugation at 48,384 g for 20 minutes, the supernatant was loaded into 10 ml of Ni-NTA-agarose beads (QIAGEN) with the Ni-NTA buffer containing PBS, 300 mM NaCl and 10 mM β -mercaptoethanol, pH 7.4. After washing with the Ni-NTA buffer containing 0-60 mM

imidazole-HCl, mUBA1 was eluted by increasing the concentration of imidazole-HCl from 80 to 300 mM in Ni-NTA buffer for one column volume, respectively (Figure 2-2). The eluted proteins were dialyzed with the buffer containing 50 mM Tris-HCl, 500 mM NaCl and 1 mM DTT, pH 8. An approximate 8 mg of mUBA1 was yielded from a 300 ml culture.

2-4. Large-scale Preparation of Polyubiquitin Chains

K48-linked polyubiquitin chains were enzymatically produced *in vitro* by using wild type ubiquitin, recombinant E1 (mUBA1) and E2 enzymes (E2-25K). For ubiquitylation, 1.2 mM ubiquitin was mixed with 1 μ M mUBA1 and 4 μ M E2-25K in a 50 mM Tris-HCl pH 8.0 buffer containing 5 mM MgCl₂, 4 mM ATP, 1 mM DTT and 20 mM phosphocreatin. After adding creatin phosphokinase and inorganic pyrophosphatase at a final concentration of 0.6 unit/ml each, the reaction solution was incubated overnight at 310 K. Then, the formation of K48-linked polyubiquitin chains from dimers was observed (Figure 2-2).

Enzymatically synthesized K48-linked polyubiquitin chains were separated by each chain length using the customized cation exchange. First, pH of the reaction mixture was adjusted to pH 4.5 in order to enhance binding of ubiquitin chains to the cation exchange column. Long polyubiquitin chains with more than 8 ubiquitin units were not efficiently obtained because they aggregated during such a pH adjustment step. The adjusted polyubiquitin chains were applied into the column equipped with 15 ml of cation exchange Source 15S (GE Healthcare) beads in 50 mM sodium acetate buffer, pH 4.5 containing 1 mM DTT. Bound ubiquitin chains were eluted by increasing salt concentration using the two sequential linear gradients (Figure 2-3): a steeper gradient (100-200 mM NaCl in 6 column volumes) and a shallow gradient (200-290 mM NaCl in 40 column volumes). This protocol can be applied to preparation of polyubiquitin chains with other linkages. Actually, K63-linked and linear polyubiquitin chains have been separated by the chain length using this method similarly to K48-linked polyubiquitin chains.

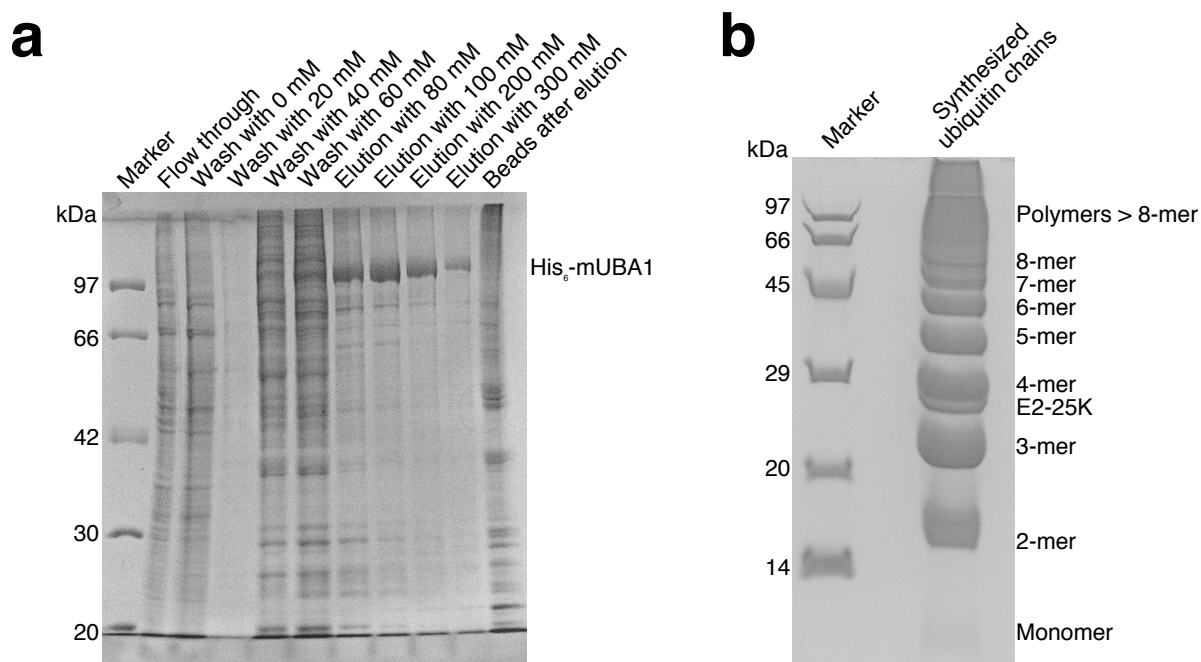


Figure 2-2. **Purification of mUBA1 overexpressed in SF9 cells and synthesis of K48-linked polyubiquitin chains by using recombinant mUBA1.** **a**, Ni-NTA purification of mUBA1. The concentrations indicated are imidazole-HCl concentrations. All experiments were performed at 277 K. **b**, *In vitro* K48-linked polyubiquitin chains synthesis.

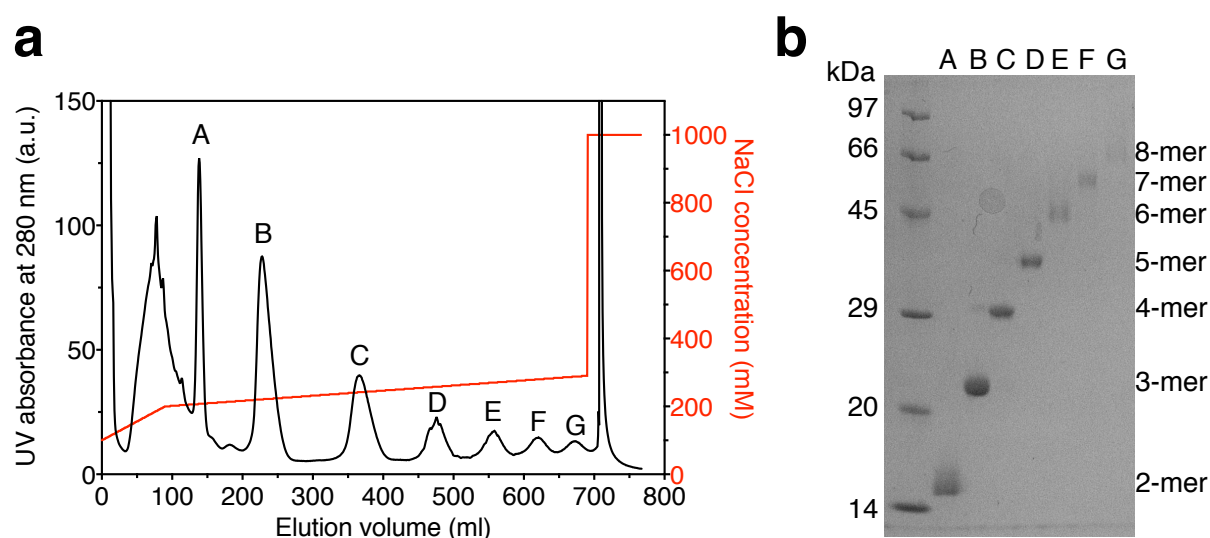


Figure 2-3. **Separation of K48-linked polyubiquitin chains by the chain length.** **a**, Isolation of polyubiquitin chains with distinct chain length by cation exchange chromatography. The black curves display UV absorption at 280 nm and the red line displays the concentration of NaCl in the buffer. The NaCl concentration gradients for elution consist of the two sequential gradients: a steeper gradient (100-200 mM NaCl in 6 column volumes) and a shallow gradient (200-290 mM NaCl in 40 column volumes). **b**, Polyubiquitin chains obtained at the peak indicated in the purification profile (**a**) are examined with a 15% SDS-PAGE gel. A, diubiquitin; B, triubiquitin; C, tetraubiquitin; D, pentaubiquitin; E, hexaubiquitin; F, heptaubiquitin; G, octaubiquitin.

2-5. Crystallization of K48-linked Polyubiquitin Chains

Purified K48-linked tetraubiquitin, hexaubiquitin and octaubiquitin were concentrated to 2.5 mg/ml, 6.0 mg/ml or 12.5 mg/ml with 3,000 MWCO PES filters (Sartorius Stedim), respectively. Then, they were subjected to crystallization screening. Crystals of K48-linked tetraubiquitin were grown in drops containing 2.5 mg/ml protein, 1.8 M ammonium sulfate, 100 mM citrate and 4 % PEG400, pH 5.4. Single crystals with dimensions of $200 \times 150 \times 150 \mu\text{m}^3$ appeared within a week at 293 K (Figure 2-4a). Crystals of K48-linked hexaubiquitin were obtained from a crystallization solution containing 12.5 mg/ml protein, 50 mM MES, 38 % PEG300 and 200 mM calcium acetate, pH 6.6. Single crystals with dimensions of $150 \times 100 \times 50 \mu\text{m}^3$ were appeared within a week at 293 K (Figure 2-4b). The crystals of K48-linked tetraubiquitin and hexaubiquitin diffracted up to 2.4 Å and 2.6 Å resolutions, respectively, on an in-house X-ray source. Purified K48-linked octaubiquitin was also crystallized in drops containing 6 mg/ml protein, 100 mM Tris-HCl, 200 mM ammonium sulfate and 20 % PEG4000, pH 7.5; drops containing 3 mg/ml protein 100 mM Tris-HCl, 200 mM ammonium sulfate and 15 % PEG8000, pH 7.5 and 8.0. These crystals with an oval shape appeared within a week at 293 K (Figure 2-4c). These crystals diffracted up to 8 Å resolution on an in-house X-ray source.

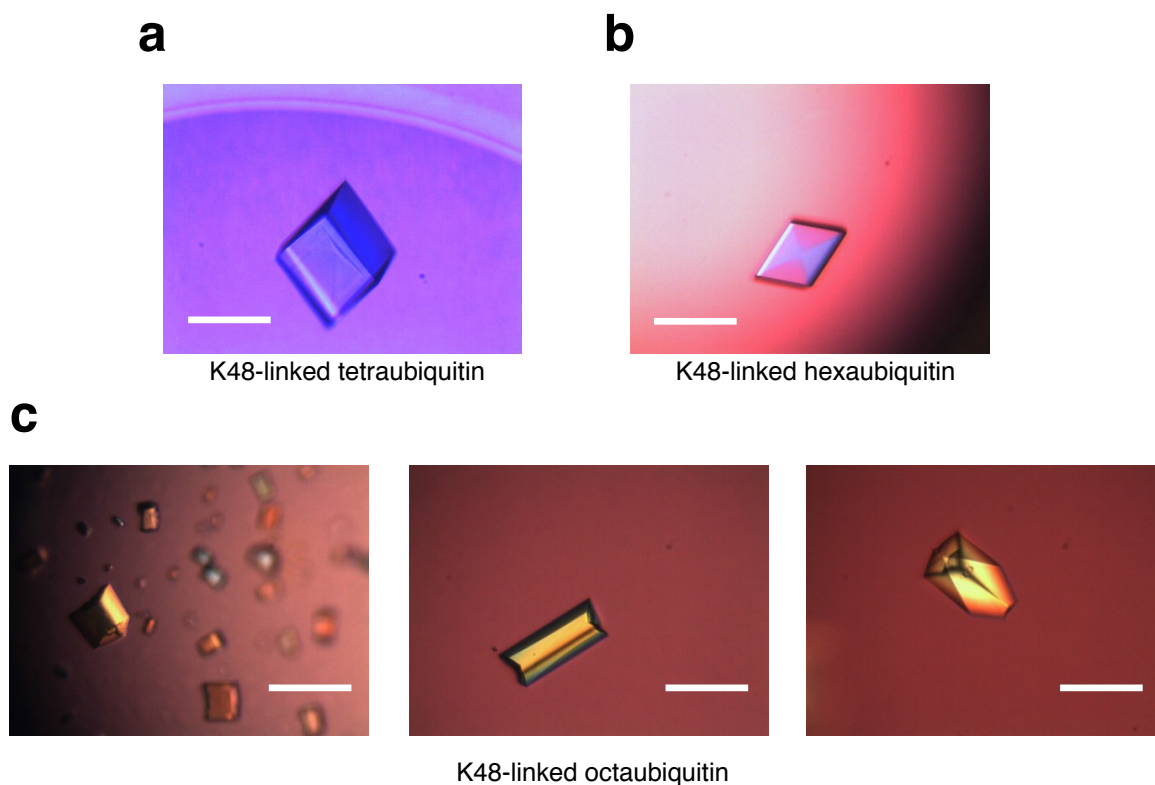


Figure 2-4. **Crystals of K48-linked polyubiquitin chains.** **a**, A crystal of K48-linked tetraubiquitin. Crystallization condition: protein concentration: 2.5 mg/ml; buffer: 1.8 M ammonium sulfate, 100 mM citrate and 4 % PEG400, pH 5.4; temperature: 293 K. **b**, a crystal of hexaubiquitin. Crystallization condition: protein concentration: 12.5 mg/ml protein; buffer: 50 mM MES, 38 % PEG300 and 200 mM calcium acetate, pH 6.6; temperature: 293 K. **c**, crystals of octaubiquitin. Crystallization condition (left): protein concentration: 6 mg/ml; buffer: 100 mM Tris-HCl, 200 mM ammonium sulfate and 20 % PEG4000, pH 7.5; temperature: 293 K. Crystallization condition: protein concentration: 3 mg/ml; buffer: 100 mM Tris-HCl, 200 mM ammonium sulfate and 15 % PEG8000, pH 7.5 (middle) and 8.0 (right); temperature: 293 K. Bars, 200 μ m.

2-6. X-ray Diffraction Data of Tetra-and Hexaubiquitin Crystals

X-ray diffraction data sets were collected by a single crystal of K48-linked tetra- and hexaubiquitin, respectively. The crystals of K48-linked tetra- and hexaubiquitin diffracted to 1.6 Å and 1.8 Å with synchrotron radiation at Photon Factory, Tsukuba, Japan. Each diffraction data set was processed using the program *HKL2000* (Otwinowski and Minor, 1997). The crystal of K48-linked tetraubiquitin belongs to space group $C222_1$ with unit cell dimensions $a = 58.795$, $b = 76.966$, and $c = 135.145$ Å. The intensity dataset contains 261,653 reflections, which cover 98.5 % of the theoretical reflections; these reflections were obtained with an overall R_{merge} of 7.5 % at 1.6 Å. The crystal of K48-hexaubiquitin belongs to space group $P2_1$ with unit cell dimensions $a = 51.248$, $b = 102.668$, $c = 51.161$ Å, and $\beta = 113.419^\circ$. The intensity dataset contains 146,701 reflections, which cover 97.2% of the theoretical reflections; these reflections were obtained with an overall R_{merge} of 7.9 % at 1.8 Å resolution. Table 2-2 summarized the crystallographic data collection statistics. These crystallographic results indicate that purity and quantity of the polyubiquitin chains purified by the novel purification method are enough not only for structural experiments such as X-ray diffraction data collections, but also for other biological and/or physicochemical experiments.

Further structural determination has been performed in collaboration with Dr. Isogai. Although native K48-linked tetraubiquitin forms a dimer of diubiquitin, the conformation of native K48-linked hexaubiquitin is a dimer of triubiquitin (data not shown). The crystal structure of native K48-linked tetraubiquitin that has been solved in this study is almost the same as that of K48-linked tetraubiquitin consisting ubiquitin mutants (Cook et al., 1994; Eddins et al., 2007). Therefore, not only interactions between Ile44 hydrophobic patches, but also ones between Ile36 patches have been observed in this K48-linked tetraubiquitin crystal

structure, similarly to the reported structure (Figure 2-1). In contrast, these inter-unit interactions have not been observed in K48-linked hexaubiquitin. K48-linked hexaubiquitin forms a dimer of triubiquitin, which contains novel inter-unit interactions. In the triubiquitin, the Ile36 hydrophobic path in a ubiquitin unit interacts with the Ile44 hydrophobic patch in the next ubiquitin unit; however, the interface is slightly different from the interface between the K6-linked diubiquitin where the Ile36 patch interacts with Ile44 one (Figure 1-4). In addition, there is little contact between the two triubiquitin in K48-hexaubiquitin. Thus, these results suggest that inter-unit interactions of K48-linked polyubiquitin chains are not the same among these different length chains. The details will be reported in the near future.

Table 2-2. Data collection statistics for K48-linked tetra- and hexaubiquitin crystals.

	K48-linked tetraubiquitin	K48-linked hexaubiquitin
Wavelength (Å)	1.0000	1.0000
Space group	$C222_1$	$P2_1$
Unit-cell parameters (Å, °)	$a = 58.795, b = 76.966, c = 135.145,$ $\alpha = 90, \beta = 90, \gamma = 90$	$a = 51.248, b = 102.668, c = 51.161,$ $\alpha = 90, \beta = 113.419, \gamma = 90$
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)

2-7. Discussion

In this chapter, the novel method to synthesize native polyubiquitin chains with distinct chain lengths has been established. Because this method uses native ubiquitin and recombinant enzymes, it is possible to prepare polyubiquitin chains with a certain chain length on a large scale. The purified polyubiquitin chains have both appropriate quality and quantity for the structural studies. For this purification of polyubiquitin chains, it is important to fine-tune the salt (NaCl) concentration gradients in cation exchange chromatography (Figure 2-3a). The appropriate combination of sequential gradients contributes to separation of polyubiquitin

chains with distinct chain lengths. In particular, the adjustment may be critical in separation of longer polyubiquitin chains because there are less electrostatic differences between them, compared with shorter ubiquitin chains. Moreover, because electrostatic profiles of ubiquitin chains depend on the chain linkage type (Figure 1-4), more precise constructions of salt concentration gradients would be necessary for ubiquitin chains other than K48-linked ones.

Furthermore, because this purification method utilizes molecular electrostatic differences, this will also be available for polymers of ubiquitin-like modifiers (Ubls) such as poly-SUMO chains and for polymeric biomolecules. Although Ubl polymers (poly-SUMO or poly-Nedd8 chains) have been observed so far (Fu et al., 2005; Ohki et al., 2009; Pichler et al., 2002; Tatham et al., 2001), their conformations have not been investigated and remained unclear. In addition, it would be possible to purify polymeric molecules that are difficult to purify by liquid chromatography in organic solvents; for example, microtubules or DNA-based polymers (nucleosomes). Thus, this purification method using several sequential salt concentration gradients will be useful for future polymeric biomolecule researches.

Chapter 3. Polyubiquitin Fibril Formation

3-1. Abstract

Ubiquitin is known to be one of the most soluble and stably folded intracellular proteins. However, ubiquitin has been often found in inclusion bodies associated with many intractable diseases including neurodegenerative disorders and cancer. To gain insight into this contradictory behavior, the physicochemical properties of ubiquitin and its polymeric chains have been examined and compared with each other. Unexpectedly, the folding stability of ubiquitin chains decreases with increasing chain length. Furthermore, this instability of ubiquitin chains results in their formation of amyloid-like fibrils. Not only polymerization of ubiquitin itself, but also ubiquitylation of substrate proteins causes chain-length-dependent fibril formation. When expressed in cells, polyubiquitin chains covalently linked to EGFP also form aggregates in a chain-length dependent manner. Remarkably, these aggregates are selectively degraded by macroautophagy. These results propose a novel model in which the physical and chemical instability of polyubiquitin chains drives the formation of fibrils, which then serve as an initiation signal for autophagy.

3-2. Introduction

Ubiquitin has an exceptional structural rigidity and high solubility *in vitro*. In fact, ubiquitin is tolerant to high temperature, high pressure and extreme pH changes. In contrast to these properties, ubiquitin has been found as a component of intracellular aggregates in diverse neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Table 1-2). Therefore, the *in vitro* character of ubiquitin cannot explain that the ubiquitin-positive inclusion bodies are the hallmark of their diseases. Importantly, in cells, ubiquitin exists not only in a monomeric form, but also in a polymeric or substrate-conjugated form; this aspect suggests that the high rigidity of mono-ubiquitin would be affected by conjugation to another ubiquitin molecule and/or other proteins. However, the physicochemical influence on ubiquitin by polymerization and conjugation to a substrate protein has not been investigated so far. Moreover, the formation mechanism and function of ubiquitin-positive aggregates have remained unclear since ubiquitin was first found in the paired helical filaments in Alzheimer's disease more than 25 years ago. Although ubiquitin regulates a variety of cellular events in a linkage-type-specific manner, the ubiquitin-positive aggregates observed in autophagy-deficient cells contain all linkage types of ubiquitin chains (Riley et al., 2010); this observation suggests that the driving force to form intracellular ubiquitin-positive aggregates would be independent of linkage-type-specific properties of ubiquitin chains.

In addition, intracellular aggregates containing ubiquitylated protein are suggested to be selectively degraded by autophagy (Komatsu et al., 2006) and loss of autophagic functions in the nervous system results in neurodegeneration, which has often been observed in Alzheimer's disease or Parkinson's disease. Therefore, the autophagic degradation of ubiquitin-positive aggregates is essential for cell homeostasis, especially for the neurons, even though there would be not any pathological mutations or additional environmental stress. However, how ubiquitin-positive aggregates form in cells and how autophagy recognizes these cargos have been largely unclear.

This chapter aims to elucidate the possibility that ubiquitylation (polymerization of ubiquitin and conjugation of ubiquitin with other proteins) has effect on physical and chemical property of ubiquitin. Here, the thermodynamic stability of ubiquitin is found to decrease when ubiquitin chains are elongated, regardless of their linkage types. This results in formation of amyloid-like fibrils, which then act as an intracellular signal for clearance by macroautophagy.

3-1. Longer Ubiquitin Chains Have Lower Thermodynamic Stability

First of all, to clarify whether ubiquitin polymerization affects thermodynamic stability of ubiquitin, the differential scanning calorimetry (DSC) analysis compared the stability of monoubiquitin with that of a polyubiquitin chain with distinct chain length. As reported previously (Wintrode et al., 1994), monoubiquitin is highly heat-stable; actually, the DSC measurement showed that its transition temperature is 368 K (Figure 3-1, Figure 3-2 and Figure 3-3). In contrast, all three types of polyubiquitin chains [Met 1 (linear), Lys 48 (K48) or Lys 63 (K63)-linked polyubiquitin chains] have a transition temperature that is more than 5 K lower (Figure 3-1a, b and c) than that of monoubiquitin. More interestingly, a longer chain has a lower transition temperature regardless of the linkage type (Figure 3-1d). Although K48-linked polyubiquitin chains are more heat-stable than K63-linked and linear polyubiquitin chains, any linkage types of hexaubiquitin have a transition temperature that is more than 15 K lower than that of monoubiquitin (Figure 3-1d). In addition, there is few thermodynamic difference between enzymatic synthesized and recombinant linear polyubiquitin chains (Figure 3-2). Thus, the stability of polyubiquitin chains significantly decreases with increasing chain length.

In addition, the DSC experiments revealed that polymerization of ubiquitin affect thermal folding reversibility. Monoubiquitin is soluble at and above the transition temperature (Figure 3-3a), which indicates that monoubiquitin is a thermally reversible protein. On the other hand, any linkage types of polyubiquitin chains form insoluble small aggregates above their transition temperatures and remain insoluble even when the temperature decreases below their transition temperatures (Figure 3-3b, c and d). These observations show that polymerization of ubiquitin molecules, namely conjugation of ubiquitin to another ubiquitin molecule, impairs the ability to refold into a native structure from a denatured form by heat denaturation. Taken together, ubiquitin form insoluble aggregates by conjugation of another ubiquitin molecule and the addition of further ubiquitin molecules results in aggregate formation of ubiquitin chains at even lower temperatures.

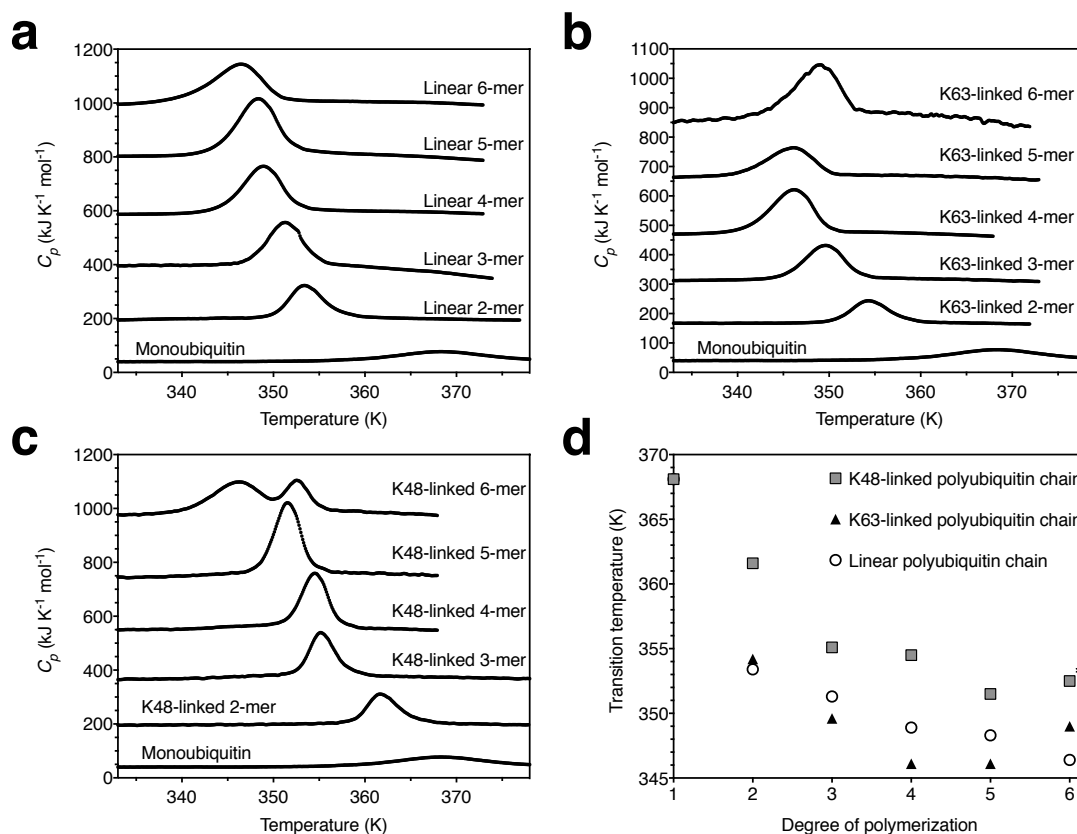


Figure 3-1. Comparative analysis of the thermodynamic stability for polyubiquitin chains of different length. Differential scanning calorimetry traces of monoubiquitin and linear polyubiquitin chains (a), K63-linked polyubiquitin chains (b) and K48-linked polyubiquitin chains (c) with up to six ubiquitin units. d, Transition temperatures are plotted against chain length for K48-linked, K63-linked and linear ubiquitin chains. (*In the thermal denaturation of K48-linked hexaubiquitin, the higher transition temperature is selected.)

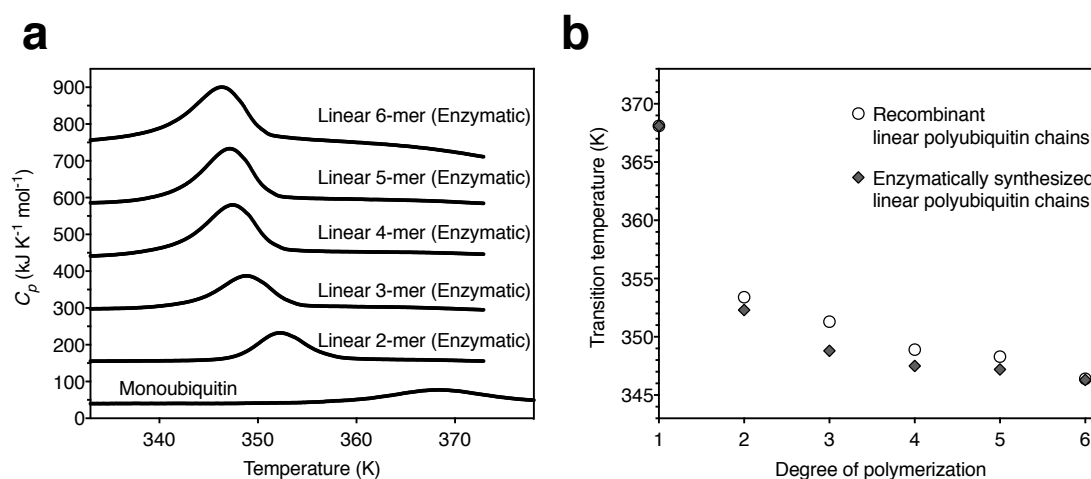


Figure 3-2. Comparative analysis of thermal stability for enzymatically synthesized polyubiquitin chains of different length. a, Differential scanning calorimetry traces of monoubiquitin and enzymatically synthesized linear polyubiquitin chains with up to six ubiquitin units. b, Transition temperatures are plotted against chain length for recombinant and enzymatically synthesized linear ubiquitin chains.

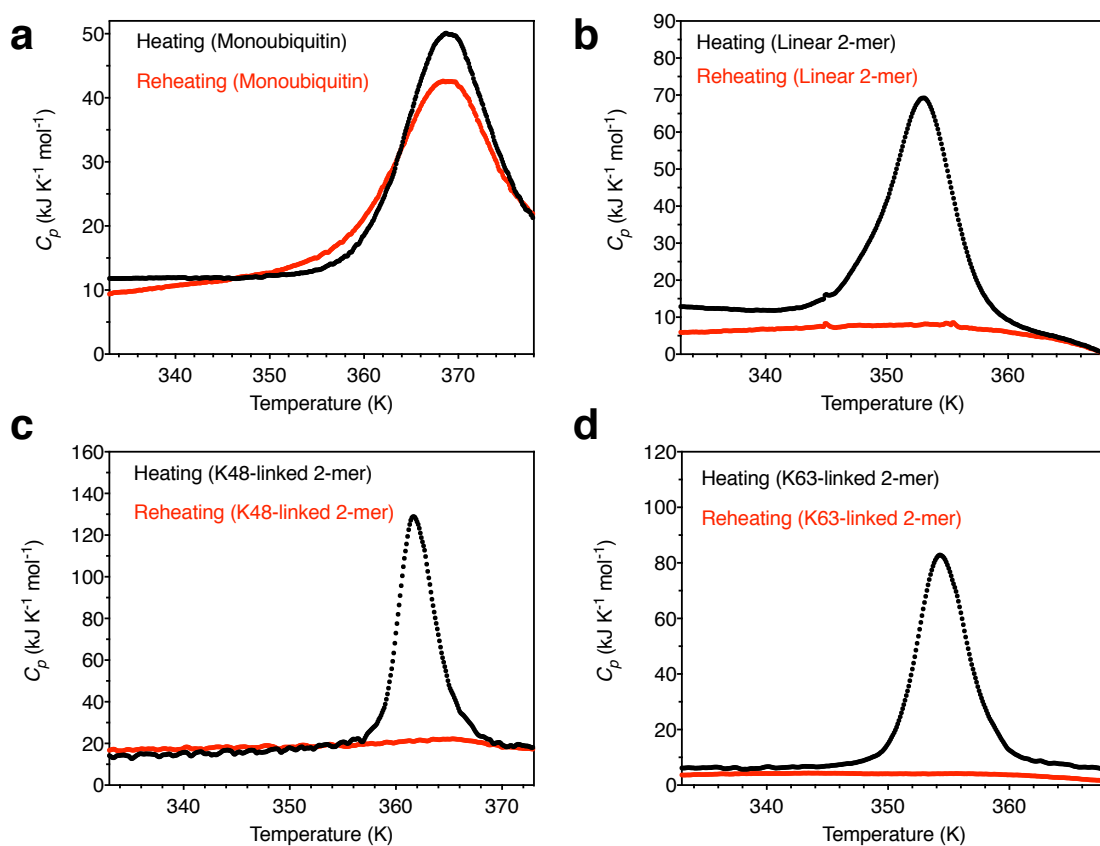


Figure 3-3. **Irreversible thermal unfolding of diubiquitin.** Differential scanning calorimetry traces of monoubiquitin (a), linear diubiquitin (b), K48-linked diubiquitin (c) and K63-linked diubiquitin (d) are shown. Black traces show initial heating and red lines represent reheating. Thermal unfolding of monoubiquitin is reversible, whereas thermal unfolding of diubiquitin is irreversible.

3-2. Polyubiquitin Chains Form Amyloid-like Fibrils By Heat

Polyubiquitin chains, not monoubiquitin, form insoluble aggregates by heat denaturation. Unexpectedly, the electron microscopy (EM) images of the aggregates of diubiquitin or hexaubiquitin showed that they consist of fibrils of up to 100 nm in length and approximately 5 nm in diameter (Figure 3-4). The fibril formation is independent of the chain linkage type (Figure 3-4); namely, linear, K48-linked and K63-linked polyubiquitin chains form fibrils by heat denaturation. Neither aggregate nor fibril in heat-treated monoubiquitin was observed by EM (Figure 3-7 left). Interestingly, the morphology of these polyubiquitin fibrils was reminiscent of amyloid-like fibrils reported previously (Olzscha et al., 2011). To examine whether polyubiquitin fibrils are amyloid-like fibrils, Thioflavin T (ThT)-binding assay and circular dichroism (CD) measurements were performed. Binding and fluorescence of ThT are considered to be indicative of amyloid-like fibrils. Polyubiquitin fibrils were all stained with ThT (Figure 3-5) although heat-treated monoubiquitin was not stained (Figure 3-5 green). Circular dichroism (CD) spectra revealed that the polyubiquitin aggregates consistently displayed β -rich secondary structure, similar to that observed in amyloid- β (A β) (1-40) fibrils (Figure 3-6). These results support the idea that polyubiquitin chains form amyloid-like fibrils by heat denaturation.

Taken together, these observations show that the irreversible transition of polyubiquitin chains by heat denaturation results in their amyloid-like fibril formation. Importantly, because a longer ubiquitin chain has a lower transition temperature (Figure 3-1, Figure 3-2), longer chains form fibrils at lower temperatures. For other fibrillogenic proteins such as α -synuclein and tau, the thermodynamic destabilization of the protein correlates closely with its aggregation propensity: its tendency to form fibrils (Chiti et al., 2003; Vassall et al., 2011). This result is consistent with the observed fibril formation of polyubiquitin chains, which suggests that length-dependent thermodynamic destabilization of polyubiquitin chains may cause fibril (aggregation) formation.

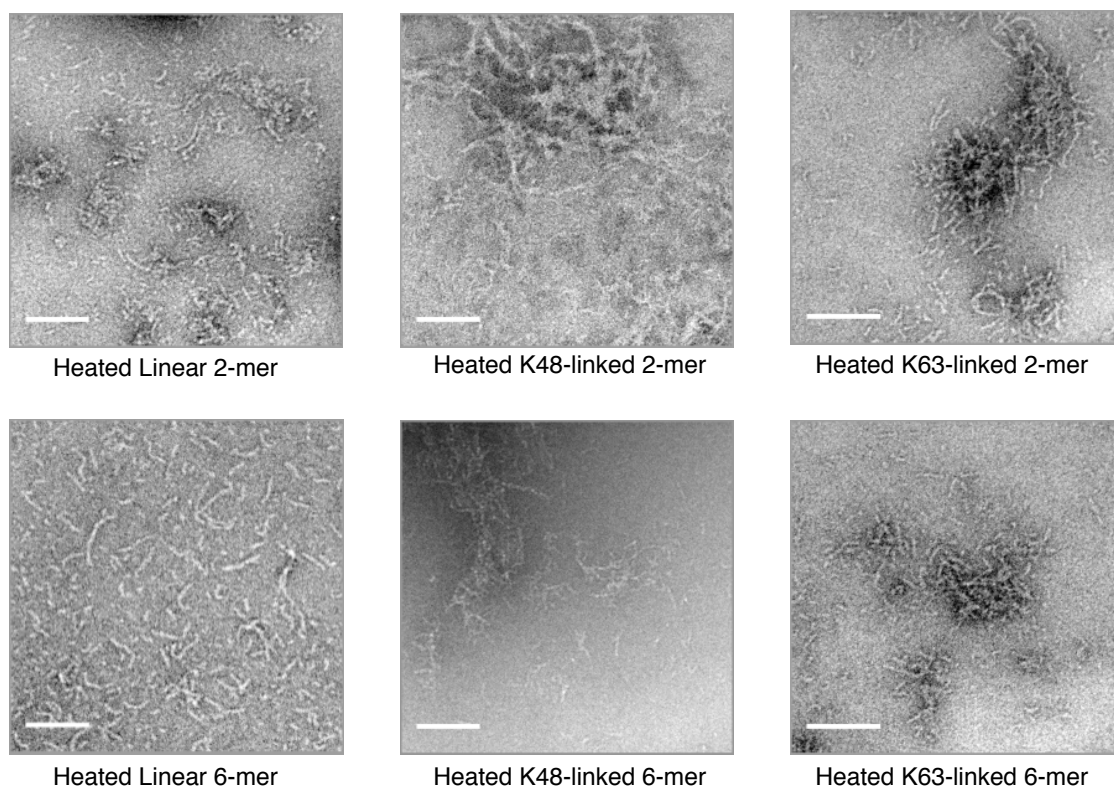


Figure 3-4. **Heat-treated polyubiquitin chains form fibrils.** Electron microscopy (EM) images of heat-treated linear diubiquitin (left upper), linear hexaubiquitin (left lower), K48-linked diubiquitin (middle upper), K48-linked hexaubiquitin (middle lower), K63-linked diubiquitin (right upper) and K63-linked hexaubiquitin (right lower). Scale bars, 100 nm.

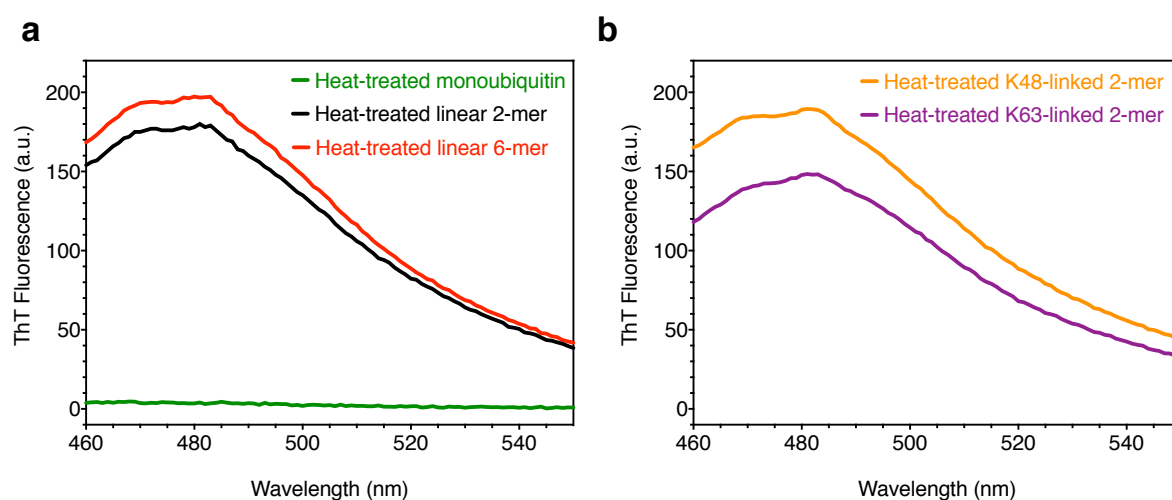


Figure 3-5. **Thioflavin T staining of polyubiquitin fibrils produced by heat-denaturation indicates that their fibrils are amyloid-like fibrils.** Thioflavin T fluorescence emission spectra of heat-treated monoubiquitin (left, green), linear diubiquitin (left, black), linear hexaubiquitin (left, red), K48-linked diubiquitin (right, orange) and K63-linked diubiquitin (right, purple).

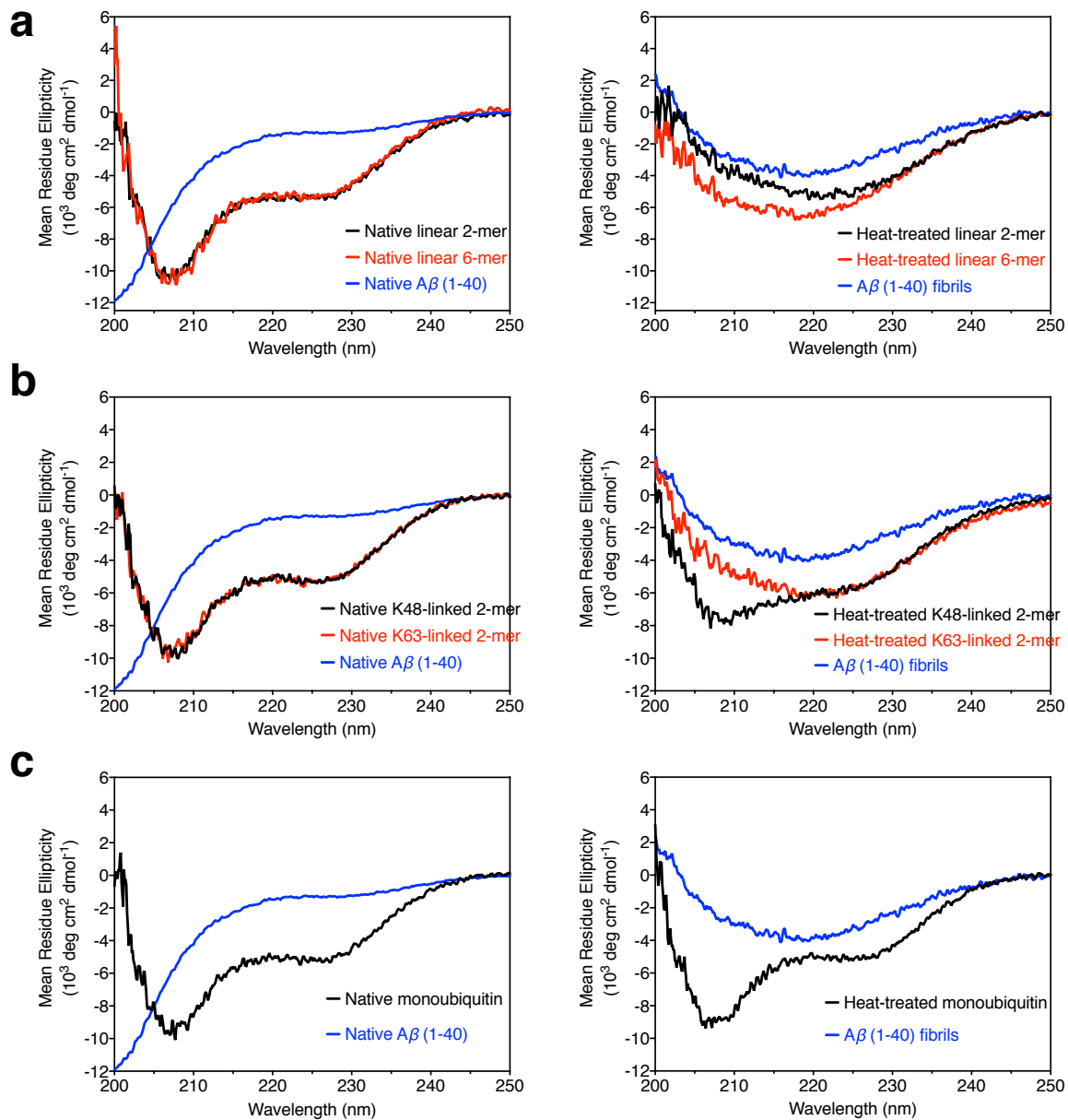


Figure 3-6. Polyubiquitin chains form β -rich secondary structures after heat treatment. Left, circular dichroism (CD) spectra of native linear diubiquitin (**a**, black), hexaubiquitin (**a**, red), K48-linked diubiquitin (**b**, black) and K63-linked diubiquitin (**b**, red), monoubiquitin (**c**, black) and A β (**c**, 1-40) (blue). Right, circular dichroism spectra of these heat-treated samples and A β (1-40) fibrils (blue). In general, α -helical proteins have negative bands at 208 nm and 222 nm and a positive at 193 nm (HOLZWARTH and DOTY, 1965). β -sheet conformations have a negative band at 218 nm and a positive band at 195 nm, whereas random coils have a negative band near 195 nm (Greenfield and Fasman, 1969). Therefore, these CD measurements indicate that heat-denaturation of polyubiquitin chains results in their secondary conformational changes: they form β -rich structures by heat-denaturation while their native structures contain both α -helices and β -sheets.

3-3. Mechanical Forces Can Induce Polyubiquitin Fibril Formation

Next, to probe other driving forces to form polyubiquitin fibrils, whether mechanical forces induce their amyloid-like fibril formation was examined. Some fibrillogenic proteins such as A β form amyloid fibrils can form amyloid fibrils simply by agitation (Dunstan et al., 2009), which suggests that the agitation or stirring could cause fibril formation of polyubiquitin chains. When monoubiquitin and polyubiquitin chains were treated with moderate agitation at a rotational speed as low as 33 s⁻¹, all polyubiquitin chains, regardless of chain length or linkage type, formed ThT-positive aggregates, whereas monoubiquitin displayed no noticeable change under the same conditions (Figure 3-7 and Figure 3-9). EM images showed that the aggregates formed by mechanical stress contain fibrils and they are similar to polyubiquitin fibrils prepared by heat-denaturation (Figure 3-4 and Figure 3-8). These results indicate that not only heat but also mechanical stress induces amyloid-like fibril formation of polyubiquitin chains.

Because the thermodynamic destabilization correlates with the aggregation propensity as mentioned above, it is possible that a longer ubiquitin chain form amyloid-like fibrils faster than shorter one. To examine this possibility, the rate of fibril formation using a ThT fluorescence assay at a rotational speed of 25 s⁻¹ was measured to compare the influence of these hydrodynamic forces on different types of ubiquitin. As expected, the order of fibril formation tendency by shear stress was the same as that observed for the transition temperature of the polyubiquitin chains, whereby linear chains aggregated at the lowest temperature, followed by K63- and then K48-linked chains (Figure 3-10a and b).

In addition, to obtain more quantitative parameters, whether polyubiquitin chains form fibrils by controlled shear stress in a Couette cell was investigated. In response to a shear rate of 470-600 s⁻¹, linear hexaubiquitin formed ThT-positive aggregates (Figure 3-11b). A more moderate shear rate of 47-60 s⁻¹ also causes fibril formation of linear hexaubiquitin (Figure 3-11c). This shear rate is of the same order as that required to induce the aggregation of other fibrillogenic proteins, such as A β , insulin and β -lactoglobulin, in a Couette cell (Bekard et al., 2011). These observations suggest that a longer ubiquitin chain form fibrillar aggregates more easily under conditions close to physiological conditions. This tendency may be caused by the increase in molecular anisotropy in polyubiquitin chains; that is, elongated molecules undergo larger anisotropic Brownian motions, and thus may be more easily affected by external mechanical stress and temperature.

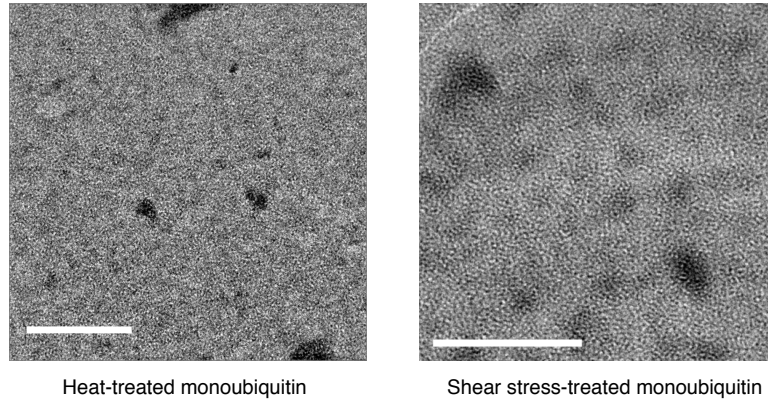


Figure 3-7. **Neither heat nor shear stress induces fibril formation of monoubiquitin .** Electron Microscopy (EM) images of heat-treated monoubiquitin (a) and shear stress-treated monoubiquitin (b). Scale bars, 100 nm.

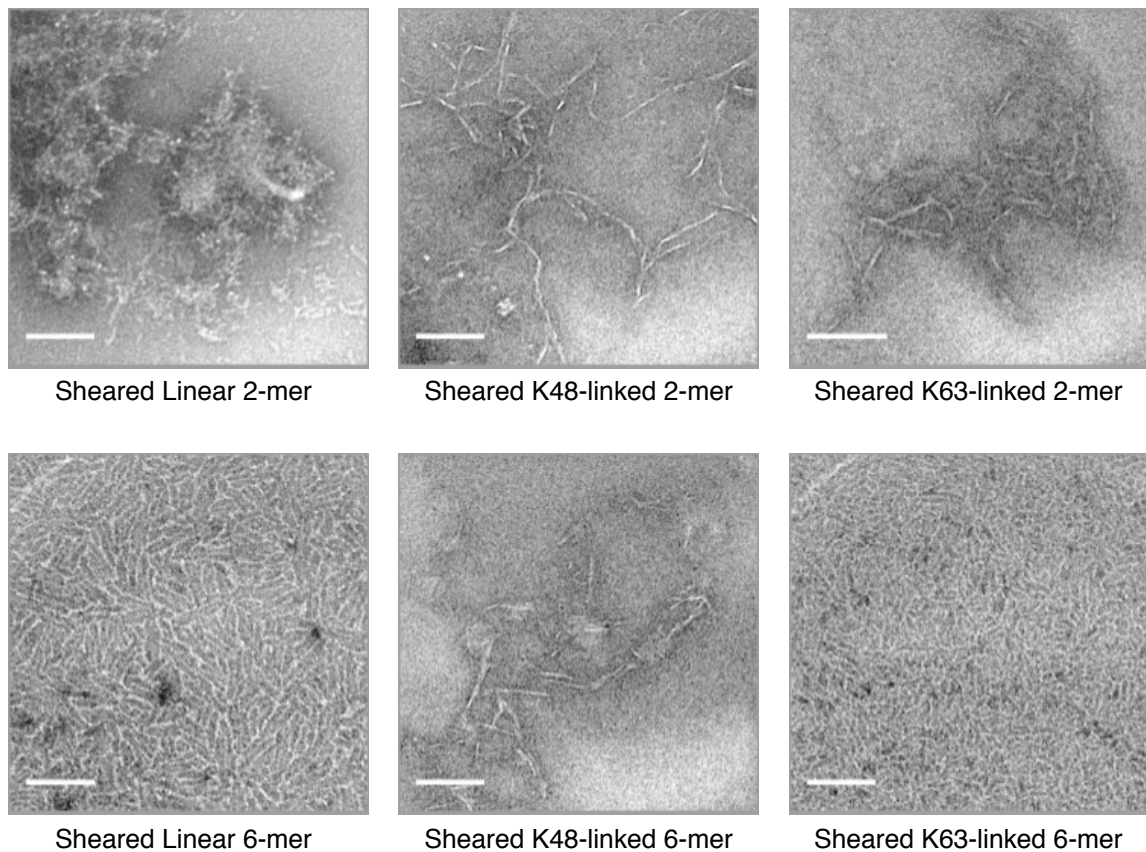


Figure 3-8. **Mechanical stress also induces the formation fibrils of polyubiquitin chains.** EM images of sheared samples: linear diubiquitin (left upper), linear hexaubiquitin (left lower), K48-linked diubiquitin (middle upper), K48-linked hexaubiquitin (middle lower), K63-linked diubiquitin (right upper) and K63-linked hexaubiquitin (right lower) show that all of these types of polyubiquitin form fibrils with amyloid-like morphology when subjected to mechanical stress. The shear stress was applied as an agitation at a rotational speed of 25 or 33 s^{-1} . Scale bars, 100 nm.

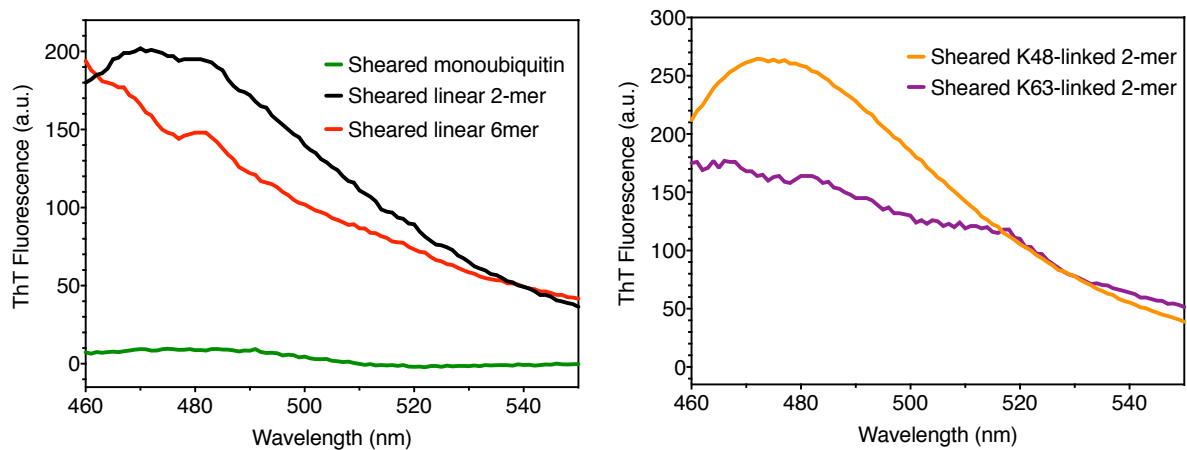


Figure 3-9. ThT binding assay for fibrils of polyubiquitin chains treated with mechanical forces. Thioflavin T fluorescence emission spectra of sheared monoubiquitin (left, green), linear diubiquitin (left, black), linear hexaubiquitin (left, red), K48-linked diubiquitin (right, orange) and K63-linked diubiquitin (right, purple). Shear stress was applied as an agitation at a rotational speed of 25 s^{-1} for 90 hours. For monoubiquitin and K48-linked diubiquitin, the applied shear stress was that of 33 s^{-1} for 90 hours.

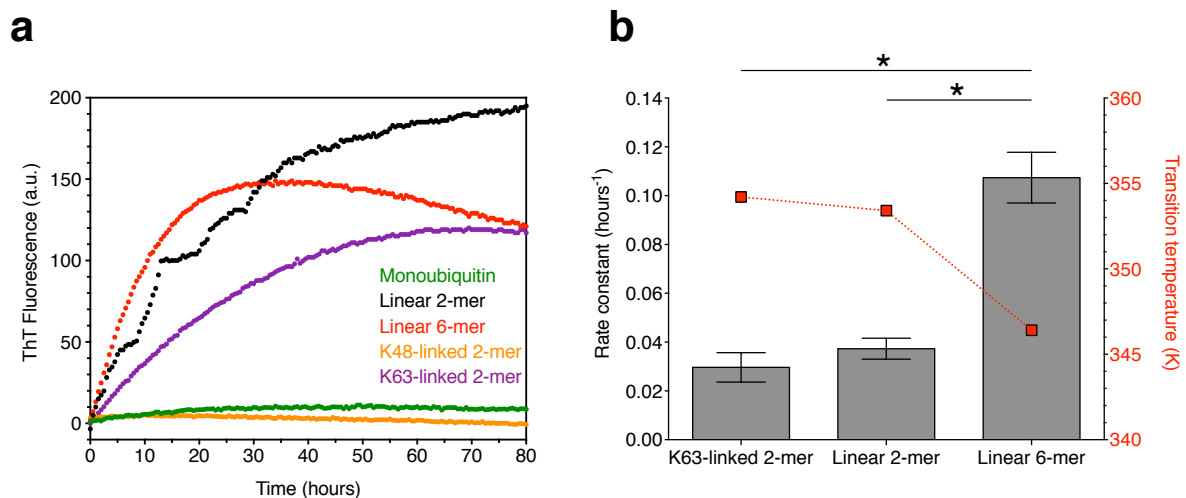


Figure 3-10. Folding destabilization of polyubiquitin chains correlates with their rate to form amyloid-like fibrils by mechanical forces. **a**, Fibril formation of sheared K63-linked diubiquitin (purple), linear diubiquitin (black) and hexaubiquitin (red), as followed by ThT fluorescence. K48-linked diubiquitin (orange) and monoubiquitin (green) did not show relevant increases in fluorescence. Shear stress was applied by agitation at a rotational speed of 25 s^{-1} . **b**, Comparative analysis of the kinetics of fibril formation by polyubiquitin chains of different linkage type and chain-length with transition temperature. Rate constants were obtained by fitting the data to first-order kinetics. The values represent the average of two independent experiments. Error bars, the standard error of the mean. $*P < 0.05$ (Student's *t*-test).

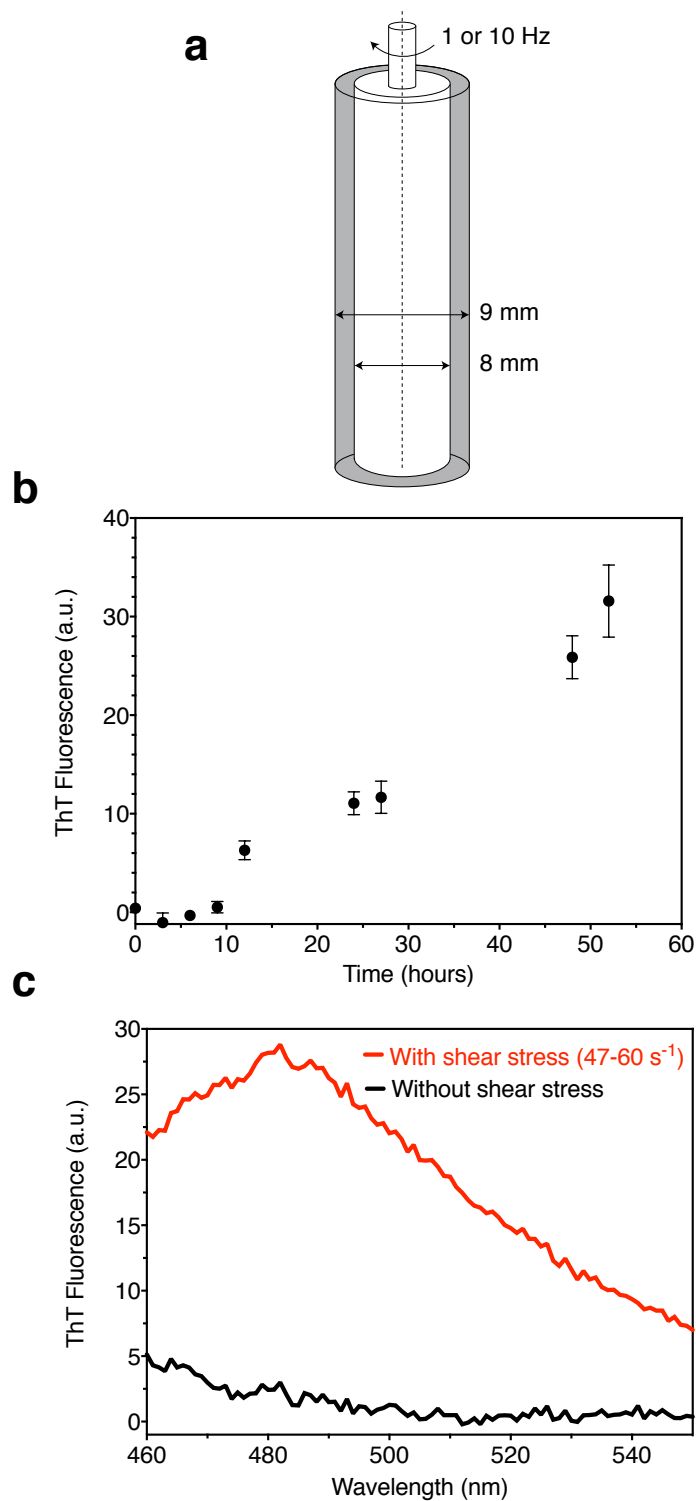


Figure 3-11. **Quantitative shear stress analysis in a Couette cell.** **a**, Schematic diagram of the rotational flow device used. **b**, Fibril formation of linear hexa ubiquitin in response to shear rates of 470-600 s⁻¹ in a Couette cell. Error bars, the standard error of the mean. **c**, Thioflavin T fluorescence emission spectra of non-treated linear hexa ubiquitin (grey) and linear hexa ubiquitin sheared in a Couette cell at a rate of 47-60 s⁻¹ for 72 hours (black).

3-4. Ubiquitylated Proteins Also Form Fibrils by Heat or Shearing

To examine whether ubiquitylated proteins also form amyloid-like aggregates under moderate conditions, their thermodynamic profiles and responses to mechanical forces have been investigated. As models for substrate proteins, rat calmodulin and human FK506-binding protein (FKBP12) are chosen: both of which are ubiquitylated *in vivo* (Laub et al., 1998; Wagner et al., 2011). The ubiquitylated forms were constructed: in which monoubiquitin or linear hexaubiquitin was attached to the amino group of the N-terminal residue of the protein (*i.e.*, Ub-calmodulin, Ub₆-calmodulin, Ub-FKBP12 and Ub₆-FKBP12).

Although both non-ubiquitylated calmodulin and FKBP12 are thermally reversible (Figure 3-12 upper), the DSC thermographs of ubiquitylated calmodulin and FKBP12 displayed irreversible thermo-transitions similar to those of ubiquitin chains. For both ubiquitylated proteins, their aggregate formation occurs not only by heat-denaturation but also by shear stress (Figure 3-12 and Figure 3-14). Although EM observations did not clearly reveal whether the aggregates contain fibrils, the CD spectra and ThT binding of their aggregates resembled to those of fibrils formed by polyubiquitin chains (Figure 3-13 and Figure 3-14). In contrast, non-ubiquitylated substrate proteins displayed no aggregates in response to heat-denaturation or mechanical forces (Figure 3-12, Figure 3-13 and Figure 3-14). Interestingly, calmodulin conjugated to a longer polyubiquitin chain formed fibrils more easily than that conjugated to mono-ubiquitin (Figure 3-15a). Such effect was limited in the case of FKBP12 (Figure 3-15b). Taken together, these results indicate that ubiquitylation has an effect on aggregation propensity and thermal reversibility of a substrate protein. In addition, the observations imply that conjugation of longer ubiquitin chains appears to enhance aggregate formation even though such effect was limited in the case of FKBP12: the effect of ubiquitin chains on fibril-formation might depend on the attached substrate protein.

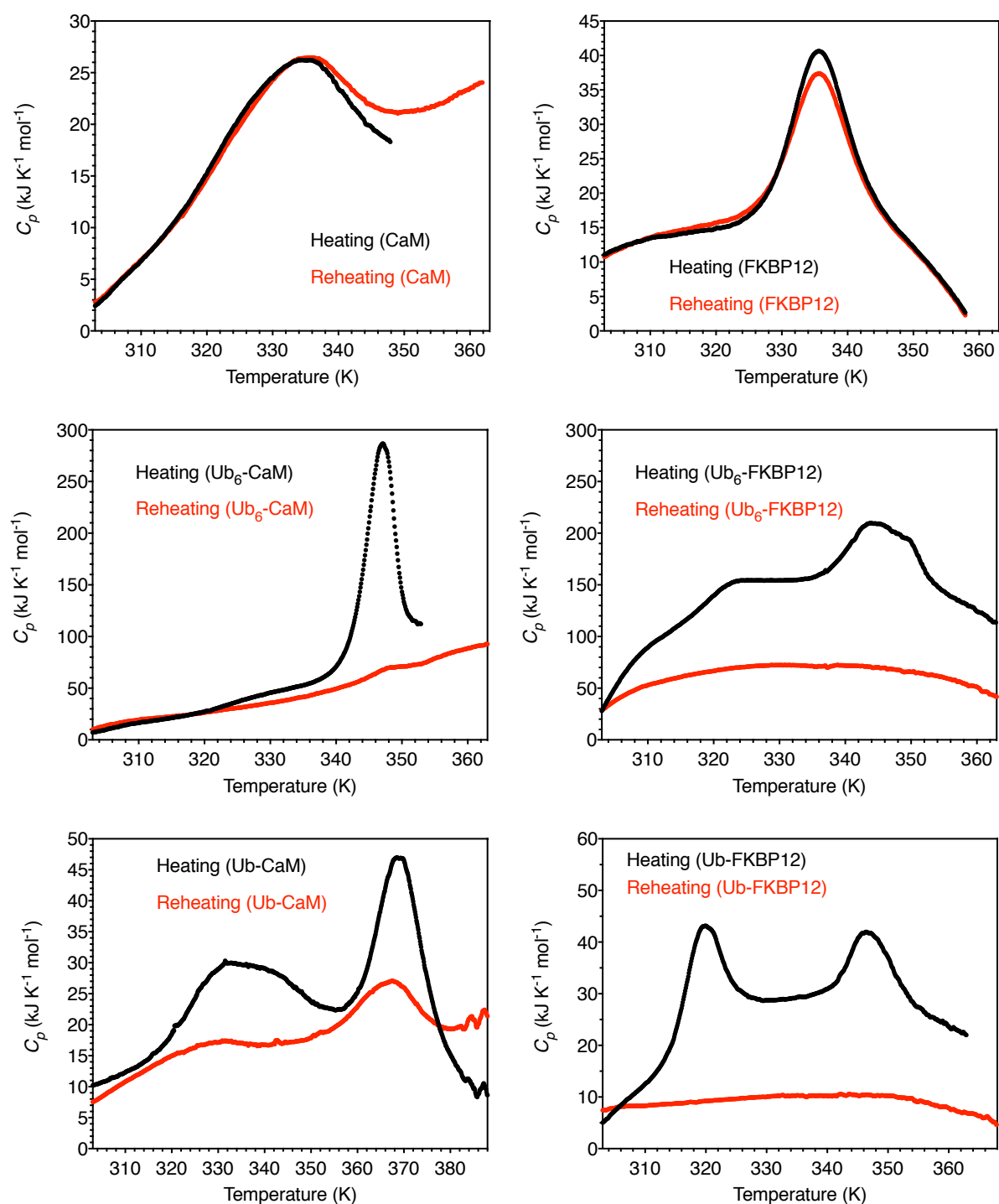


Figure 3-12. Ubiquitylation impairs the folding reversibility of proteins. Differential scanning calorimetry (DSC) traces of calmodulin (CaM) (left upper) and FKBP12 (right upper), showing that the thermal unfolding of these proteins is reversible. DSC traces of Ub_6 -CaM (left middle), Ub_6 -FKBP12 (right middle), Ub-CaM (left lower) and Ub-FKBP12 (right lower), indicating that the thermal unfolding of ubiquitylated proteins is irreversible. Black traces represent initial heating and red traces show reheating.

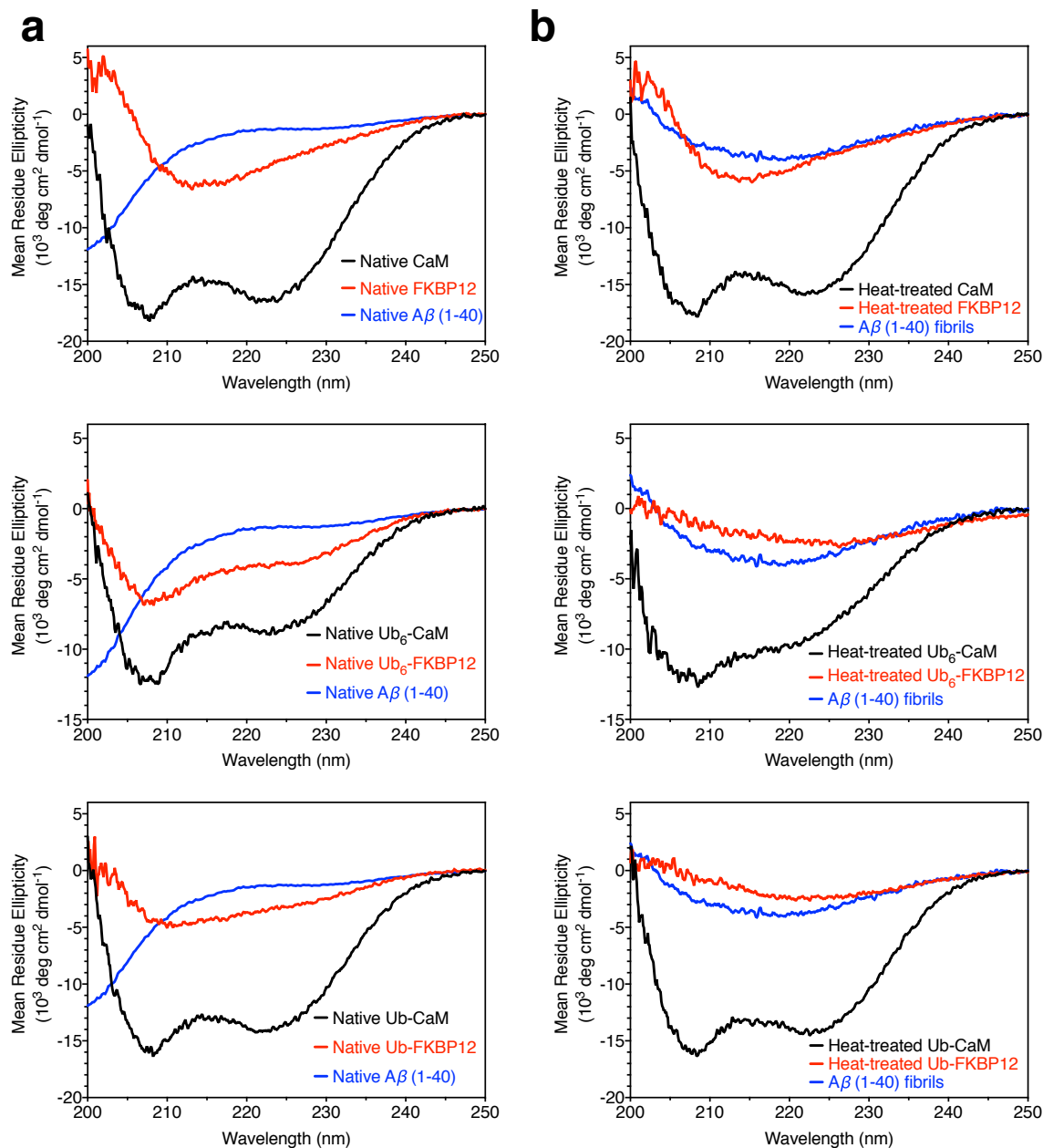


Figure 3-13. **Heat-treated ubiquitylated proteins form β -rich secondary structures after heat treatment.** Circular dichroism spectra of native (a) and heated (b) CaM (upper, black), FKBP12 (upper, red), Ub₆-CaM (middle, black), Ub₆-FKBP12 (middle, red), Ub-CaM (lower, black) and Ub-FKBP12 (lower, red). For comparison, spectra of native A β (1-40) or A β (1-40) fibrils (blue) are shown in each spectrum.

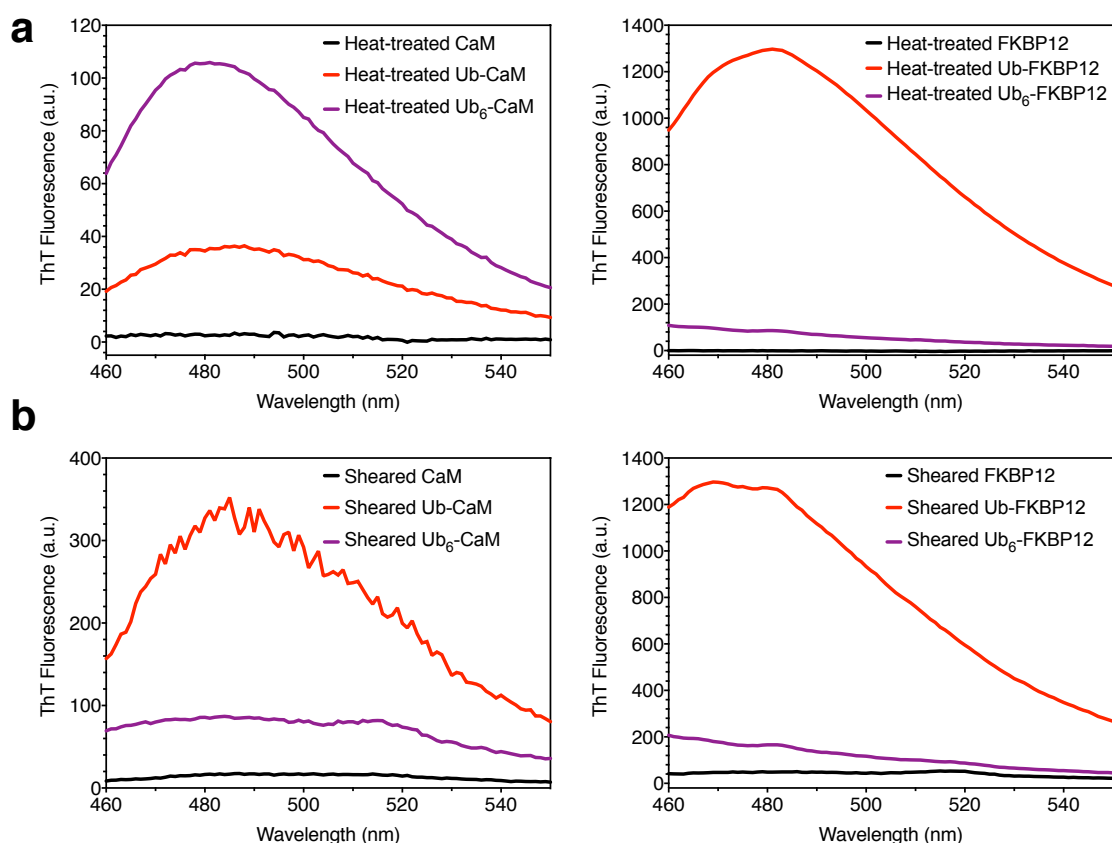


Figure 3-14. Thioflavin T fluorescence emission of heat-treated ubiquitylated proteins. **a**, Thioflavin T fluorescence emission spectra of heated (upper) and sheared (lower) CaM (left, black), Ub-CaM (left, red), Ub₆-CaM (left, purple), FKBP12 (right, black), Ub-FKBP12 (right, red) and Ub₆-FKBP12 (right, purple). Shear stress was applied as an agitation at a rotational speed of 25 s⁻¹ for 90 hours.

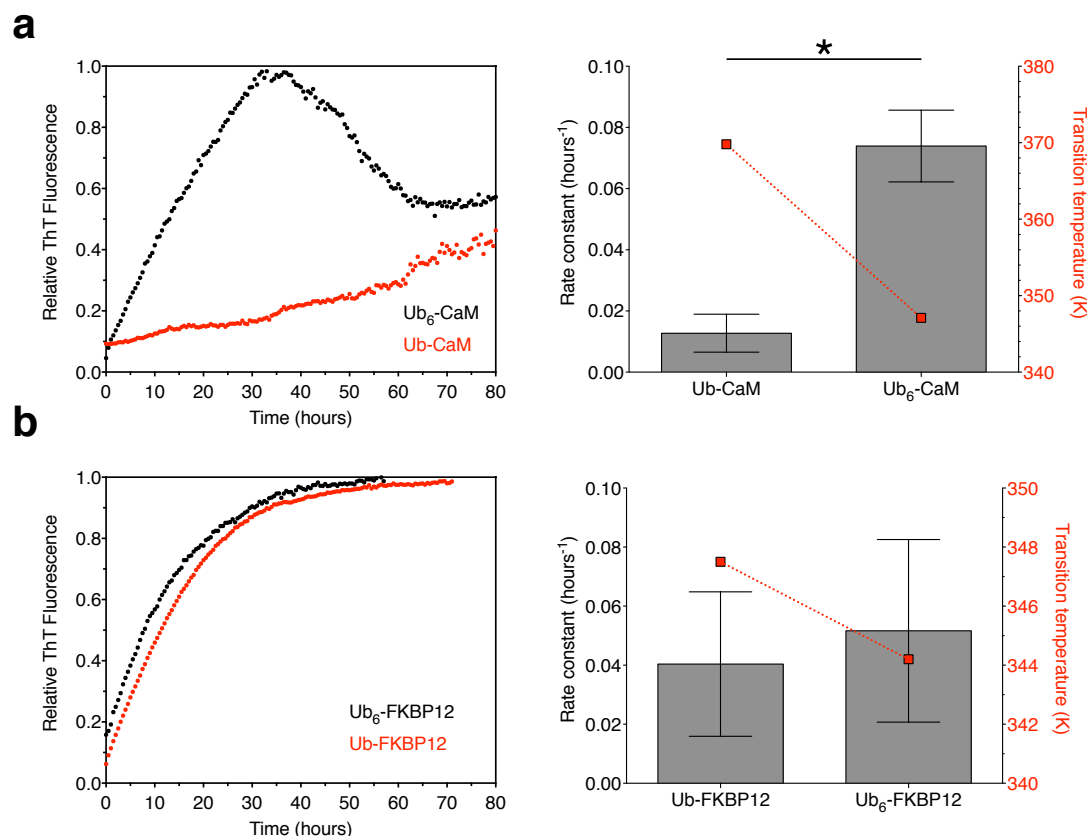


Figure 3-15. Ubiquitylation induces formation of amyloid-like fibrils in a chain-length dependent manner. Fibril formation of sheared Ub-CaM (left upper, red), Ub₆-CaM (left upper, black), Ub-FKBP12 (left lower, red) and Ub₆-FKBP12 (left lower, black) as followed by ThT fluorescence. Comparative analysis of the fibril formation kinetics of ubiquitylated CaM (right upper) and FKBP12 (right lower) with transition temperature. Shear stress was applied as an agitation at a rotational speed of 25 s⁻¹. Rate constants were obtained by fitting the data to first-order kinetics. The values represent the average of two independent experiments. Error bars, the standard error of the mean. **P* < 0.05 (Student's *t*-test).

3-5. Polyubiquitin Chains Form Intracellular Aggregates

The *in vitro* chain-length-dependent aggregation might be related to the formation of ubiquitin-positive inclusion bodies in cells. To elucidate this possibility, whether ubiquitylated proteins form intracellular aggregates was tested. EGFP attached to linear hexaubiquitin were expressed in mouse embryonic fibroblasts (MEFs). The C-terminal di-amino acids (G-G) of each ubiquitin unit in the hexaubiquitin were replaced by V-V (Ub^{VV}₆-EGFP) to prevent cleavage of ubiquitin chains by DUB enzymes. The MEFs expressing Ub^{VV}₆-EGFP displayed several EGFP-positive intracellular plaques of up to 2 μm in diameter (Figure 3-16 upper). In addition, expression of linear hexaubiquitin (Ub^{VV}₆) without any protein attached also resulted in formation of intracellular insoluble aggregates (Figure 3-17). In contrast, in MEFs expressing monoubiquitin-attached EGFP (Ub^{VV}-EGFP), the EGFP fluorescence was smoothly distributed across the cytosol and the nucleus, as observed in the cells expressing EGFP, except for the occasional formation of a few small spots in a small fraction of cells (Figure 3-16 middle, bottom and Figure 3-17). Thus, these observations indicate that whether ubiquitin chains form intracellular aggregates or not is dependent on their chain-length.

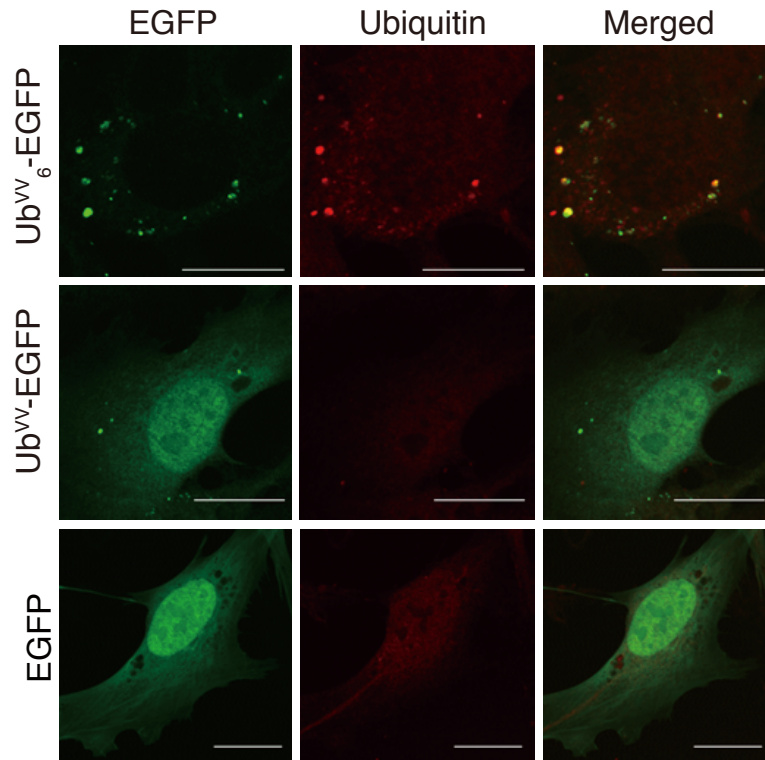


Figure 3-16. ***In vivo* chain-length dependent aggregation of polyubiquitin chains.** MEFs were transiently transfected with Ub^{V6}-EGFP (top), Ub^V-EGFP (middle) or EGFP (bottom) and imaged 48 hours after transfection. No ubiquitin-positive aggregates were detected in cells expressing Ub^V-EGFP and EGFP, whereas ubiquitin-positive aggregates of up to 2 μ m in diameter were observed in cells expressing Ub^{V6}-EGFP. Scale bars, 20 μ m. Data are representative of three independent experiments.

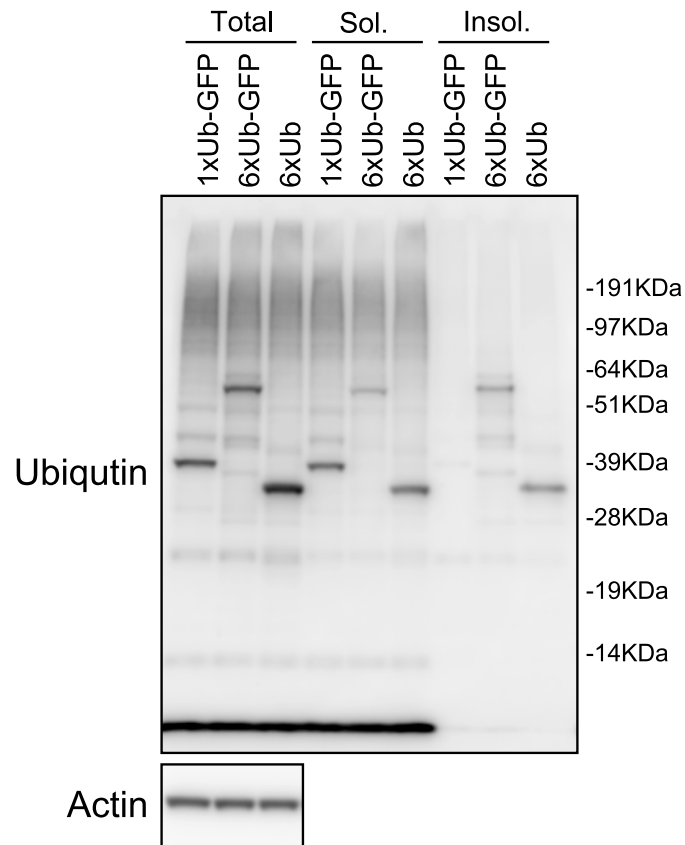


Figure 3-17. **Aggregate formation of polyubiquitin chains in cells is independent of conjugation to other proteins.** HeLa cells were transiently transfected with pcDNA 3.1 (+) vectors encoding Ub^{VV}-EGFP (left), Ub^{VV}₆-EGFP (middle) and Ub^{VV}₆ (right). Total, soluble (Sol.), and insoluble (Insol.) fractions were subjected to immunoblotting with the indicated antibodies. Data are representative of two independent experiments.

3-6. Autophagy Specifically Degraded Polyubiquitin Aggregates

Clearance of ubiquitin-positive aggregates has been shown to depend on a type of selective autophagy, which degrades aggregated proteins (aggrephagy) (Kirkin et al., 2009b; Komatsu et al., 2006). In aggrephagy, ubiquitin on the aggregated structures leads to the assembly of ubiquitin-adaptor proteins such as p62 and NBR1, which then induces formation of autophagosome by core Atg proteins (Kirkin et al., 2009b). These suggest that such ubiquitin-adaptor proteins could recognize ubiquitin-positive aggregates; *i. e.*, polyubiquitin fibrils.

To determine whether p62 and NBR1 have the ability to bind to the fibrillar form of polyubiquitin or not, the association was examined by a method of solution NMR (Fawzi et al., 2012), in which the exchange process (binding and dissociation) is imprinted onto the transverse relaxation rates of the free UBA domain. Estimation of transverse relaxation rates ($^{15}\text{N}-R_2$) from heteronuclear single quantum coherence (HSQC) spectra of the respective free UBA domain and the UBA domain titrated with polyubiquitin fibrils indicated that both UBA domains interact with polyubiquitin fibrils. When the ^{15}N -labeled NBR1 domain was titrated with non-labeled polyubiquitin fibrils, its average $^{15}\text{N}-R_2$ increased by $5.32 \pm 1.53 \text{ s}^{-1}$ (S.D.) in a residue-specific manner (Figure 3-18a). In the case of the UBA domain of p62, the increase was $2.94 \pm 1.10 \text{ s}^{-1}$ (S.D.) (Figure 3-18b). Indeed, it has been shown that the interaction of amyloid- β monomers with the surface of amyloid- β protofibrils leads to an increase in $^{15}\text{N}-R_2$ of the monomeric form by approximately 2 s^{-1} , which is of the same order as the results (Fawzi et al., 2011). It is proposed that both UBA domains interact with the surface of polyubiquitin fibrils, but it will be necessary to quantitatively probe the interaction kinetics in the near future.

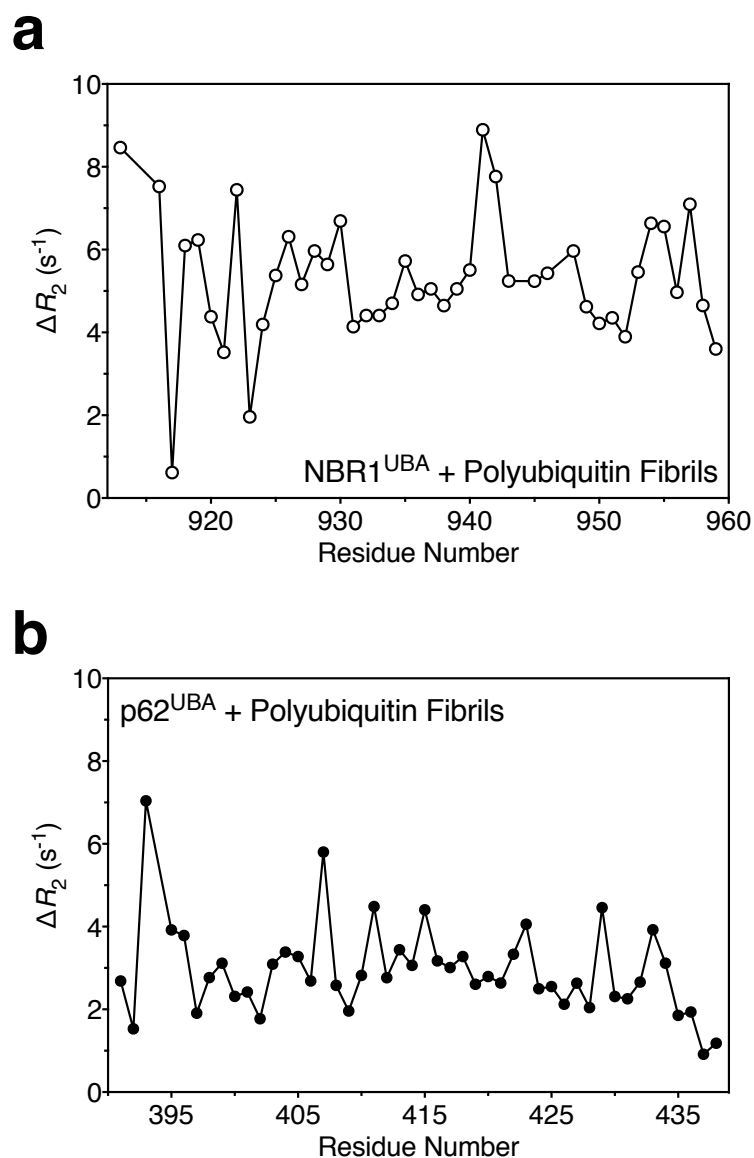


Figure 3-18. Interaction of the UBA domains of p62 and NBR1 with polyubiquitin fibrils. Estimation of ΔR_2 , the difference of the transverse relaxation rates ($^{15}\text{N}-R_2$) of free UBA domains and UBA domains titrated with polyubiquitin fibrils, at each residue position (Fawzi et al., 2012). 200 μM of ^{15}N -labeled UBA domains of p62 (**a**) or NBR1 (**b**) were titrated with four fold molar excess of non-labeled polyubiquitin fibrils formed by heat-denaturation of linear hexaubiquitin V-V mutants. The concentration of polyubiquitin chains was defined as the concentration of monomeric ubiquitin subunits in a polymer. $^{15}\text{N}-R_2$ values were obtained from the full-width at half height (FWHH) of each peak in heteronuclear single quantum coherence (HSQC) spectra using NMRPipe (Delaglio et al., 1995). Resonance assignments of the UBA domains were based on the previous works (Isogai et al., 2011; Walinda et al., 2014).

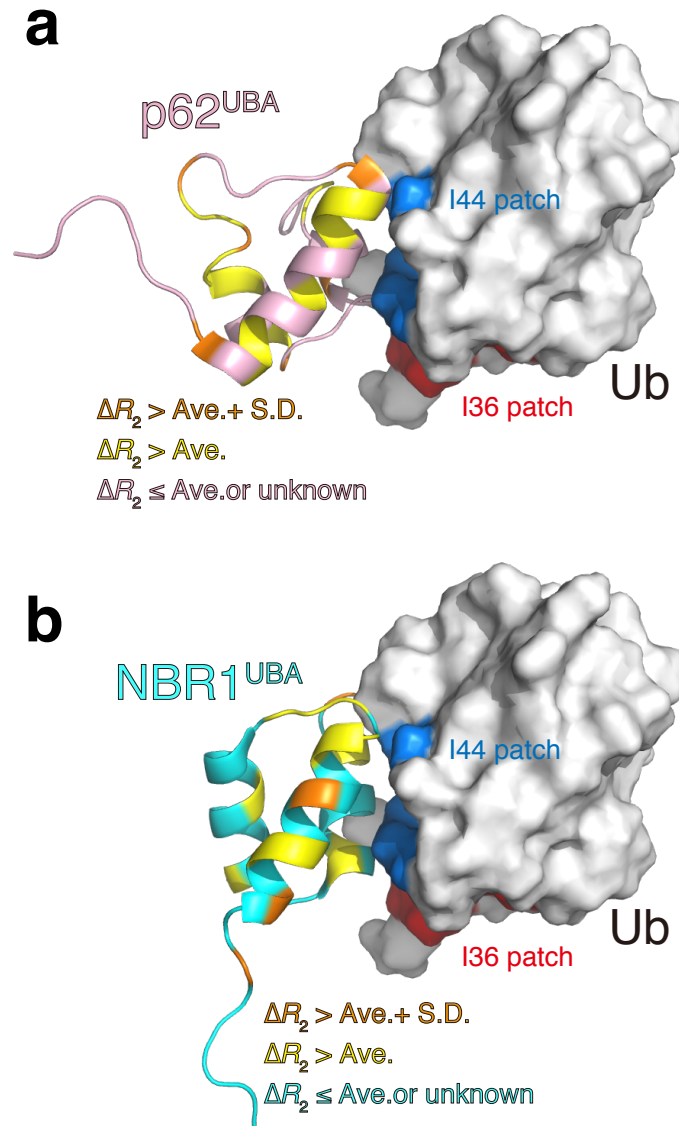


Figure 3-19. **Interface between the UBA domain and polyubiquitin fibrils.** Mapping of measured ΔR_2 for UBA domains onto the individual complex structures with mono-ubiquitin. **a**, 2RRU for the complex of p62 UBA domain with mono-ubiquitin (Isogai et al., 2011) and **b**, 3MJ5 for the complex of NBR1 UBA domain with mono-ubiquitin (Walinda et al., 2014) in PDB database are used. The two of hydrophobic patches in ubiquitin are indicated.

Finally, the possibility that the Ub^{VV}₆-EGFP aggregates serve as an initiation signal for aggrephagy was examined. Autophagic flux assays revealed that expression of Ub^{VV}₆-EGFP did not affect turnover of LC3-II which represents autophagosome formation (Kabeya et al., 2000), as did expression of Ub^{VV}-EGFP (Figure 3-20a). By contrast, degradation of Ser351-phosphorylated p62 in lysosomes, a hallmark of aggrephagy (Ichimura et al., 2013), was induced by expression of Ub^{VV}₆-EGFP, but not Ub^{VV}-EGFP (Figure 3-20a). Endogenous LC3 were extensively co-localized with aggregates positive for Ub^{VV}₆-EGFP (Figure 3-20b left). As expected (Kirkin et al., 2009b), p62 was also recruited to these structures, and found to be phosphorylated at Ser351 (Figure 3-20b right). Loss of autophagy-related 7 (*Atg7*), an essential gene for autophagy, inhibited degradation of Ub^{VV}₆-EGFP, p62 and Ser351-phosphorylated p62 (Figure 3-21a). Expression of wild-type *Atg7*, but not the active site mutant *Atg7* C567S (Tanida et al., 1999) restored degradation of these proteins (Figure 3-21b). In accordance with these biochemical data, immunofluorescence staining revealed Ub^{VV}₆-EGFP aggregate structures positive for Ser351-phosphorylated p62 in MEFs expressing the *Atg7* C567S mutant (Figure 3-21c). Although soluble linear-ubiquitylated proteins are partly degraded by the ubiquitin-proteasome system (Kirisako et al., 2006), the results indicate that aggrephagy mainly contributes to clearance of insoluble ubiquitin-positive aggregates. In support of the data, it has been recently found that aggregates formed in hepatocytes with proteasome deficiency were selectively entrapped by autophagosomes, and pathological features of livers with impaired proteasome activity were exacerbated by simultaneous suppression of autophagy (Kageyama et al., 2014). Remarkably, a highly sensitive polyubiquitin chain quantification method (Tsuchiya et al., 2013) revealed that insoluble proteins from livers with impaired proteasome activity contained all linkage types of polyubiquitin chains (Figure 3-22b). Likewise, insoluble polyubiquitin chains accumulating in autophagy-deficient livers showed no linkage specificity (Riley et al., 2010) (Figure 3-22b). These *in vivo* analyses suggest that all types of polyubiquitin chains on proteins have the potential to induce their fibrillar aggregate formation followed by induction of autophagy, which serves a cytoprotective function.

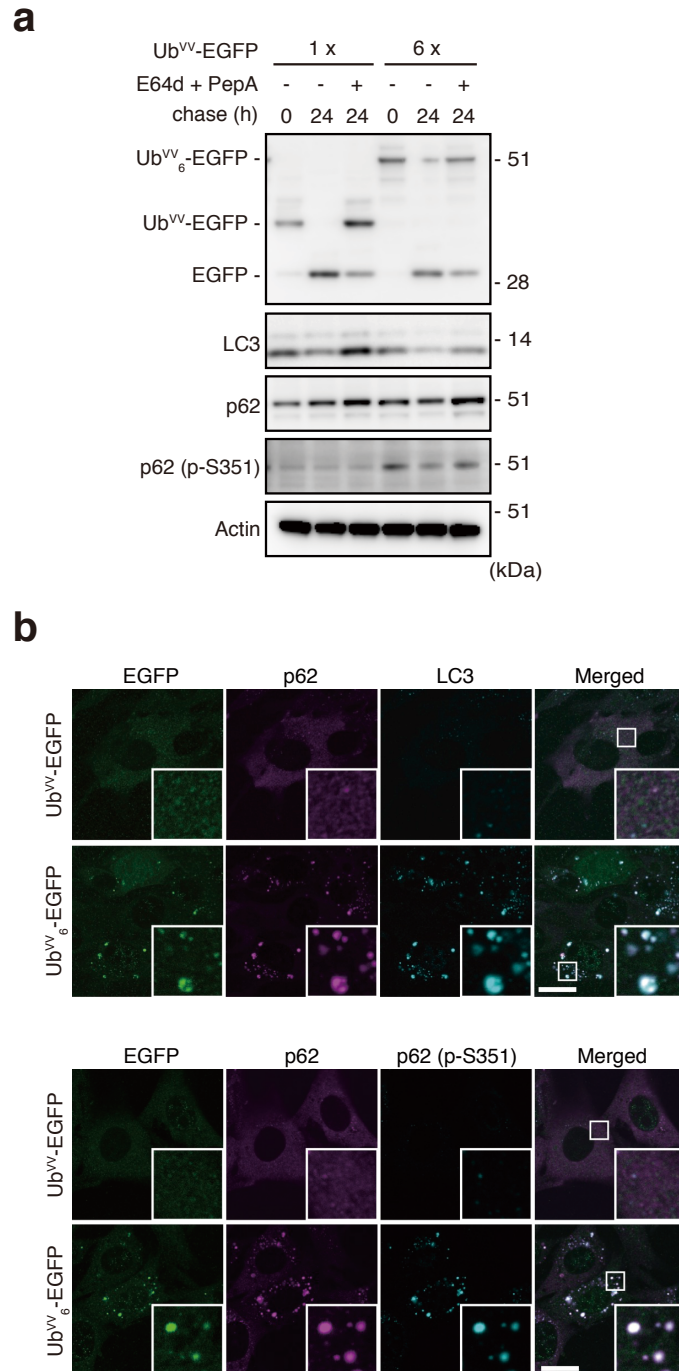


Figure 3-20. Degradation of polyubiquitin aggregates and their co-localization with autophagy-related proteins. **a**, Immortalized wild-type MEFs harboring two regulator-gene cassettes, CAG-rtTA and either TRE-Ub^{VV}₆-EGFP or TRE-Ub^{VV}-EGFP, were cultured for 24 hours in the presence of Dox to induce expression of Ub^{VV}₆-EGFP or Ub^{VV}-EGFP. Subsequently, the cells were cultured in the absence of Dox for 24 hours. E64d and pepstatin were added as indicated. The cell lysates were prepared and immunoblotted with the indicated antibodies. Data are representative of two independent experiments. **b**, The MEFs shown in (a) were cultured for 24 hours in the presence of Dox to induce expression of Ub^{VV}₆-EGFP or Ub^{VV}-EGFP, and then immunostained with p62 and LC3 antibodies or p62 and Ser351-phosphorylated p62 antibodies. Bars: 20 μ m. Data are representative of three independent experiments.

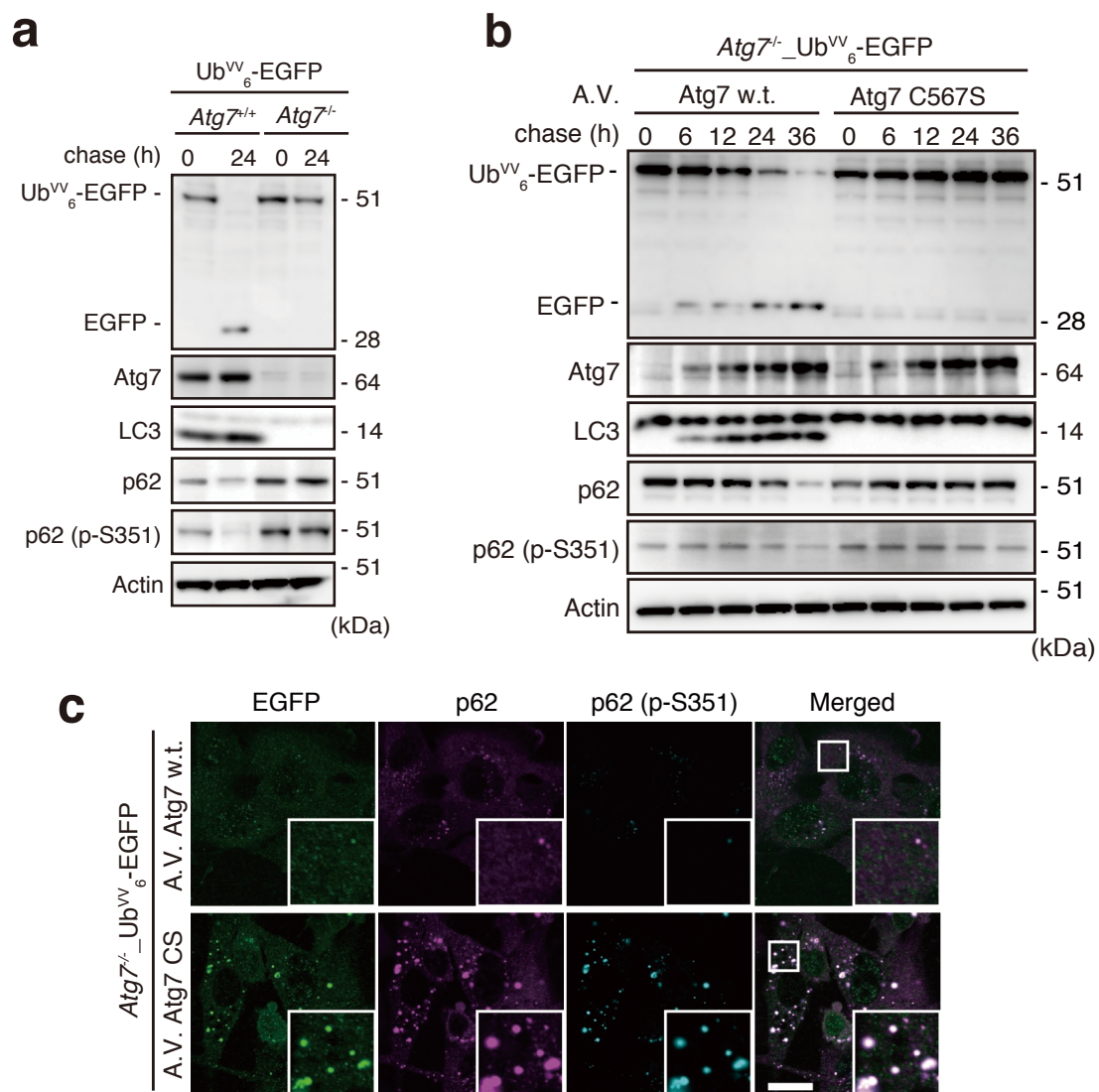


Figure 3-21. Macroautophagy selectively degrades intracellular polyubiquitin aggregates. **a**, Immortalized wild-type and *Atg7*-deficient MEFs were stably transfected with two regulator-gene cassettes, CAG-rtTA and TRE-Ub^{VV}₆-EGFP. The cells were cultured for 24 hours in the presence of Dox to induce expression of Ub^{VV}₆-EGFP. Subsequently, the cells were cultured in the absence of Dox for 24 hours. The cell lysates were prepared and immunoblotted with the indicated antibodies. Data are representative of two independent experiments. **b**, After induction of Ub^{VV}₆-EGFP in the *Atg7*-knockout MEFs as shown in (a), *Atg7* or *Atg7* C567S was expressed using an adenovirus system. At the indicated time points, cell lysates were prepared and immunoblotted with the indicated antibodies. Data are representative of two independent experiments. **c**, MEFs treated as shown in (b) were immunostained with p62 and Ser351-phosphorylated p62 antibodies, 36 hours after infection with the indicated adenovirus vectors. Bar: 20 μ m. Data are representative of three independent experiments.

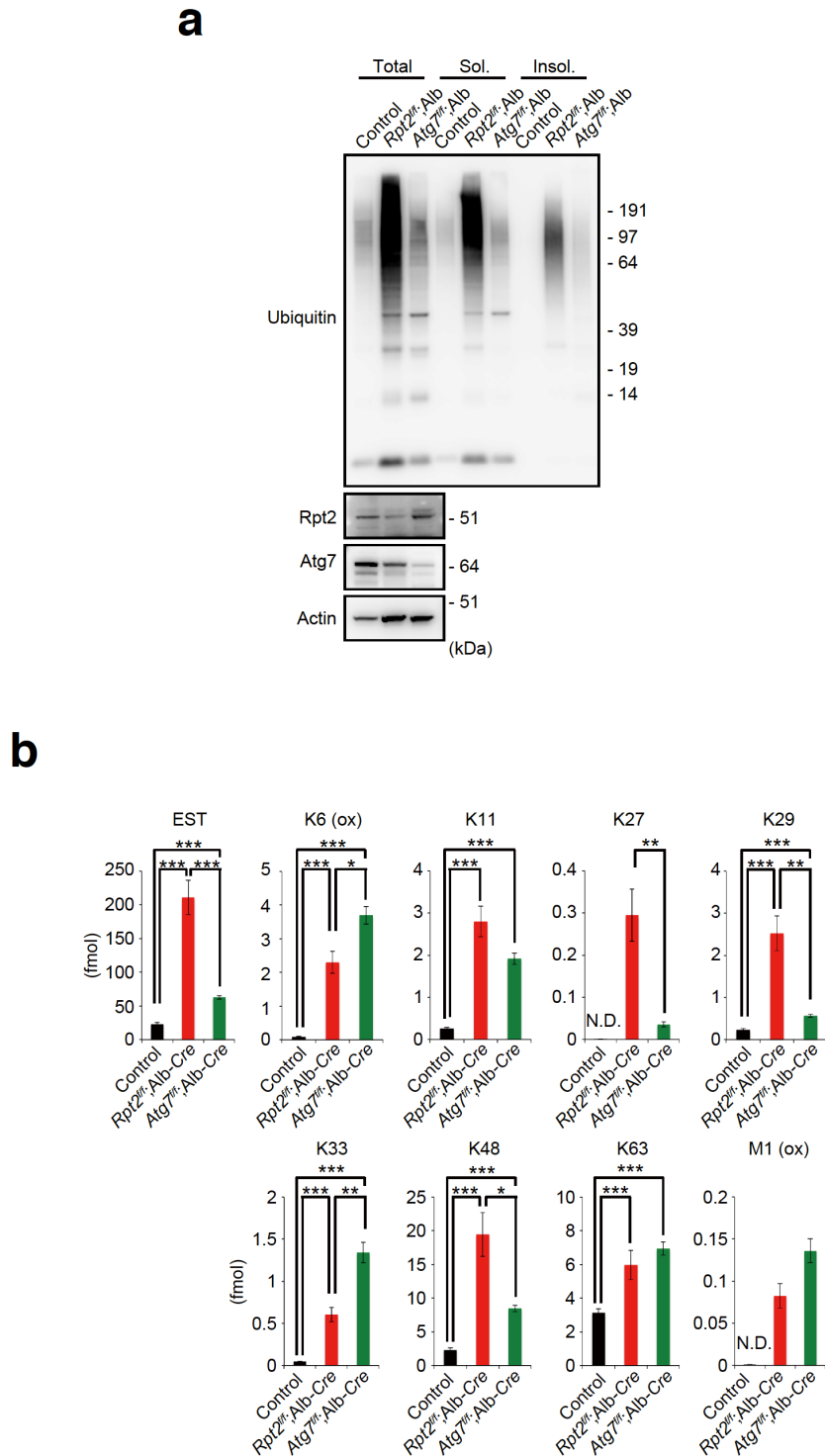


Figure 3-22. Characterization of ubiquitylated aggregates in *Rpt2^{lox/lox}*;Alb-Cre and *Atg7^{lox/lox}*;Alb-Cre livers. (a) Liver homogenates were prepared from *Rpt2^{lox/lox}*;Alb-Cre and *Atg7^{lox/lox}*;Alb-Cre mice at postnatal day 30. Total, soluble (Sol.), and insoluble (Insol.) fractions were subjected to immunoblotting with the indicated antibodies. Data were obtained from three independent experiments. (b) Absolute quantitation of total polyubiquitin chains (EST) and of each type of polyubiquitin chains in the insoluble fractions of control ($n = 4$), *Rpt2^{lox/lox}*;Alb-Cre ($n = 4$) and *Atg7^{lox/lox}*;Alb-Cre ($n = 5$) livers. Error bars represent the standard error of the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (Student's t -test).

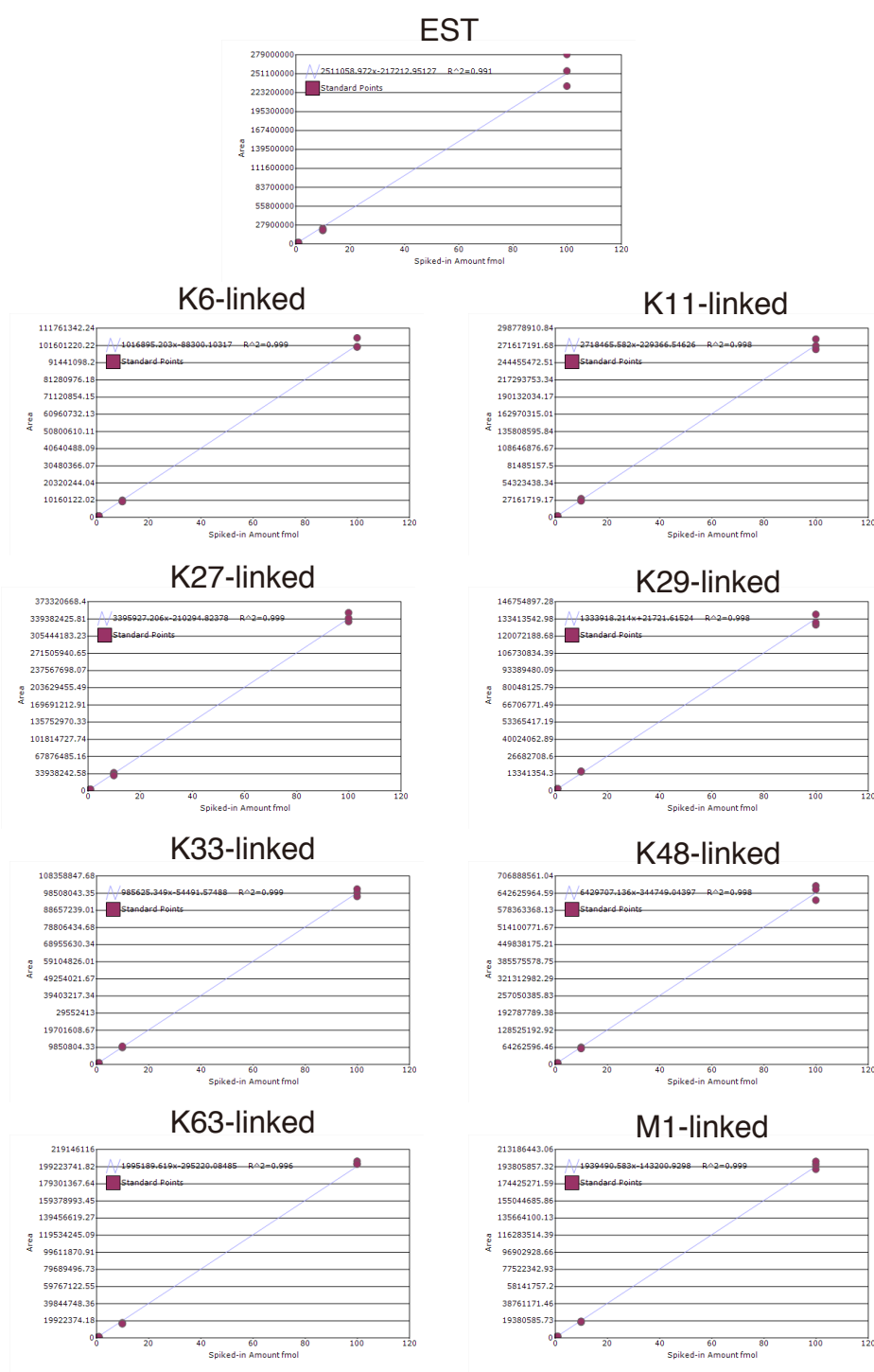


Figure 3-23. Establishment of standard curves for ubiquitin peptides. All nine ubiquitin peptides, including eight ubiquitin linkages and ESTLHVLR (EST), were spiked into the *E. coli* matrix (250 ng on column, MassPREP, Waters) and analyzed by PRM in triplicate. Standard curves were established covering the range from 100 amol to 100 fmol in the *E. coli* matrix. The calculated standard curve regression lines for ubiquitin-linkage peptides are presented ($R^2 = 0.991$ – 0.999). The acceptable range of variation was set by the FDA guidelines for the lower limit of quantification ($\pm 20\%$).

Table 3-1. **List of the ubiquitin peptides used in PRM analysis.** In the peptide sequences, the following abbreviations are used: EST, K6, K11, K27, K29, K33, K48, K63, and M1 for the corresponding ubiquitin peptides; Heavy for isotopically labeled ($^{13}\text{C}^{15}\text{N}$) amino acids (e.g., “HeavyL” denotes isotopically labeled leucine); di-GlyGly for the ubiquitylation site; and Oxid for Met oxidation. For each peptide, the optimal precursor ions, product ions, and retention times are listed.

Abbreviation	Peptide Sequence	Precursor m/z (charge state)	Product ions for PRM	RT min
EST	ESTLHLVLR	534.314 (+2)	y3, y4, y5, y6, y7	41.69
	ESTLHLVL[HeavyL]R	537.83 (+2)		
K6 (ox)	M[Oxid]QIFVK[di-GlyGly]TLTGK	465.927 (+3)	y5, y6, y7	41.96
	M[Oxid]QIFVK[di-GlyGly]TL[HeavyL]TGK	468.266 (+3)		
K11	TLTGK[di-GlyGly]TITLEVEPSDTIE NVK	801.427 (+3)	y8, y9, y10, y11, y12, y13	59.81
	TLTGK[di-GlyGly]TITLEVEPSDTIE N V[HeavyV]K	803.431 (+3)		
K27	TITLEVEPSDTIENVK[di-GlyGly]AK	701.039(+3)	y6, y10, y11, y12	54.18
	TITLEVEPSDTIENV[HeavyV]K[di-GlyGly]AK	703.044 (+3)		
K29	AK[di-GlyGly]IQDK	408.732(+2)	y3, y4, y5	23.34
	AK[di-GlyGly]I[HeavyI]QDK	412.241(+2)		
K33	IQDK[di-GlyGly]EGIPPDQQR	546.613 (+3)	y3, y4, y6	28.72
	IQDK[di-GlyGly]EGIPP[HeavyP]DQQR	548.618 (+3)		
K48	LIFAGK[di-GlyGly]QLEDGR	487.6 (+3)	y4, y5, y6, y7, y8, y9	43.53
	LIFAGK[di-GlyGly]QL[HeavyL]EDGR	489.9391 (+3)		
K63	TLSDYNIQK[di-GlyGly]ESTLHLVLR	748.738 (+3)	y5, y6, y7, y8, y9, y10	62.23
	TLSDYNIQK[di-GlyGly]ESTLHLVL[HeavyL]R	751.077 (+3)		
M1 (ox)	GGM[Oxid]QIFVK	448.2389 (+2)	y3, y4, y5, y6	32.78
	GGM[Oxid]QIFV[HeavyV]K	451.2458 (+2)		

3-7. Discussion

In this chapter, a novel and unexpected nature of ubiquitin was shown; that is, in contrast to the rigid structure of a single ubiquitin, ubiquitin becomes thermodynamically unstable when it is conjugated to another ubiquitin molecule or to another protein. This is unexpected because proteins with repeating identical domains have been considered to show increasing folding stability with an increasing number of domains (Cortajarena and Regan, 2011).

So why does length-dependent destabilization occur in polyubiquitin chains? One possibility is domain swapping, in which a secondary or tertiary element of a monomeric protein is replaced by the corresponding element of another protein molecule (Bennett et al., 2006). Recently, it has been reported that connecting of immunoglobulin domains in tandem causes fibril formation via a domain swapping mechanism (Borgia et al., 2011; Wright et al., 2005). Intriguingly, a similar domain swapping event has been proposed for diubiquitin (Xia et al., 2011). Therefore, elongation of ubiquitin into a ubiquitin chain may promote the formation of both intra- and inter-molecular domain-swapped structures. This may explain the length-dependent thermodynamic destabilization in polyubiquitin chains.

The thermodynamic stability of monoubiquitin is exceptionally high. Even polyubiquitin chains may have a higher thermodynamic stability than other cytosolic proteins. However, the temperature at which long polyubiquitin chains form fibrils is comparable with that at which several other amyloid-prone proteins form fibrils: myoglobin (horse skeletal muscle) forms fibrils by heating to 338 K (Fändrich et al., 2003) and β -lactoglobulin does so by heating to 353 K (Arnaudov et al., 2003). In addition, it is noteworthy that long polyubiquitin chains form insoluble fibrils at moderate shear stress under physiological temperature and pH (Figure 3-11b and c), implying that ubiquitin fibrils may form under physiological conditions even though the experimental conditions did not exactly mimic *in vivo* situations. It is currently not well understood, in what capacity melting temperatures of proteins determined *in vitro* correlate with protein aggregation *in vivo*. As the intracellular environment markedly differs from the test tube system in which these unfolding temperatures were determined, it is plausible that additional factors trigger the aggregation of a given ubiquitin chain in living cells.

An involvement of ubiquitylation in the sequestration and degradation of misfolded proteins has been already described (Kaganovich et al., 2008; Shiber et al., 2013) and it is reported that monoubiquitylation triggers sequestration of α -synuclein aggregates (Rott et al., 2008). However, the mechanism of how ubiquitylation induces aggregation-associated

sequestration has remained elusive. The *in vitro* data suggest that ubiquitylation has an effect on aggregation propensity and thermal folding reversibility of a substrate protein (Figure 3-12), which could be connected to the sequestration and degradation of misfolded proteins by ubiquitylation. But, how monoubiquitylation causes protein aggregation and why the kinetics of fibril formation depends on a substrate protein have been elusive; therefore, further analysis is needed to clarify these critical issues. In addition, the *in vivo* data indicate that simple overexpression of EGFP attached to linear hexaubiquitin, but not monoubiquitin, was sufficient for the formation of aggregate structures in cells (Figure 3-16a). This observation underscores that fibril formation occurs *in vivo*, when polyubiquitylated proteins, which accumulate owing to proteasome dysfunction or dysregulated deubiquitylation, are exposed to intracellular forces arising from cytoplasmic streaming, macromolecular crowding or (non-)specific protein interactions over a period of time. It will be necessary to determine the kind of intracellular forces involved and the length of time needed for ubiquitin fibril formation.

In selective autophagy, namely aggrephagy, mitophagy and xenophagy, different target proteins are ubiquitylated by distinct E3s such as Chip, Parkin, and LRSAM1 (Johansen and Lamark, 2011), implying that polyubiquitin chains of varying topology give rise to different types of selective autophagy. Nevertheless, in any of these types of selective autophagy, ubiquitylation triggers a common transduction signal — namely, the assembly of core Atg proteins and adaptor proteins such as p62 around the autophagic cargo. Therefore, the observation that the formation of fibrils by polyubiquitin chains was linkage-type independent (Figure 3-1d) suggests that the ubiquitin fibril plays a critical role in selective autophagy. It is proposed that polyubiquitin chains have two distinct biological roles: one is a linkage-specific signal for proteasome-mediated degradation and other non-proteolytic pathways. The other role is a linkage-independent, but length-dependent inducer of fibrillar structures for selective clearance by autophagy. The latter pathway would be cytoprotective: such insolubilization would prevent any undesired activities of the substrate proteins before protein aggregates start to accumulate in cells (Bjørkøy et al., 2005; Zhou et al., 2009).

In healthy cells, protein aggregates sequestered by ubiquitylation are degraded by selective autophagy before they form large inclusions (Figure 3-20a). On the other hand, the activity of the ubiquitin-proteasome system decreases with aging (Carrard et al., 2002) and loss (*i.e.*, dysfunction or inactivation) of autophagy has been previously described in senescent cells (Cuervo et al., 2005; Rubinsztein et al., 2011). Accordingly, most elderly

patients suffering from neurodegenerative diseases may have insufficient activity of autophagy – namely the capacity to specifically degrade intracellular ubiquitin-positive aggregates (ubiquitin fibrils). As a result, aging- and/or disease-associated inactivation of the proteasome and/or autophagy pathways may result in cytotoxic accumulation of ubiquitin-positive aggregates, even in the absence of aggregate-prone proteins related to conformational diseases. Ultimately, such ubiquitin-positive aggregates may be sequestered into so-called inclusion bodies, particularly in non-dividing cells such as neurons and myocytes. It is proposed that this is why most inclusion bodies observed in neurodegenerative diseases that have been reported contain ubiquitin as a major constituent (Mori et al., 1987). A unique feature of polyubiquitin chains is that it is the wild-type protein that forms fibrils; this feature is in stark contrast to other amyloid-forming proteins, many of which are truncated or carry mutations such as A β or SOD1 (Chiti and Dobson, 2009; Chiti et al., 2003). Therefore, the proposed model would take into account the pathological hallmarks of human sporadic proteinopathies without genetic mutations.

Collectively, intracellular ubiquitylation not only aids recruitment of proteins to the proteasome in solution, but also shields the cell from undesired activities of substrate proteins by encapsulating them in solid aggregates. Lastly, the structure of the ubiquitin fibrils acts as an initiation signal for autophagy. These observations are the first to report that ubiquitin chains function as a driving force to form fibrillar aggregates that have chain-length dependency but no linkage specificity and that these aggregates, whose accumulation disturbs cellular homeostasis, can act as direct substrates for removal by selective autophagy. The two biological roles of polyubiquitin chains (*i.e.*, a canonical linkage-specific signal and an inducer of fibril formation) may function independently and, accordingly, future studies should aim to elucidate the underlying mechanism that discriminates between them in a spatiotemporal fashion.

Chapter 4. Folding Destabilization by Ubiquitylation

4-1. Abstract

Compared with non-protein post-translational modifiers such as acetyl, methyl, and phosphate groups, ubiquitin is physically much larger. This property suggests that ubiquitylation may have a direct physical influence such as increasing the molecular weight/volume and molecular shape anisotropy of substrate proteins. A recent molecular dynamics analysis implies that ubiquitylation might cause partial unfolding of its substrate proteins. Nevertheless, this destabilization has not been investigated experimentally and the mechanism remains unclear. To get experimental evidence, the folding stability of ubiquitylated proteins was compared with that of their non-ubiquitylated counterparts. Tryptophan fluorescence spectroscopy reveals a global folding destabilization of the two different substrate proteins (FKBP12 and FABP4) due to ubiquitylation. Furthermore, comparative analysis of the respective spectral density functions showed the ubiquitylation-induced micro- to millisecond-scale structural fluctuations in both proteins examined. Thus, these results suggest that the structural fluctuation induced by ubiquitylation may be one of the factors to cause thermodynamic destabilization of ubiquitylated proteins. The folding destabilization induced by ubiquitin-conjugation may particularly contribute to efficient proteasomal protein degradation.

4-2. Introduction

Ubiquitylation (conjugation of ubiquitin molecule/chains) induces recognition of attached ubiquitin molecules by downstream proteins and then specific signal transduction occurs in a manner similar to other post-translational modifications such as phosphorylation. One of the most representative cellular functions of ubiquitylation is ATP-dependent protein degradation (Hershko and Ciechanover, 1992). Polyubiquitin-tagged substrate proteins are targeted to the 26S proteasome, followed by their unfolding and degradation by the proteasome in an ATP-dependent manner (Schrader et al., 2009). Recently, the ubiquitin-independent proteasomal protein degradation has also been observed in the case of some proteins (Orlowski and Wilk, 2003), whereas ubiquitylation plays a main role in intracellular protein turnover by the proteasome. In addition, ubiquitylation has also non-proteolytic functions such as regulations of protein activity and localization (Komander and Rape, 2012). Therefore, as other post-translational modifications, ubiquitylation participates in many cellular processes via the control of protein behavior.

In contrast, ubiquitin (8.6 kDa) and ubiquitin-like modifiers (8-20 kDa: Table 1-1) are relatively large modifiers, compared with other post-translational modifiers such as acetyl (43 Da), methyl (15 Da) and phosphate (97 Da) groups. This physical difference implies that conjugation of a ubiquitin molecule to a substrate protein would more directly affect the physical properties of the substrate protein such as molecular weight, molecular volume and molecular shape anisotropy. Indeed, a recent molecular dynamics analysis by Hagai & Levy showed that ubiquitylation might directly cause global unfolding of a substrate protein (Hagai and Levy, 2010). Thus, these imply that ubiquitylation may have not only biological effect, but also physical effect on a substrate protein. However, no experimental evidence for such physical effect by ubiquitylation has been obtained and its mechanism has remained unclear.

In the Chapter 3, the thermodynamic stability of ubiquitin itself decreases due to polymerization (Figure 3-1). Therefore, it is possible to hypothesize that a ubiquitylated protein would be thermodynamically destabilized via the same molecular mechanism as polyubiquitin chains. To obtain experimental evidence, in this chapter, the folding stability of a ubiquitylated protein and structural effect by ubiquitylation were investigated. Compared with a non-ubiquitylated protein, the ubiquitylated protein is thermodynamically destabilized and global structural fluctuations occur, which may cause such folding instability.

4-3. N-terminal and Site-specific Chemical Ubiquitylation

First, ubiquitylated proteins were prepared by two distinct kinds of ubiquitylation: N-terminal ubiquitylation (Ciechanover and Ben-Saadon, 2004b) and site-specific chemical ubiquitylation at a native ubiquitylation site. As models for substrate proteins, human FK506-binding protein (FKBP12) and human fatty acid binding protein 4 (FABP4) were chosen: both of which are ubiquitylated *in vivo*: FKBP12 and FABP4 are ubiquitylated at residue Lys53 (Wagner et al., 2011) and Lys121 (Wagner et al., 2012), respectively.

N-terminally ubiquitylated forms of these substrate proteins were constructed by genetic attachment monoubiquitin or linear hexaubiquitin to the N-terminal amino group of the protein (*i.e.*, Ub-FKBP12, Ub₆-FKBP12, Ub-FABP4 and Ub₆-FABP4). On the other hand, multiple methods to prepare chemically ubiquitylated proteins have been reported previously (Baker et al., 2013; Chatterjee et al., 2010; Chen et al., 2010; Eger et al., 2011; Kumar et al., 2011; Virdee et al., 2011). Some of them employ a disulfide conjugation of ubiquitin with a target protein (Baker et al., 2013; Chatterjee et al., 2010; Chen et al., 2010); in addition, any of these disulfide-mediated ubiquitylation protocols mimic native ubiquitylation. In this study, a simple and efficient protocol to prepare chemically ubiquitylated proteins has been established to avoid complicated intermediates and byproducts. Using activation of a cysteine thiol group by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Chen et al., 2010), the disulfide bridge is specifically formed between the C-terminus of ubiquitin and the native ubiquitylation site of a substrate protein (Figure 1b). To apply this protocol, a mutant of ubiquitin (G76C) and mutants of substrate proteins in which the lysine residue at the native ubiquitylation site is replaced by a cysteine residue (FKBP12 C22S K53C and FABP4 C2A C118A K121C) were prepared.

First, ubiquitin (G76C) was treated with DTNB resulting in activation of the C-terminal cysteine thiol group because there are no cysteine residues in native ubiquitin (Figure 4-1). This activation facilitates the subsequent disulfide bond formation with a free thiol group. A completely reduced substrate protein (FKBP12 C22S K53C or FABP4 C2A C118A K121C) was mixed with a 3-fold molar excess of activated ubiquitin G76C-DTNB for 1 hour, resulting in the formation of ubiquitylated proteins.

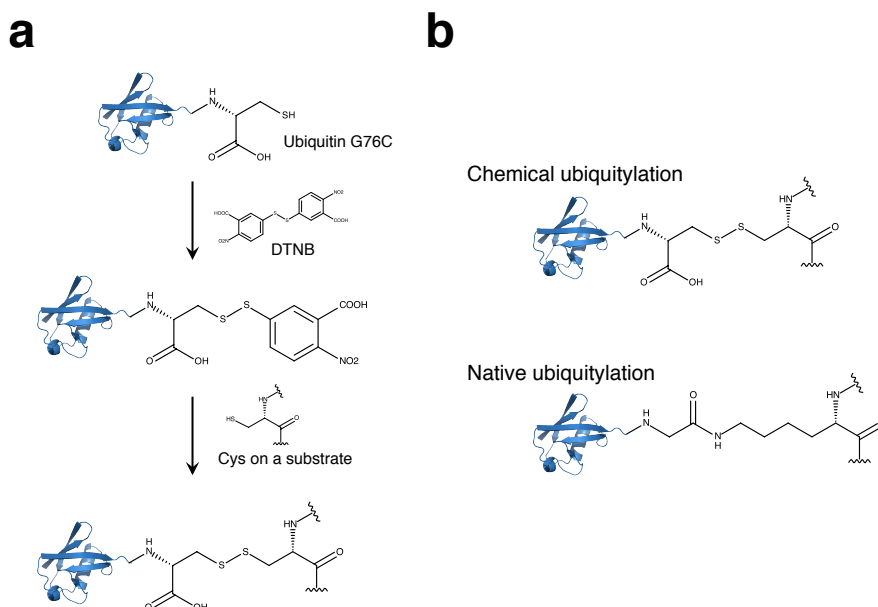


Figure 4-1. Synthesis of chemically ubiquitylated proteins. **a**, Scheme for conjugation of ubiquitin to a substrate protein via a disulfide bond. **b**, In this chemical ubiquitylation, the distance between ubiquitin and the substrate is shorter by two carbon-carbon bond distances than in native ubiquitylation.

To remove residual ubiquitin and disulfide-conjugated diubiquitin, it is necessary to perform additional purification. When the isoelectric point (pI) and/or the molecular weight of a substrate protein sufficiently differ from those of ubiquitin and diubiquitin, ion exchange chromatography and/or size-exclusion chromatography is available for separation of the product as reported previously (Baker et al., 2013; Chen et al., 2010). However, both FKBP12 C22S K53C and FABP4 C2A C118A K121C have a similar pI and molecular weight as ubiquitin; it is therefore difficult to isolate ubiquitylated proteins from the mixture with diubiquitin. For this reason, it is appropriate to employ hydrophobic interaction chromatography (HIC), which exploits the difference in protein hydrophobicity, for isolation; therefore, separation of several proteins whose electrostatic properties and/or molecular size are similar can be performed by application of an appropriate salt concentration gradient. Indeed, both ubiquitylated FKBP12 C22S K53C and FABP4 C2A C118A K121C were obtained in high purity as shown in Figure 4-2 and analysis by mass spectrometry supported this result (data not shown).

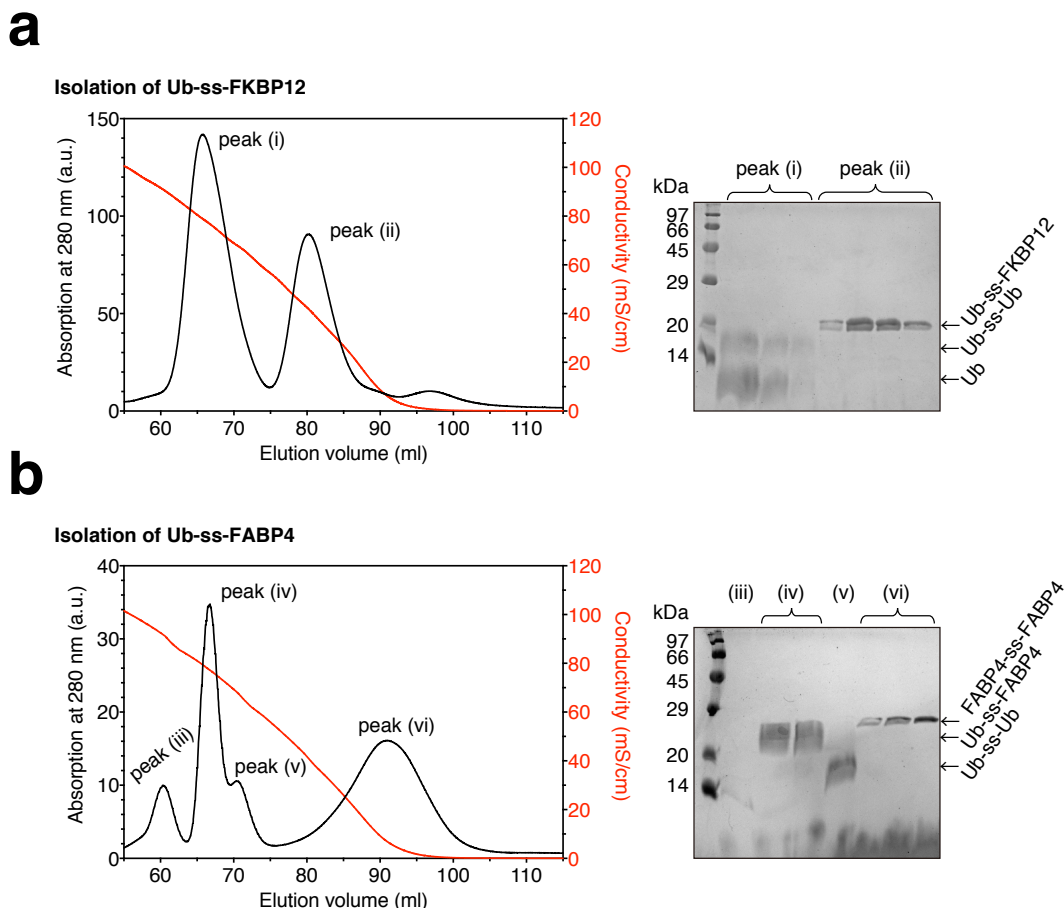


Figure 4-2. **Isolation of chemically ubiquitylated proteins.** Hydrophobic interaction chromatography profiles of chemically ubiquitylated FKBP12 (**a**) and FABP4 (**b**). The black lines represent UV absorption at 280 nm and the red lines display the conductivity of solution. Right, each peak in the purification profile was subjected to SDS-PAGE.

4-4. Ubiquitylation Causes Folding Destabilization of Substrates

To investigate the physical effect of ubiquitylation, it is necessary to compare the folding stability of ubiquitylated proteins with that of the non-ubiquitylated form. Native ubiquitin has no tryptophan residue, whereas both substrate proteins (FKBP12 and FABP4) have several tryptophan residues. Therefore, tryptophan emission spectra of ubiquitylated proteins will specifically reflect the chemical environment of the tryptophan residues in the substrate proteins. When a tryptophan residue participates in hydrogen bonding and/or is exposed to water, its emission shifts to longer wavelengths; therefore, the wavelength of tryptophan fluorescence emission selectively changes in response to conformational changes and/or denaturation in the substrate protein. As Figure 4-3a indicates, tryptophan fluorescence emission shifts were clearly observed in heat-treated FKBP12.

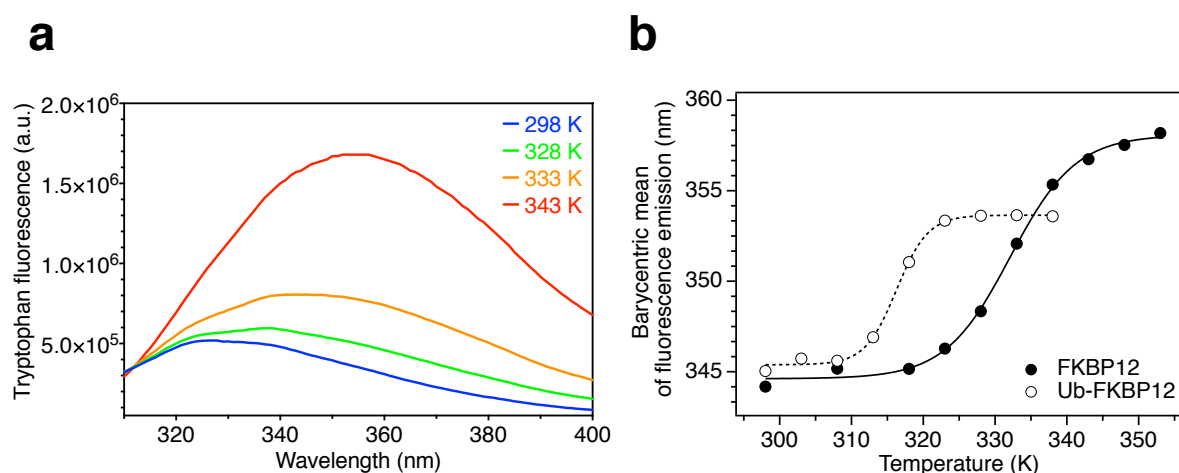


Figure 4-3. **Temperature-dependent changes in the tryptophan fluorescence emission spectra of FKBP12.** **a**, The tryptophan fluorescence emission spectra of FKBP12 at 298 to 343 K in PBS containing 0.5 mM TCEP. The emission maxima in the fluorescence spectra show a red-shift with increasing temperature. **b**, Series of fluorescence emission wavelengths (barycentric mean) of FKBP12 (filled circles) and N-terminally mono-ubiquitylated FKBP12 (open circles). The connecting line represent fitting to a sigmoidal equation. Similar profiles for the remaining samples are shown in the Figure 4-4.

The thermal denaturation transitions of FKBP12, FABP4 and their ubiquitylated forms were investigated by tryptophan fluorescence spectroscopy (Figure 4-3 and Figure 4-4). Fluorescence analysis shows that N-terminal mono-ubiquitylation decreases the thermal transition point of both substrate proteins by more than 5 K (Figure 4-5). In stark contrast, a simple mixture of a protein and ubiquitin (without covalent conjugation) does not appear to affect the transition temperature of that protein (Figure 4-5). Interestingly, N-terminal poly-ubiquitylation (attachment of linear hexa-ubiquitin) further enhanced unfolding of the substrate protein by ubiquitylation to a given extent (*ca.* 1.5 K) (Figure 4-5). Furthermore, thermodynamic analysis indicates that the enthalpy change between the folded and unfolded conformations of a ubiquitylated protein at physiological temperature (310K) was larger than that of the non-ubiquitylated form (Figure 4-6). In particular, because the Gibbs energy change between the folded and unfolded conformations of a ubiquitylated FKBP12 was smaller than that of the non-ubiquitylated FKBP12, the folding destabilization may be an entropy-driven phenomenon (Figure 4-6), which suggests that a substrate protein conjugated to a longer ubiquitin chain may be more destabilized. However, such a tendency seems to be limited in the case of FABP4 and chemically ubiquitylated proteins; therefore, the

thermodynamic parameters in folding destabilization may depend on the substrate protein and the way to conjugate.

Similarly to N-terminal ubiquitylation, chemical ubiquitylation decreased the thermal denaturation transition point of both substrate proteins (Figure 4-5). However, the effect of chemical ubiquitylation on the folding stability of a substrate protein appears to be smaller than that of N-terminal ubiquitylation. Taken together, covalent conjugation of (poly-)ubiquitin to a substrate protein decreased its folding stability and the degree of destabilization depends on the chain-length and the site of ubiquitylation.

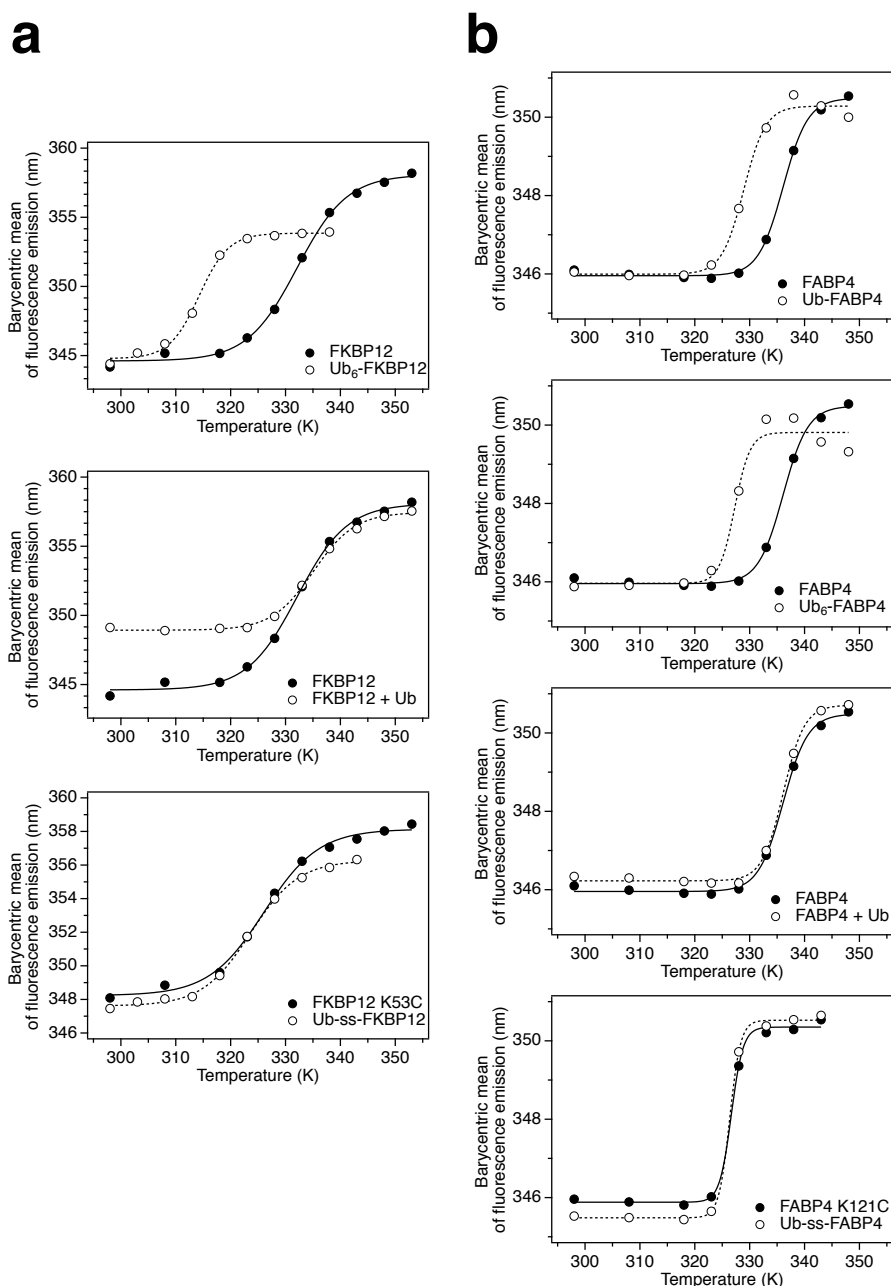


Figure 4-4. **Temperature induced tryptophan fluorescence red-shift in (poly-)ubiquitylated proteins.** The averaged fluorescence wavelength at each temperature was estimated by calculation of the barycentric means of the tryptophan fluorescence emission spectrum. The temperature-dependent fluorescence emission wavelengths of substrate proteins (filled circles), (poly-)ubiquitylated substrate proteins (open circles) and substrate proteins mixed with ubiquitin (open circles) were fitted to the sigmoidal equation (FKBP12, **a**; FABP4, **b**). The profile for Ub-FKBP12 is shown in Figure 4-3. Data are representative of three independent experiments.

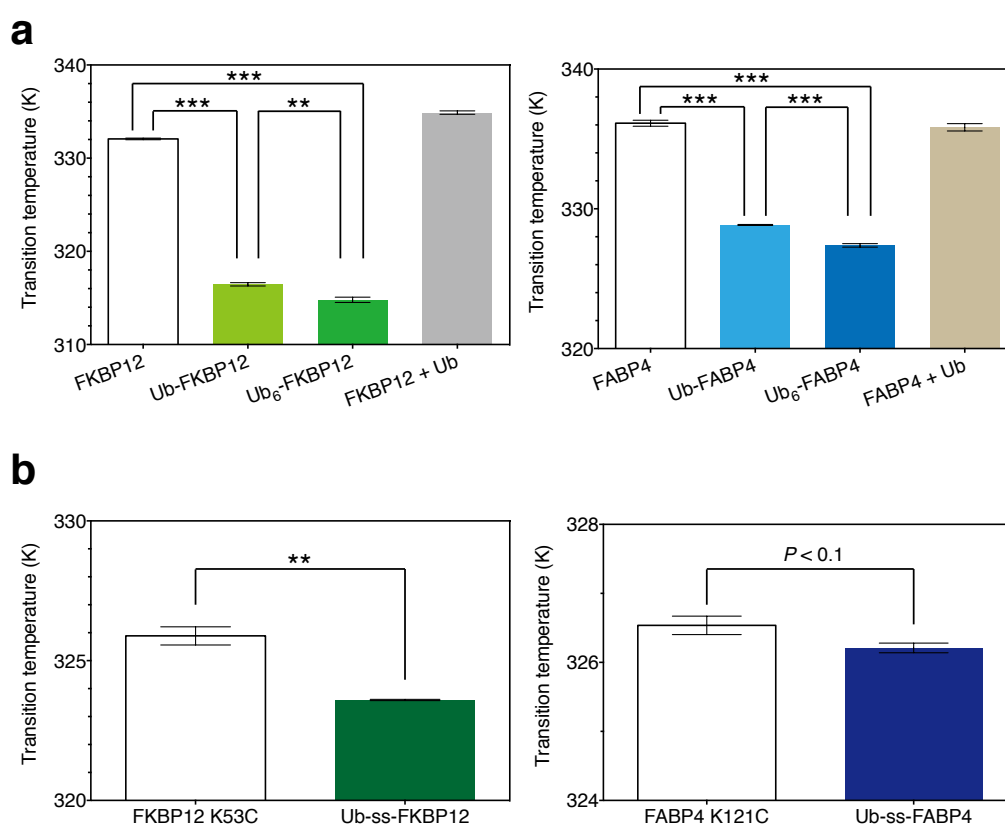


Figure 4-5. **Folding destabilization of a substrate proteins by (poly-)ubiquitylation.** **a**, Comparative analysis of thermal denaturation transitions for N-terminal (poly-)ubiquitylated FKBP12 (left) and FABP4 (right). Simple addition of ubiquitin to a substrate protein had little effect on its thermostability. *** $P < 0.001$ and ** $P < 0.01$ (Student's t test). **b**, Comparative analysis of thermal denaturation transitions for chemically ubiquitylated FKBP12 (left) or FABP4 (right). FKBP12 C22S K53C is referred to as FKBP12 K53C and FABP4 C2A C118A K121C is referred to as FABP4 K121C. ** $P < 0.01$ (Student's t test). All values represent the average of three independent experiments. Error bars, the standard error of the mean.

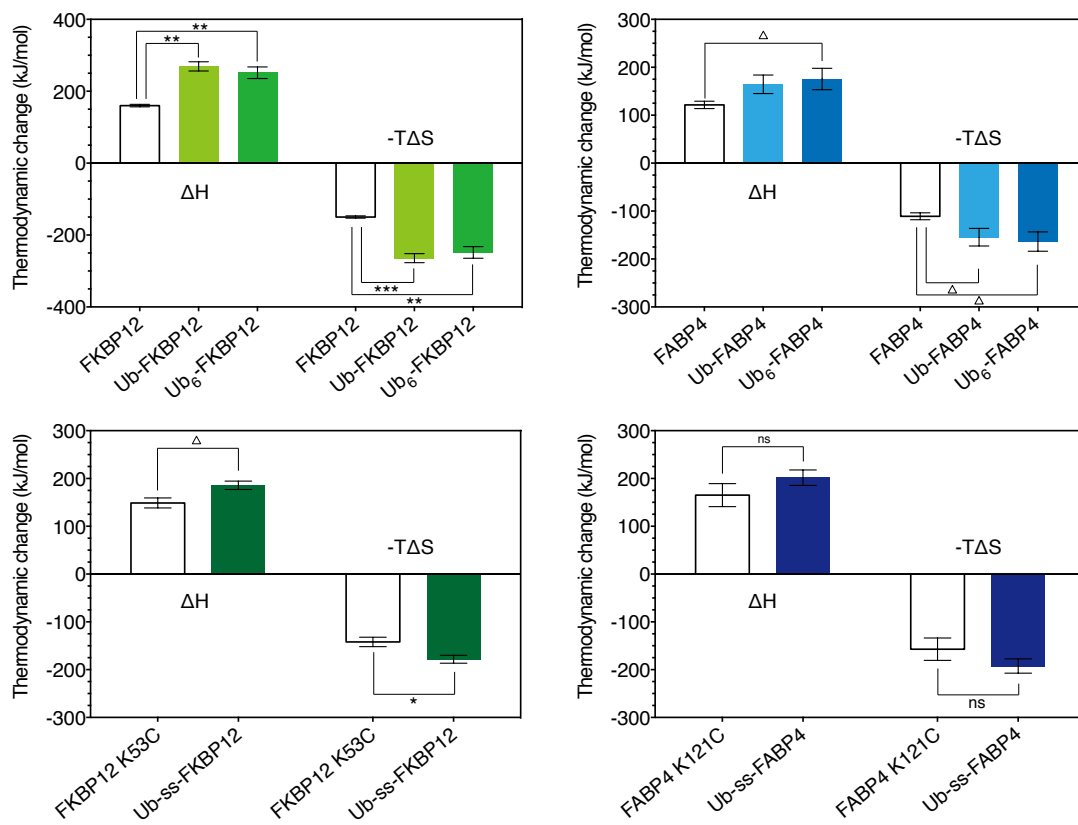
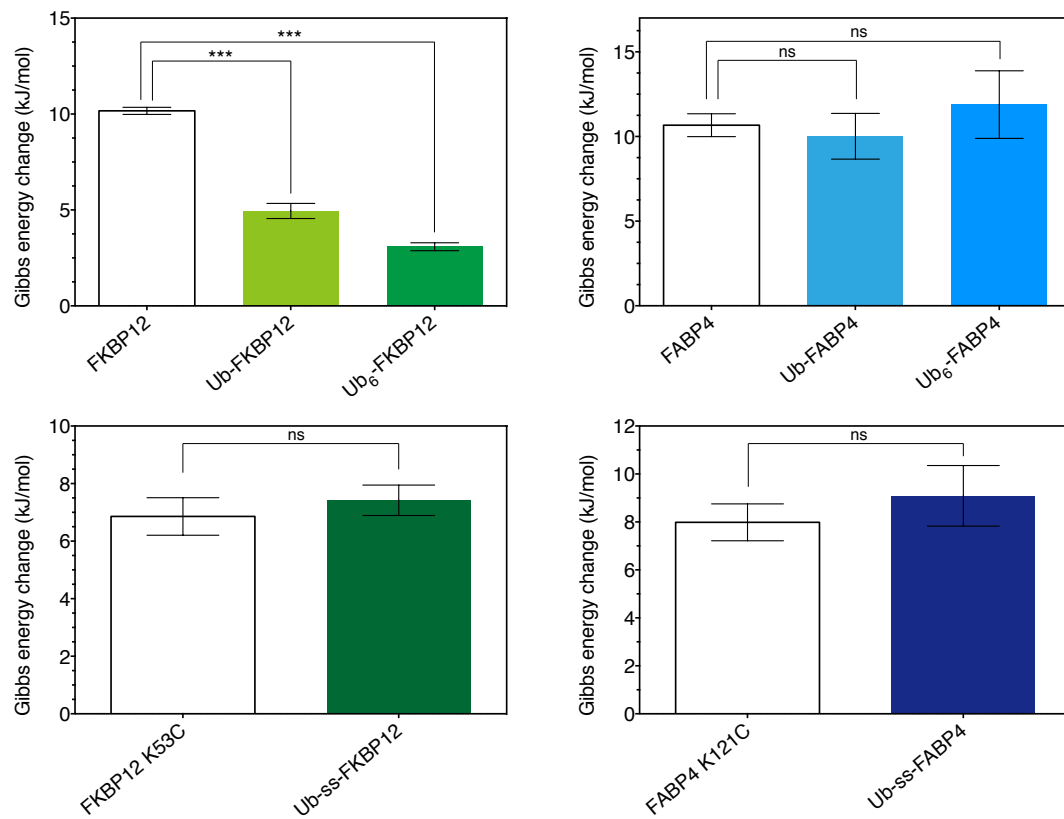
a**b**

Figure 4-6. **Thermodynamic parameters for heat-denaturation of ubiquitylated proteins.** Enthalpy and entropy changes between the folded and unfolded conformations of a protein at 310 K were obtained for N-terminally ubiquitylated (**a**, upper) and chemically ubiquitylated proteins (**a**, lower). The fraction denatured f_D at each temperature was estimated from the equation $f_D = \frac{\lambda - \lambda_N}{\lambda_D - \lambda_N}$ where λ is the tryptophan fluorescence emission wavelength at this temperature, λ_N is that of the native protein and λ_D is that of the completely denatured protein. λ_N and λ_D were obtained from sigmoidal fitting of the temperature-dependent fluorescence emission wavelengths (PACE and LAURENTS, 1989). The equilibrium constant K_{eq} of the heat denaturation reaction was calculated from the equation $K_{eq} = \frac{f_D}{1 - f_D}$, followed by estimation of the difference in Gibbs energy ($\Delta G = -RT \ln K_{eq}$). The enthalpy and entropy differences were obtained by fitting the ΔG values obtained to the linear function $\Delta G = \Delta H - T\Delta S$. The ΔG values at 310K obtained from respective ΔH and ΔS values are shown in **b**. All values represent the average of three independent experiments. Error bars, the standard error of the mean. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, $^{\Delta}P < 0.1$ and ns (not significant) (Student's t test).

4-5. Ubiquitin Traps an Unfolded State of FKBP12

Thermal unfolding of FKBP12 is reversible; in contrast, N-terminally ubiquitylated FKBP12 displays an irreversible transition (Figure 3-12). Because ubiquitin loses its thermal folding reversibility by polymerization and/or conjugation to other proteins (Figure 3-12), it is obscure whether FKBP12 in the ubiquitylated form is just entrapped by insoluble ubiquitin aggregates when heat-denatured, or it also loses folding reversibility. To probe the folding reversibility of FKBP12 in ubiquitylated form, we investigated whether its differential scanning calorimetry (DSC) profile could be obtained again after only FKBP12 was selectively thermally unfolded was investigated. Because the thermal transition point of FKBP12 is approximately 20 K lower than that of (poly-)ubiquitin, it is possible to selectively denature the FKBP12 moiety in Ub-FKBP12 or Ub₆-FKBP12. Although the thermal transition point of FKBP12 itself was found to be 335.5 K in the DSC measurements, the transition of FKBP12 in the (poly-)ubiquitylated form was approximately 10 K lower (Figure 3-12), which confirmed the thermodynamic destabilization of FKBP12 by N-terminal ubiquitylation in the tryptophan fluorescence experiments (Figure 4-5). When Ub-FKBP12 was heated up to 330 K, mildly cooled down to room temperature and then reheated, the DSC peak corresponding to FKBP12 was hardly recovered in the reheating thermograph, but the peak corresponding to ubiquitin was readily detected (Figure 4-7a upper). Similarly to Ub-FKBP12, the DSC peak corresponding to FKBP12 could not be obtained in the reheating

thermograph of Ub₆-FKBP12, but that of linear hexaubiquitin was detected (Figure 4-7a lower). These results indicate that (poly-)ubiquitylation causes proteins which show thermal folding reversibility to lose this reversibility. Interestingly, heat-treated (poly-)ubiquitylated FKBP12 was soluble and DSC analysis indicated that the selective heat-denaturation of FKBP12 did not affect the folding of (poly-)ubiquitin.

To investigate what structural changes occur in the selective heat-treatment of ubiquitylated FKBP12, it is necessary to measure its ¹H-¹⁵N hetero-nuclear multiple quantum coherence (HMQC) spectrum. The spectrum of non-heated (poly-)ubiquitylated FKBP12 displayed signals derived from both FKBP12 and ubiquitin. In contrast, no peaks corresponding to folded FKBP12 but those of (poly-)ubiquitin were detected in the spectrum of selectively heat-treated (poly-)ubiquitylated FKBP12 (Figure 4-7b and Figure 4-8). Instead of a simple disappearance of folded-FKBP12-derived signals, new peaks with random coil chemical shifts (Wishart et al., 1995) appeared due to the selective heat treatment, which confirmed that the selective heat-denaturation of FKBP12 in (poly-)ubiquitylated FKBP12 caused irreversible unfolding of FKBP12, but not of ubiquitin. Taken together, these results indicate that ubiquitylation does not only destabilize the structural fold of a substrate protein, but also stabilizes an unfolded state of the substrate protein which may be a consequence of the high solubility of ubiquitin.

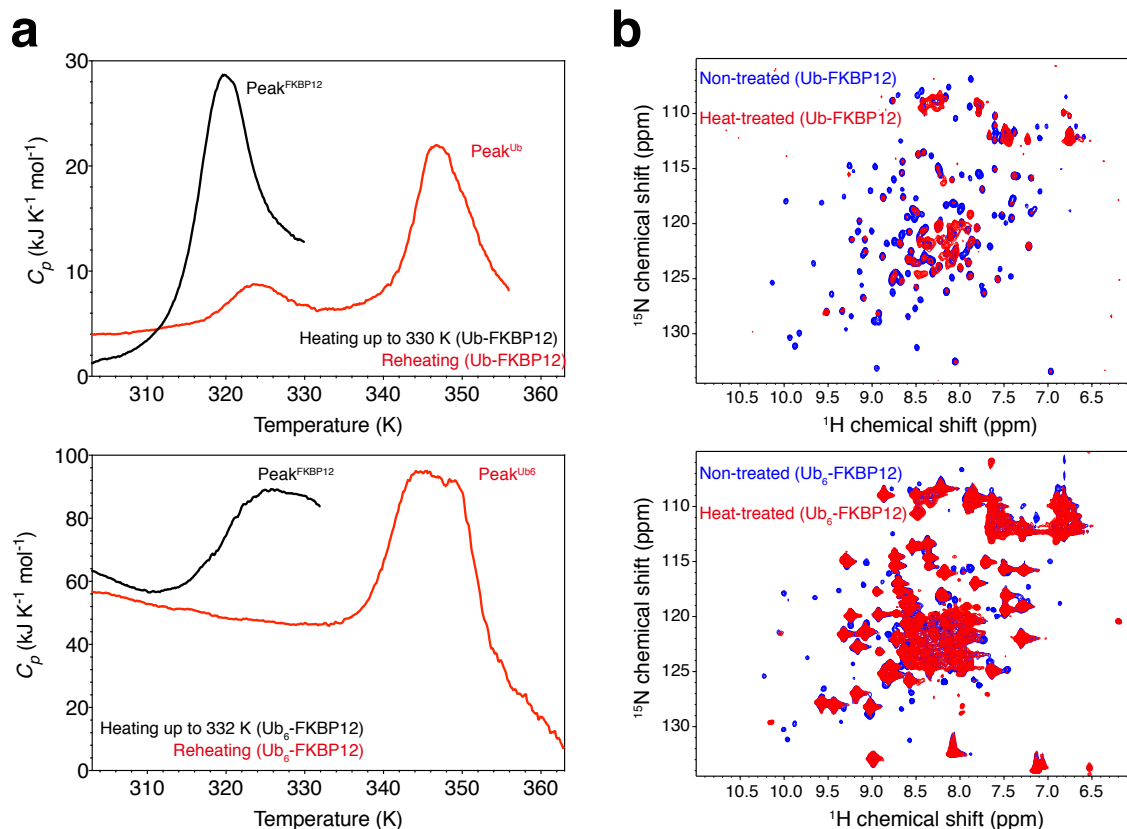


Figure 4-7. Irreversible thermal unfolding of FKBP12 in the N-terminally ubiquitylated form. **a**, Differential scanning calorimetry (DSC) traces of Ub-FKBP12 (upper) and Ub₆-FKBP12 (lower). Black traces show initial heating and red lines represent reheating. Each reheating experiment was performed after initial heating of Ub-FKBP12 to 330 K (upper) and after heating of Ub₆-FKBP12 to 332 K (lower). Peak assignments are based on the DSC measurements of FKBP12 and (poly-)ubiquitin in Figure 3-1 and Figure 3-12. **b**, ¹H-¹⁵N hetero-nuclear multiple quantum coherence (HMQC) spectra of non-heated (blue) and heated (335K) (red) ubiquitylated FKBP12. Most of the peaks of FKBP12 were not detected in the spectra of selectively heat-treated ubiquitylated FKBP12, however, (poly-)ubiquitin was still folded after the heat-treatment. The HMQC spectra of 40 μM Ub-FKBP12 were acquired in the PBS buffer containing 5 mM EDTA and 1 mM DTT at 298 K; those of 48 μM Ub₆-FKBP12 were measured in PBS buffer at 310K.

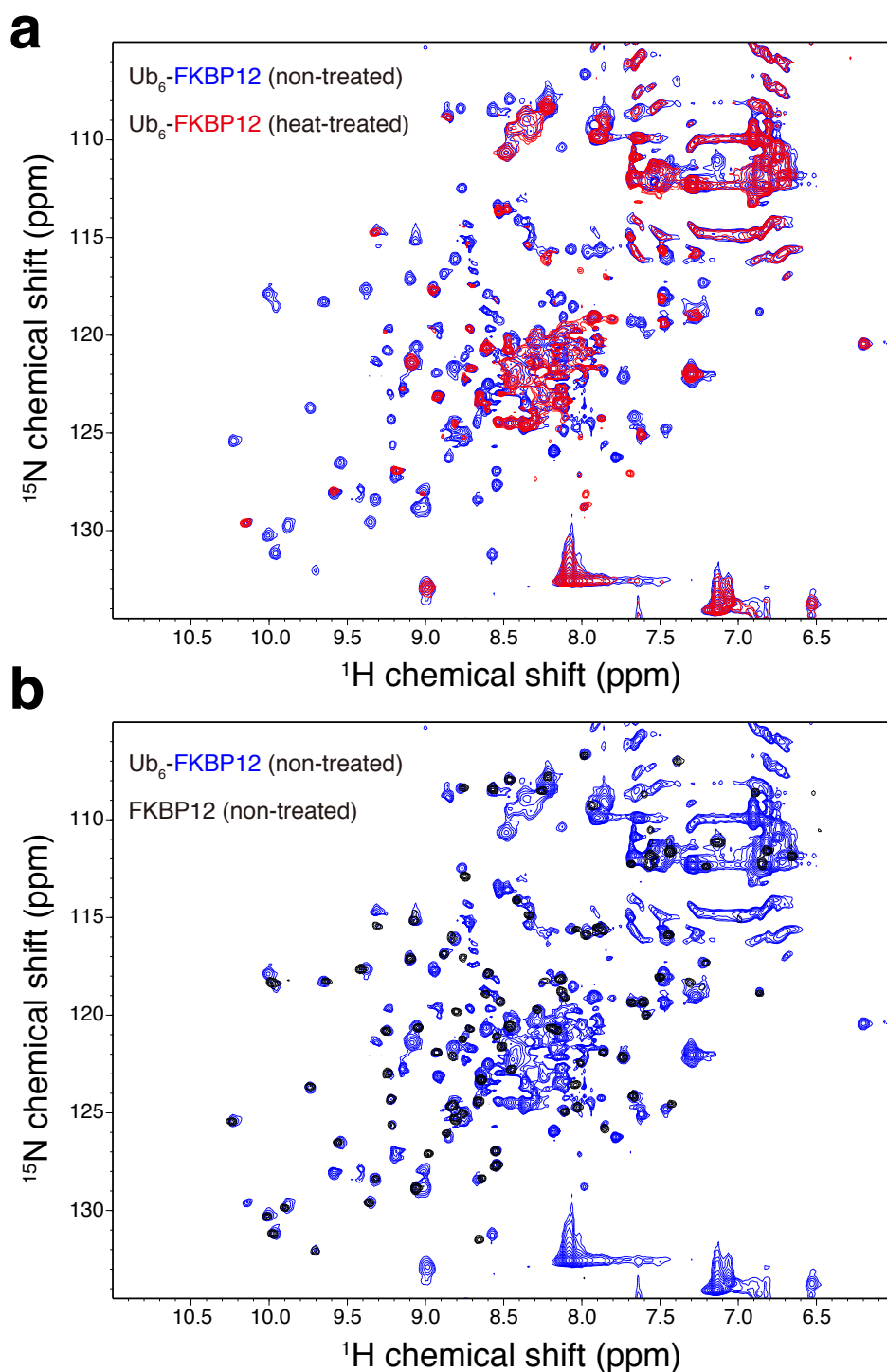


Figure 4-8. **Irreversible thermal unfolding of FKBP12 in Ub_6 -FKBP12.** **a**, ^1H - ^{15}N heteronuclear multiple quantum (HMQC) spectra of non-heated (blue) and heated (335K, red) Ub_6 -FKBP12. These spectra were obtained by subtracting the HMQC spectrum of Ub_6 from each spectrum of Ub_6 -FKBP12. **b**, Comparative analysis of the spectrum of the non-heated Ub_6 -FKBP12 with that of non-heated FKBP12. The HMQC spectrum of 40 μM FKBP12 was measured in the PBS buffer containing 0.5 mM TCEP at 310 K.

4-6. Ubiquitylation Induces Global Structural Fluctuations

To gain insight into the destabilization mechanism of substrate proteins by ubiquitylation, it is necessary to precise structural and dynamical parameters of ubiquitylated proteins. First, selectively labeled ubiquitylated proteins, in which only the substrate protein was isotope-labeled, were prepared because spectral overlap makes it difficult to separate NMR cross-peaks of a substrate protein from those of ubiquitin. Using unlabeled ubiquitin (G76C) and ^{15}N -labeled substrate proteins, ubiquitylated proteins were prepared by the method described in Figure 4-1. ^1H - ^{15}N hetero-nuclear single quantum coherence (HSQC) spectra showed little chemical shift perturbation in the spectra of ubiquitylated proteins, compared to those of the non-ubiquitylated form (Figure 4-9). This observation indicates that ubiquitylation did not significantly alter the overall structure of a given substrate protein.

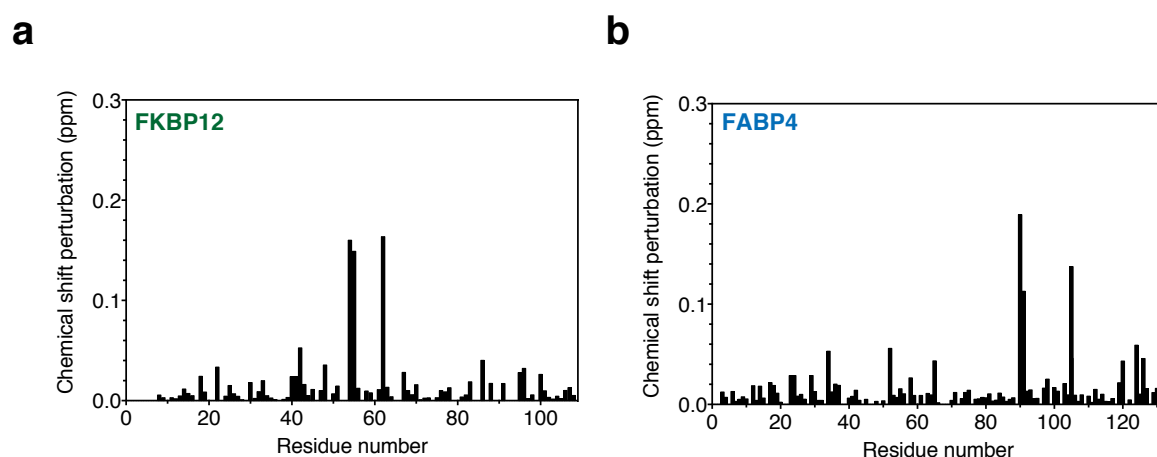


Figure 4-9. Ubiquitylation has little effect on the ground state structure of substrate proteins. Chemical shift perturbations due to chemical ubiquitylation of FKBP12 (a) and FABP4 (b) as obtained from the equation $\delta_{\text{wt}} = [\delta_{\text{HN}}^2 + (\delta_{\text{N}} / 5)^2]^{0.5}$ where δ_{HN} and δ_{N} are the ^1H and ^{15}N chemical shift changes between non-ubiquitylated and ubiquitylated proteins. The chemical shifts were obtained from hetero-nuclear single quantum coherence (HSQC) spectra of 500 μM protein in the phosphate buffer (20 mM KPO_4 , 5 mM KCl, 1 mM EDTA, 50 mM NaCl, 5 mM DTT, pH 6.8) at 298 K.

In contrast, steady-state ^1H - ^{15}N heteronuclear NOE (hnNOE) measurements detected changes in global structural fluctuations induced by ubiquitylation. The hnNOE values of ubiquitylated proteins were compared with those of the non-ubiquitylated form (Figure 4-10 upper). The hnNOE values of the ubiquitylated proteins were lower than those of the non-ubiquitylated form for both substrate proteins in the majority of regions. The ratio of the hnNOE values of ubiquitylated to non-ubiquitylated protein is mapped onto the respective structure in Figure 4-10 lower. Residues with an hnNOE ratio of less than the average were located not only in the vicinity of the ubiquitylation site, but found to be distributed rather globally (Figure 4-10 lower). Taken together, because the hnNOE values reflect protein backbone motion in a residue specific manner, these data indicate that ubiquitylation induces global structural fluctuations in substrate proteins.

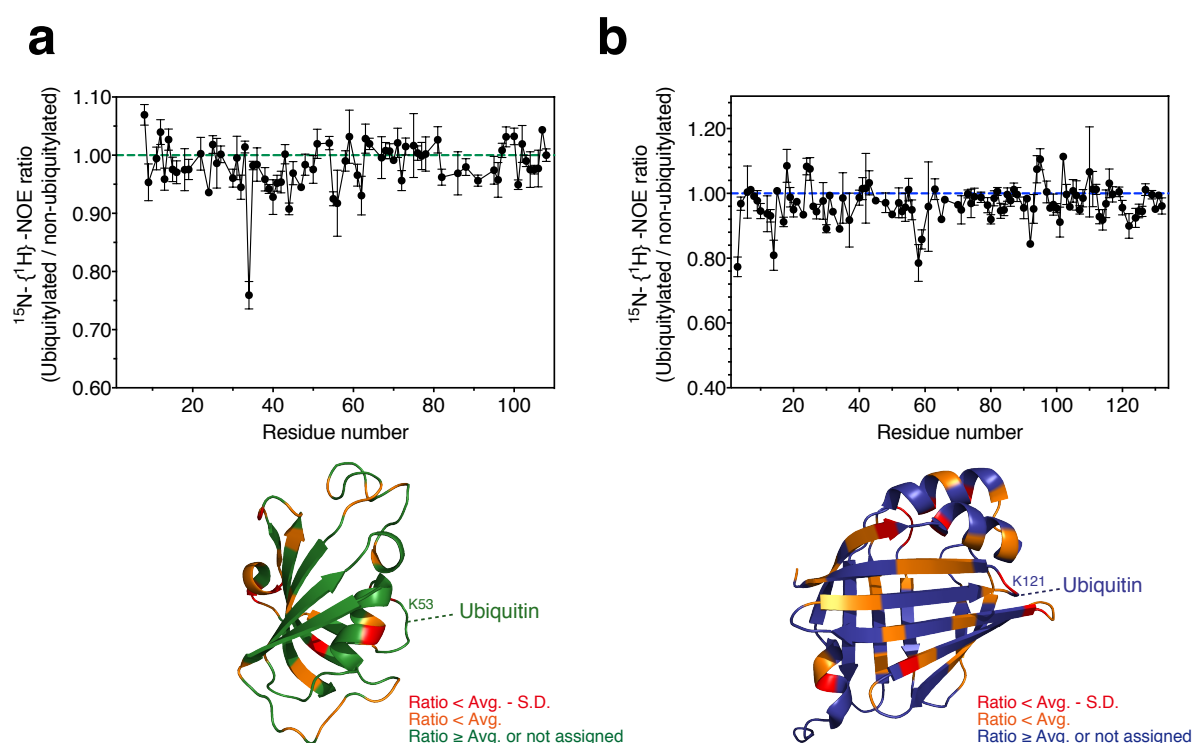


Figure 4-10. Ubiquitylation causes structural fluctuations in substrate proteins. Comparative analysis of ^1H - ^{15}N hetero-nuclear NOE values between non-ubiquitylated and ubiquitylated FKBP12 (a) or FABP4 (b). Upper panels show the ratio of the hnNOE values of ubiquitylated to non-ubiquitylated protein. Lower panels display the mapping of this hnNOE ratio onto the individual structures (2PPN for FKBP12 and 3RZY for FABP4 in PDB database were used). The ubiquitylation sites are indicated. Errors bars are obtained by error propagation.

Furthermore, it is important to probe the effect of ubiquitylation on protein backbone dynamics at three different frequencies by deriving the spectral density functions, $J(0)$, $J(\omega_N)$, $J(0.87 \omega_H)$ from ^{15}N T_1 , T_2 relaxation rate constants and ^1H - ^{15}N hnNOE values (Figure 4-11; the respective relaxation rate constants are shown in Figure 4-12). The spectral density function $J(\omega)$ quantitatively report on dynamics on a variety of timescales (pico- to millisecond) (Farrow et al., 1995). Because the increase in molecular weight by ubiquitylation affects the overall molecular tumbling of the substrate proteins in solution, ubiquitylation might in principle change the spectral density function $J(\omega)$ even if there were no differences in the structural dynamics between ubiquitylated and non-ubiquitylated proteins (see the equation 1-3 in the Materials methods). Thus, in general, it may be difficult to evaluate the effect of ubiquitylation on the spectral density function; nevertheless, more dispersed spectral density function of both ubiquitylated proteins was observed as compared with the deviation of the non-ubiquitylated forms (with the exception of $J(\omega_N)$ of (non-)ubiquitylated FKBP12) (Figure 4-11). In particular, the most remarkable differences between ubiquitylated and non-ubiquitylated proteins in the $J(0)$ values were detected (Figure 4-11). The average values of $J(0)$ for FKBP12 in the non-ubiquitylated and ubiquitylated form were 4.2 ± 1.3 (S.D.) nanoseconds (variance: 1.6 as^2) and 11.4 ± 6.0 (S.D.) nanoseconds (variance: 27.2 as^2), respectively; the individual values for FABP4 were 5.2 ± 1.8 (S.D.) nanoseconds (variance: 3.2 as^2) and 8.0 ± 2.2 (S.D.) nanoseconds (variance: 4.9 as^2). Thus, the variance in the $J(0)$ values of the substrate proteins increased due to ubiquitylation (Figure 4-11). This may reflect the possibility that ubiquitylation partially disturbs the dynamical integrity of a protein by causing integral protein dynamics to vary from residue to residue. Furthermore, residues for which $J(0)$ was larger than the average were distributed slightly more globally in the ubiquitylated proteins (Figure 4-13). Because $J(0)$ is sensitive to slow protein motions (micro- to millisecond), ubiquitylation may induce fluctuations in the dynamics of substrate proteins on this timescale. Unfortunately, the increase in molecular weight by ubiquitylation affects the overall molecular tumbling of the substrate proteins in solution; therefore, it is difficult to obtain their generalized order parameters by the model-free analysis in this study (LIPARI and SZABO, 1982a, b). These observations suggest that in substrate proteins global rigidity was relieved due to ubiquitylation, which indicates that these global fluctuations may lead to a decreased stability in the structural fold of the protein.

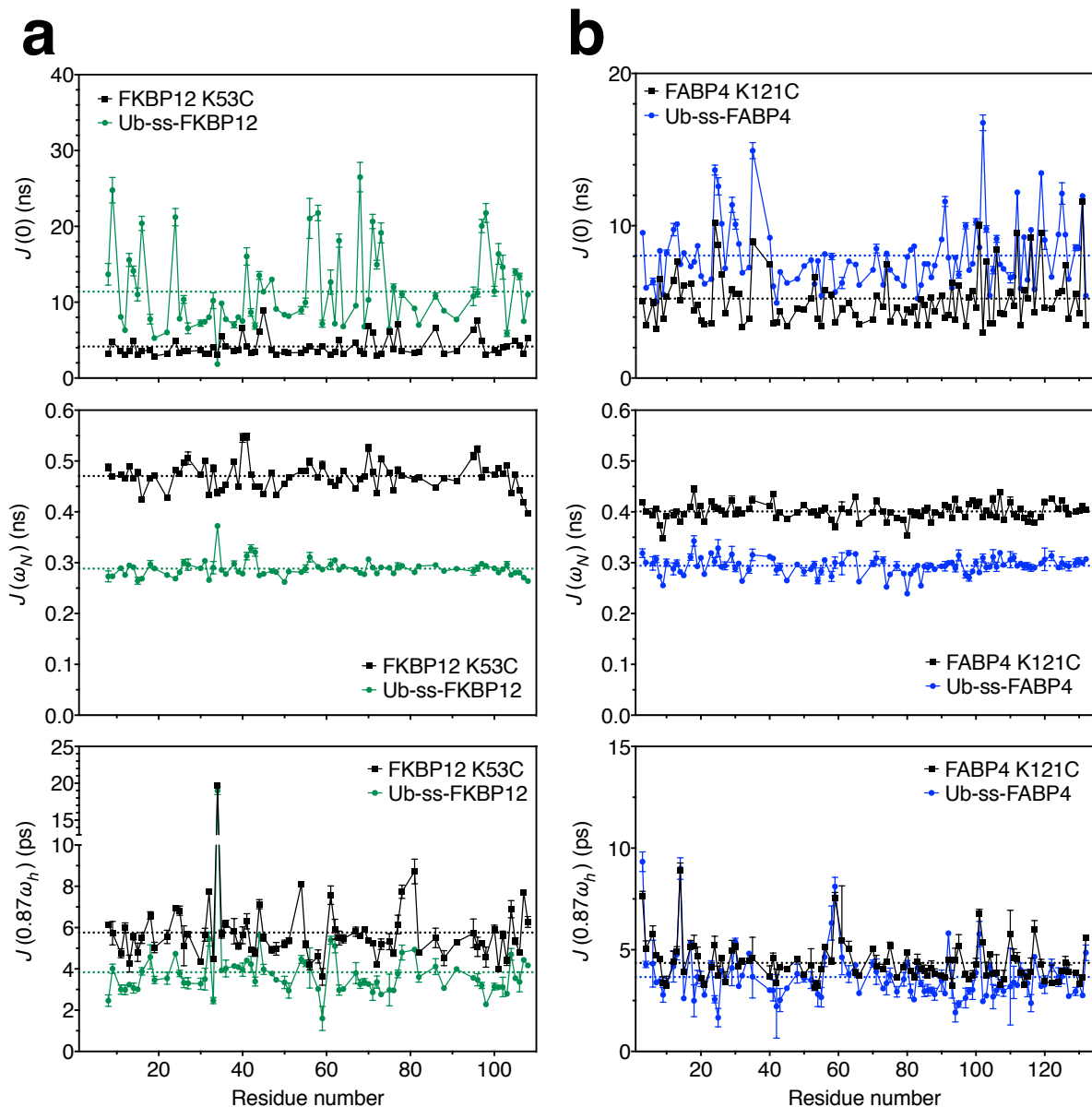


Figure 4-11. Increase in structural fluctuations of substrate proteins due to ubiquitylation. Spectral density functions at three different frequencies, $J(0)$, $J(\omega_N)$, $J(0.87\omega_h)$. **a**, comparative analysis of the $J(\omega)$ values for FKBP12 (black) and chemically ubiquitylated FKBP12 (green). **b**, comparative analysis of FABP4 (black) and chemically ubiquitylated FABP4 (blue). Dashed lines indicate the average of the respective $J(\omega)$ values. Errors bars for the spectral density functions are obtained by error propagation. FKBP12 C22S K53C is referred to as FKBP12 K53C and FABP4 C2A C118A K121C is referred to as FABP4 K121C.

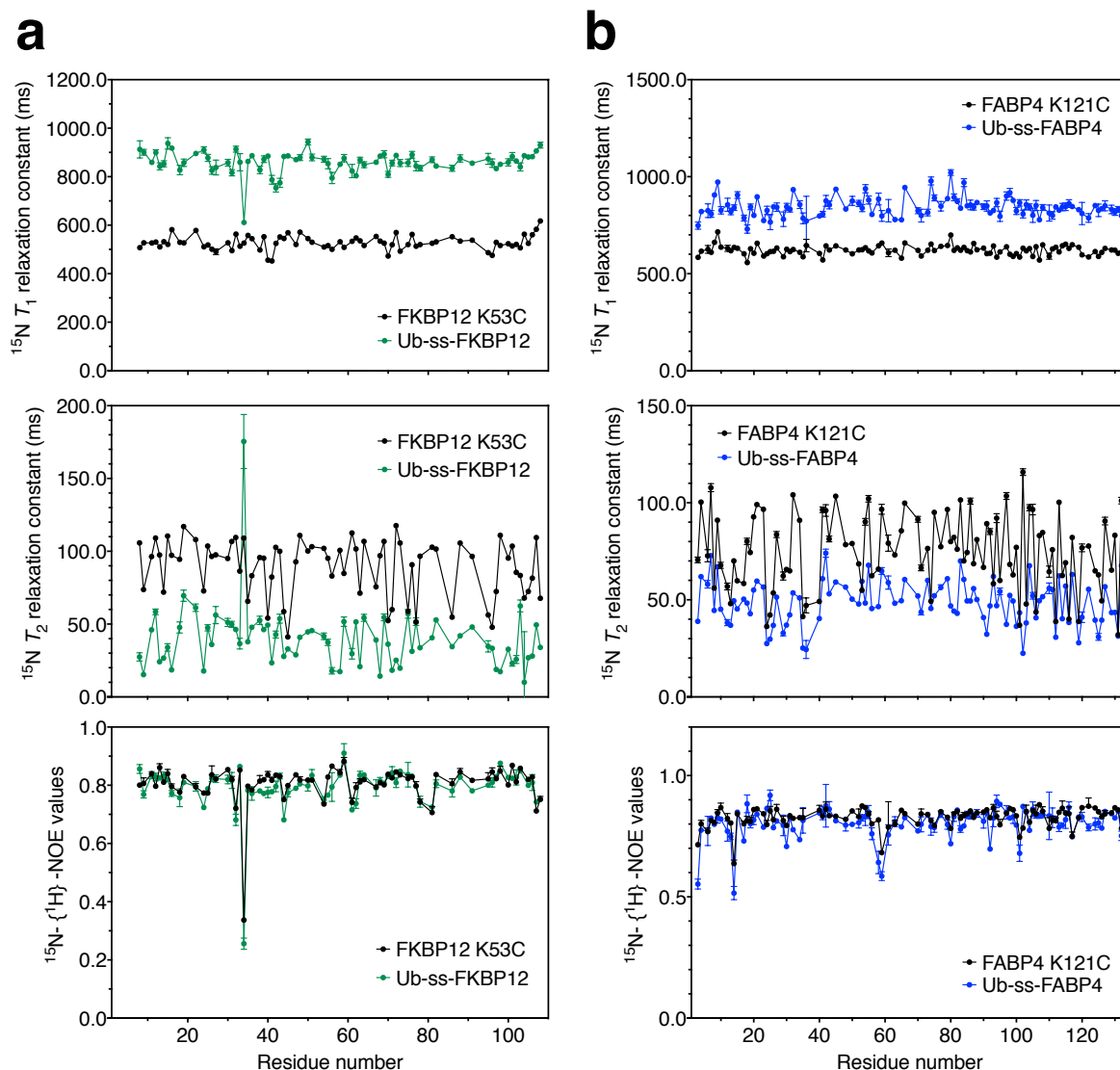
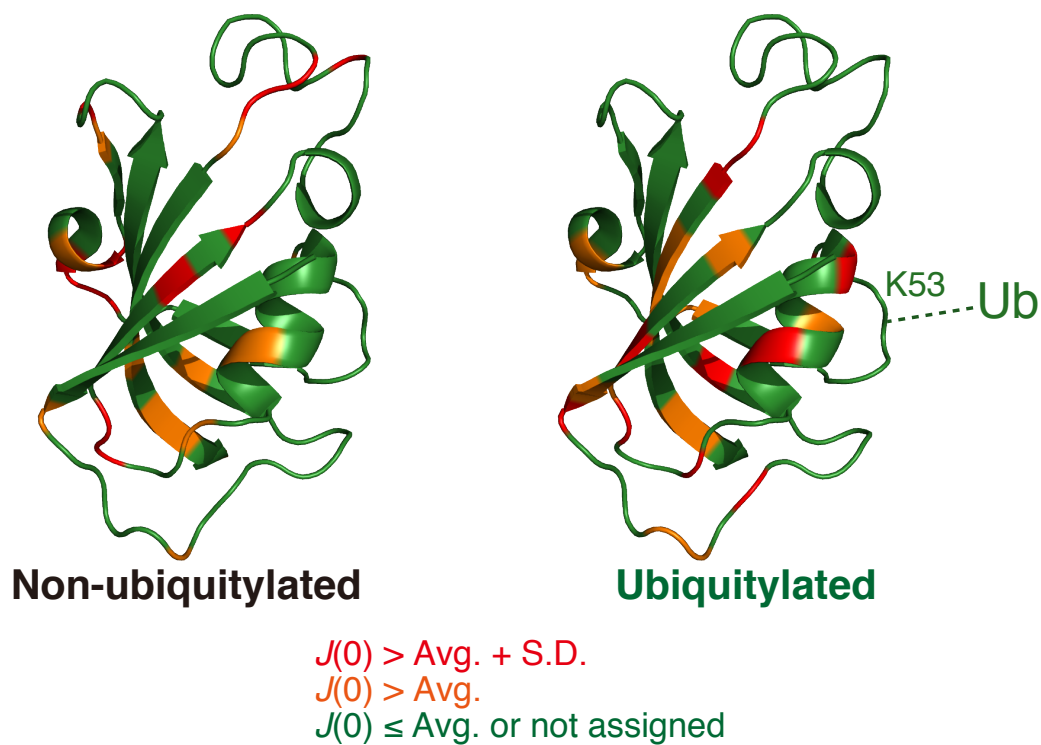


Figure 4-12. Relaxation rate constants of (non-)ubiquitylated proteins. Comparative analysis of ^{15}N T_1 , T_2 relaxation constants and ^1H - ^{15}N hetero-nuclear NOE values of non-ubiquitylated and ubiquitylated FKBP12 (**a**) and FABP4 (**b**). Experiments were performed in 20 mM KPO_4 , 5 mM KCl , 1 mM EDTA and 50 mM NaCl , pH 6.8 at 298 K. The buffer used for non-ubiquitylated proteins contained an additional 5 mM DTT . Error bars for T_1 and T_2 relaxation constants are calculated by the jackknife and Monte Carlo methods (Sugase et al., 2013) and error bars for hnNOE represent the standard error of the mean of two independent experiments. FKBP12 C22S K53C is referred to as FKBP12 K53C and FABP4 C2A C118A K121C is referred to as FABP4 K121C.

a



b

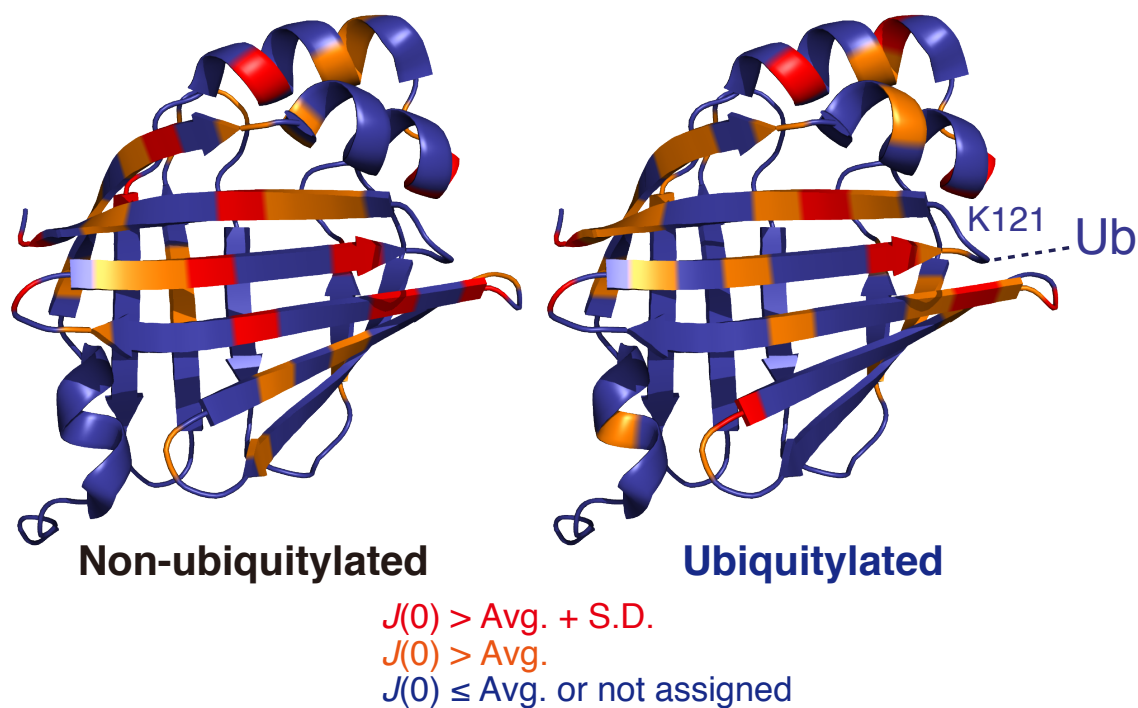


Figure 4-13. **Structural fluctuations of substrate proteins due to ubiquitylation estimated by spectral density function.** Mapping of $J(0)$ values of (non-)ubiquitylated substrate proteins onto the individual structures (2PPN for FKBP12 and 3RZY for FABP4 in PDB database were used). The respective ubiquitylation site is indicated.

4-7. Discussion

These observations present for the first time experimental evidence for a ubiquitylation-induced destabilization of the structural fold of proteins. For both substrate proteins examined, covalent conjugation of (poly-)ubiquitin to a substrate protein decreased the folding stability of that protein. Based on our observations, this ubiquitylation-mediated folding destabilization depends on the modification site of a substrate protein (Figure 4-5). Both FKBP12 and FABP4 form β -sheet structures at their N-terminal regions, whereas their native ubiquitylation sites are located at loops of the structure. This suggests that secondary structure elements at ubiquitylation sites may correlate with the degree of destabilization. On the other hand, the ubiquitylation-induced structural fluctuations were located not only at the ubiquitylation site but distributed rather globally. It will be necessary to investigate how ubiquitylation causes structural fluctuations and how these fluctuations in turn induce folding destabilization.

Ubiquitylated proteins are recognized by down-stream ubiquitin binding proteins, which induces specific intracellular signal transduction. The most common example of ubiquitin-mediated signal transduction is proteasomal degradation, which is primarily induced by K48-linked polyubiquitylation (Hershko and Ciechanover, 1992). Although K48-linked polyubiquitin chains play a central role in the ubiquitin-proteasome system, not only other linkage-type polyubiquitin chains, but also monoubiquitin induces proteasomal degradation, albeit less frequently (Kageyama et al., 2014; Shabek et al., 2012; Xu et al., 2009). In the course of proteasomal degradation, (poly-)ubiquitin tags mainly function in the delivery of substrate proteins to the 26S proteasome via their recognition by diverse ubiquitin receptors (Elsasser and Finley, 2005). Then, some of the delivered ubiquitylated proteins directly bind to the proteasome through association with the 19S regulatory particles subunits such as Rpn10 (Elsasser et al., 2004), Rpn13 (Husnjak et al., 2008), and Rpt5 (Lam et al., 2002). Other delivered ubiquitylated proteins indirectly associate with the proteasome by adaptor proteins such as Rad23 (Elsasser et al., 2004). Next, the substrate protein on the proteasome is unfolded by the AAA+ ring of the proteasome powered by ATP hydrolysis (Sauer and Baker, 2011). The protein is subsequently translocated to the proteasomal core, where it is degraded by the core protease machinery of the proteasome (Schrader et al., 2009).

One of the biological functions of the ubiquitylation-induced direct destabilization revealed in this study is suggested to be the contribution to the efficient proteasomal degradation. In the initiation of proteasomal degradation, the degron amino acid sequences

and/or intrinsically disordered tails/regions in substrate proteins (Hagai et al., 2011; Prakash et al., 2004) trigger unfolding of the entire structure. Many intracellular proteins have these unstructured regions for initiation of degradation, but a substantial fraction of proteins (about 25%) have few of such tails/loops (Hagai et al., 2011). Actually, such a protein lacking a sufficient amount of disordered tails/loops can also be degraded by the proteasome because folded domains are in equilibrium with their unfolded states. However, some additional steps or factors to unfold that protein may be needed for initiation of degradation. Therefore, it is possible to hypothesize that the direct partial unfolding by ubiquitylation could be one of the mechanisms to assist the unfolding of substrates. This direct partial unfolding by ubiquitylation would also decrease the amount of ATP required by proteasome to achieve complete denaturation of proteins.

Considering this hypothesis, it is necessary to discuss how long (poly-)ubiquitin tags keep to be conjugated with substrate proteins during their proteasomal unfolding and degradation. Because there are a large amount of deubiquitinating enzymes (DUBs) in cells, conjugation of (poly-)ubiquitin tags with substrate proteins may be dynamic: they can be cleaved and recycled in cells (Komander et al., 2009a). Indeed, the conjugated (poly-)ubiquitin tags of the recruited protein can also be cleaved off by the DUBs associated with the proteasome (Ubp6 and Uch13 in yeast) when unfolding of substrate proteins (Komander et al., 2009a; Schrader et al., 2009). However, the ubiquitin cleaving function of DUBs inhibits the initiation of proteasomal degradation because the substrate protein lacking (poly-)ubiquitin tags will be released from the proteasome machinery due to the decreased affinity with the 19S proteasome. The *in vitro* analysis for ubiquitin chain length indicates that polyubiquitin signals with more than four units of ubiquitin are more efficient for the degradation (Thrower et al., 2000); therefore, the association of ubiquitin tags with the proteasome is important for its degradation. On the other hand, a ubiquitin ligase Hul5 functions as an opposite role to the DUBs: this ligase is a component of the proteasome and can elongate ubiquitin chains on the substrate protein (Crosas et al., 2006). Thus, ubiquitin tags on the proteins delivered to the proteasome are in equilibrium between cleavage and elongation. However, it is reported that (poly-)ubiquitin chains are entirely cleaved off by hydrolyzing the isopeptide bond between the lysine residue of the substrate protein and the C-terminus of the first ubiquitin moiety in the tag by a 19S subunit Rpn11 in yeast when the substrate protein is committed for degradation (Verma et al., 2002). The cleaved ubiquitin molecules are released from the proteasome and recycles. Thus, although the chain length of

ubiquitin tags is variable dependently on the enzymatic activities of DUBs and/or ubiquitin ligases associated with the proteasome, the ubiquitin tags continue to be conjugated with the substrate protein just before the protein is degraded in the proteasome. Therefore, it is possible that the ubiquitylation-induced folding destabilization may participate in unfolding of the substrate protein. Future studies will need to focus on elucidating the underlying mechanism and quantitatively examining the effect of ubiquitylation of proteins. In addition, it is also necessary to investigate how the reaction of elongation and cleavage of ubiquitin tags with a substrate protein dynamically affect its folding stability.

Furthermore, the ubiquitylation-induced folding irreversibility observed in this study also may assist the proteasomal degradation. Generally, the folded state of a protein is thermodynamically more stable than its unfolded state. Therefore, unfolding of a folded protein needs some additional energy and/or enzyme catalysis; actually, the AAA+ ring of the proteasome is powered by ATP hydrolysis (Sauer and Baker, 2011). This suggests that the amount of ATP for unfolding and the following degradation depend on the folding stability of a substrate protein. Therefore, it is possible that some substrate proteins would be refolded even after they are once partially unfolded by the proteasome. This study has revealed that conjugation of ubiquitin tags with a substrate protein inhibits its refolding process, which suggests that ubiquitylation would avoid additional consumption of ATP by the proteasome. However, no biological evidence has been observed and therefore, it will be necessary to investigate how ubiquitin tags are associated with unfolding and refolding of a substrate protein for its proteasomal degradation.

Indeed, the ubiquitylation-induced folding destabilization and folding irreversibility observed in this study would lead to intracellular aggregate formation. If the (poly-)ubiquitin tagged proteins are not degraded by the proteasome appropriately or their ubiquitin molecules are not cleaved efficiently, they would accumulate in cells and form aggregates due to the thermodynamic destabilization by ubiquitylation. Taken together with polyubiquitin fibril formation caused by elongation of ubiquitin chains (Chapter 3), polyubiquitylated proteins may form insoluble aggregates either due to the chain-length-dependent destabilization of polyubiquitin chains or the ubiquitylation-induced destabilization of the substrate proteins. This indicates that ubiquitylation is closely associated with intracellular protein aggregation and that the relationship between ubiquitylation and human proteinopathies should be further elucidated.

Chapter 5. Materials and Methods

5.1. Protein Preparation

Mouse E1 enzyme UBA1 was expressed in Sf9 cells, yeast K63-linked E2 enzyme Ubc13-Mms2 complex was expressed in *Escherichia coli* strain BL21 Codon plus (DE3) RIL and all other recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3) in LB or M9 minimal media containing 99% ^{15}N -labeled ammonium chloride (Cambridge Isotope Laboratories). Untagged human ubiquitin was purified by cation exchange and size-exclusion chromatography (Tenno et al., 2004). Human 12-kDa FK506-binding protein (FKBP12) was expressed as a fusion protein with an N-terminal glutathione *S*-transferase (GST) and small ubiquitin-like modifier (SUMO)-1 protein tag. After cleavage of the protein tag by GST-SEN2 protease, FKBP12 was further purified by ion exchange and size-exclusion chromatography (Tsukiji et al., 2009). Untagged rat calmodulin was purified by Phenyl Sepharose Fast Flow (GE Healthcare) and size-exclusion chromatography (Hayashi et al., 1998). Human FABP4 was expressed as a fusion protein with an N-terminal hexa-histidine (His_6) SUMO-1 protein tag. After cleavage of the His-SUMO tag by GST-SEN2 protease, FABP4 was further purified by size-exclusion chromatography. Separation of FABP4 and bound ligands has not been performed. N-terminal hexa-histidine (His_6) tagged mouse UBA1, K48-linked E2 enzyme E2-25K and linear E2 enzyme UbcH7 was purified by Ni-NTA affinity chromatography (Kirisako et al., 2006; Tenno et al., 2004). Yeast Ubc13 was co-expressed with N-terminal His_6 -tagged yeast Mms2, followed by purification by Ni-NTA affinity and size-exclusion chromatography (Tenno et al., 2004). Human HOIL-1L (1-191) was co-expressed with N-terminal His_6 -tagged human HOIP (476-1072) and the HOIL-1L-HOIP complex (human linear E3 ligase) was purified by Ni-NTA affinity chromatography (Kirisako et al., 2006). K48-linked and K63-linked polyubiquitin chains were enzymatically synthesized by the appropriate E1, E2 and E3 enzymes shown above. Linear polyubiquitin chains were both expressed recombinantly and synthesized enzymatically (Kirisako et al., 2006). Separation of cyclic and non-cyclic K48-linked polyubiquitin chains has not been performed. Met1-monoubiquitylated and hexaubiquitylated FKBP12, which contain an additional DGGS sequence between ubiquitin and FKBP12, and an HRV3C-cleavable C-terminal His_6 -tag, were expressed and purified by Ni-NTA affinity chromatography. After cleavage of the C-terminal His_6 -tag by HRV3C protease, the fusion proteins were further purified by ion exchange and size-exclusion chromatography. Met1-mono- and

hexaubiquitylated calmodulin containing an HRV3C-cleavable C-terminal His₆-tag were expressed and purified in the same manner. Calcium ions, to which calmodulin and ubiquitylated calmodulin bind with high affinity, were removed by EGTA during the purification.

5.2. Crystallization

Crystallizations were carried out using the hanging-drop vapor-diffusion method at 293 K. K48-linked tetra- and hexaubiquitin chains were each screened in various conditions from commercially available kits. For each condition, 1 μ l of the protein solution was mixed with 1 μ l of the reservoir solution, and the mixture was equilibrated against 500 μ l of reservoir solution.

5.3. 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 Micro-max7 rotating anode X-ray generator (Rigaku) with an Osmic mirror system operating at 40 kV and 20 mA. X-ray diffraction data sets were collected at a wavelength of 1.000 Å on a beamline BL5A at the Photon Factory (Tsukuba, Japan). Crystals were flash-cooled at 100 K in cryoprotectant containing 20% glycerol.

5.4. Differential Scanning Calorimetry

Thermal denaturation curves were acquired by a Nano DSC instrument (TA Instruments Inc.). The scan rate was 1 K min⁻¹, and protein concentrations ranged from 0.2 to 1 mg ml⁻¹. The buffer used in the calorimetric experiments of (poly-)ubiquitin and (Met1-ubiquitylated-)calmodulin was PBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4). In the case of (Met1-ubiquitylated-)FKBP12, 0.1 or 0.5 mM TCEP was included to prevent cysteine-mediated dimerization. Reheating experiments were performed in the same manner after heating to the target temperature and subsequent mild cooling to room temperature. Analysis was performed using CpCalc (TA Instruments Inc.) and data were

reported as heat capacity ($\text{kJ K}^{-1} \text{mol}^{-1}$). The transition temperature was defined as the temperature corresponding to the peak top. However, precise thermodynamic parameters could not be acquired owing to the irreversibility of the melting reaction *in vitro*.

5.5. Transmission Electron Microscopy

TEM images were obtained using a JEM-1011 transmission electron microscope (JEOL). All samples were diluted to $100 \mu\text{g ml}^{-1}$ in 25 mM Tris-HCl, pH 8.0, and 150 mM NaCl, loaded onto a carbon grid and negatively stained with 2% (w/v) uranyl acetate or PTA (phosphotungstic acid). Scale bars were estimated by measuring Tobacco mosaic virus (TEV) under identical conditions. Images were analysed via ImageJ 1.45s.

5.6. Fluorescence Spectroscopy for Thioflavin-T binding Assay

Fluorescence was quantified on a FluoroMax4 (HORIBA) spectrometer at 298 K by excitation at 440 nm with acquisition of emission spectra over wavelengths of 460 to 550 nm with the slit width set at 5 nm. Monomeric samples were diluted to a final concentration of $5.8 \mu\text{M}$ in analysis buffer containing 25 mM Tris-HCl pH 8.0, 150 mM NaCl and $25 \mu\text{M}$ Thioflavin T. Polyubiquitin chains and ubiquitylated proteins were diluted to an equimolar concentration ($5.8 \mu\text{M}$) of monomeric ubiquitin subunits. In the case of (Met1-ubiquitylated-)FKBP12, 0.5 mM TCEP was included. The spectral contribution of the buffer was subtracted from the acquired spectra. To monitor fibril growth, the ThT fluorescence intensity at 480 nm was measured every 30 minutes with continuous stirring at 25 s^{-1} by a stirrer bar (Bel-Art Products). The fluorescence intensities at time t after the start, $I(t)$, were fitted to the equation $I(t) = I(t_{\infty}) [1 - \exp(-kt)]$ to obtain the rate constant k .

5.7. Tryptophan Fluorescence Spectroscopy

Tryptophan fluorescence was selectively measured by excitation at 300 nm with acquisition of emission spectra over wavelengths of 310 to 400 nm with the slit width set at 5 nm. All samples were diluted to a final substrate protein concentration of $20 \mu\text{M}$ in PBS buffer. In the

case of (Met1-ubiquitylated-)FKBP12 and FABP4, 0.5 mM TCEP was included. The spectral contribution of the buffer was subtracted from the acquired spectra. The peak shift was evaluated by calculation of the barycentric mean of the fluorescence emission spectrum. The

barycentric means was obtained from the equation $\lambda_{\text{bcm}} = \frac{\sum F(\lambda) \times \lambda}{\sum F(\lambda)}$. $F(\lambda)$ is the

tryptophan fluorescence intensity at λ nm. Using Igor Pro 6 (WaveMetrics), transition points were obtained by fitting a series of tryptophan emission wavelengths to the sigmoid equation

$$\lambda = \lambda_N + \left[\frac{\lambda_D - \lambda_N}{1 + \exp \left\{ \frac{T_{\text{half}} - T}{(rate)} \right\}} \right], \text{ in which } \lambda \text{ is the tryptophan fluorescence emission wavelength}$$

at temperature T Kelvin, λ_N is that of the native protein, λ_D is that of the completely denatured protein, T_{half} is the midpoint temperature of a sigmoidal curve and the *rate* is a parameter to determine slope of the curve.

5.8. Circular Dichroism Spectroscopy

Circular dichroism spectra were collected on a J-820 spectrophotometer (JASCO) from 250 to 200 nm in 0.1-nm intervals. Data were measured at 298 K with a 1-mm path length in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, containing 0.5 mM TCEP in the case of (Met1-ubiquitylated-)FKBP12. The concentration of all proteins used in the measurements was adjusted to 0.2 mg ml⁻¹. Data were reported as mean residue ellipticities. The spectral contributions by the buffer were subtracted.

5.9. Shear Stress in a Couette Cell

Controlled shear stress (Hill et al., 2006) was produced by an SM-101 instrument (As One), which incorporated an iron inner cylinder (8 mm) in a custom-built quartz cell (9 mm) at room temperature (298 K) (Figure 3-11a). Samples treated with shear stress were collected from the shear cell at each time point. Linear hexa-ubiquitin was diluted to an equimolar concentration (116 μ M) of monomeric ubiquitin subunits in analysis buffer containing 25 mM Tris-HCl, pH 8.0, and 150 mM NaCl. The samples were exposed to a moderate shear

rate of 47-60 or 470-600 s⁻¹. The shear rate in the Couette cell is calculated as

$$\dot{\gamma}(r) = \frac{2\Omega}{r^2(R_i^{-2} - R_o^{-2})}, \text{ where } \Omega \text{ is the angular frequency of the rotor (rad s}^{-1}\text{), } R_i \text{ is the radius}$$

of the rotor and R_o represents the radius of the quartz cell (Macosko; Ohgo et al., 2008).

5.10. Transfection

MEFs were maintained in DMEM (Gibco) with 10% fetal bovine serum, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). The cells were detached from the dish with 0.025% trypsin, and 2×10⁶ cells were centrifuged at 100×g for 10 min. The cell pellet was gently suspended in 100 µl of MEF Nucleofector solution (Amaxa) with 10 µg of expression plasmid pcDNA 3.1 cloned with EGFP, Ub^{VV}-EGFP or Ub^{VV}₆-EGFP, which contain an additional MASH sequence in front of ubiquitin and an additional GGSG sequence between ubiquitin and EGFP. Ub^{VV} indicates a C-terminal Val-Val ubiquitin mutant with the sequence MQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLAD YNIQKESTLHLVLRRLRVV. The cell suspension was electroporated by using the T-020 program (Amaxa).

5.11. Immunoblot Analysis

Samples were separated using 12% NuPAGE Bis-Tris gels (Invitrogen) in MOPS-SDS buffer, followed by transferring to polyvinylidene difluoride membranes. Antibodies against ubiquitin (Santa Cruz Biotechnology, Inc., P4D1), p62 (Progen Biotechnik, GP62-C), LC3B (Cell Signaling Technology, #2775), GFP (Invitrogen) and Actin (Chemicon International, Inc., MAB1501R) were purchased from the indicated suppliers. Anti-phosphorylated p62 polyclonal antibody was raised in rabbits using the peptide Cys+KEVDP(pS)TGELQSL as an antigen (Ichimura et al., 2013). The polyclonal antibody against Atg7 was raised in rabbits using the synthetic peptide VVAPGDSTRDRTLDDQQ which corresponds to the amino acid residues 556-571 as an antigen (Tanida et al., 1999). For blotting, indicated antibodies were used at a dilution of 1:500.

5.12. Immunocytochemistry

Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 or 50 $\mu\text{g ml}^{-1}$ digitonin in PBS. After washing with PBS, the coverslips were blocked with PBS containing 10% normal goat serum (Jackson Immuno Research) for 1 hour or 0.1% (w/v) gelatin (Sigma-Aldrich) in PBS for 30 min, and then incubated overnight with 150 or 200-fold diluted solution of primary antibodies against ubiquitin (Dako, Z 0458), p62 (Progen Biotechnik, GP62-C), Ser351-phosphorylated p62 and/or LC3B (Cell Signaling Technology, #2775). After washing with PBS, the coverslips were incubated with a 1000-fold diluted solution of Alexa Fluor-conjugated goat anti-guinea pig and/or anti-rabbit IgG secondary antibodies (Invitrogen) for 1 hour. Images were taken by confocal laser scanning microscopy using an FV1000 microscope (Olympus). Z-projection stack images were acquired with z-steps of 0.5 μm . Image contrast and brightness were adjusted using Photoshop CS4 (Adobe Systems, Inc.).

5.13. Mice

Rpt2^{ff} mice (Bedford et al., 2008) and *Atg7^{ff}* mice (Komatsu et al., 2006) were crossbred with Albumin-*Cre* transgenic mice (Postic et al., 1999) to generate *Rpt2^{ff};Alb-Cre* and *Atg7^{ff};Alb-Cre* mice, respectively. Mice were housed in specific pathogen-free facilities, and the Ethics Review Committee for Animal Experimentation of the Tokyo Metropolitan Institute of Medical Science approved the experimental protocols.

5.14. Quantitation of ubiquitin chains by mass spectrometry

Ubiquitin chains were quantitated by parallel reaction monitoring (PRM), a MS-based high-resolution quantification method (Tsuchiya et al., 2013). In brief, insoluble proteins (20 μg) prepared as above were separated by SDS-PAGE on 4–12% NuPAGE Bis-Tris gels (Life Technologies) with a short run (3 cm). The gel region corresponding to a molecular weight of above 62 kDa was excised, diced into 1-mm³ pieces, and subjected to in-gel trypsinization. Trypsinized peptides were extracted, spiked with nine ubiquitin AQUA peptides (K6-, K11-, K27-, K29-, K33-, K48-, K63-, and M1-linkages, and ESTLHVLR [EST]), and oxidized with

0.05% H_2O_2 in 0.1% trifluoroacetic acid at 4°C overnight. The peptides were analyzed in targeted MS/MS mode on a Q Exactive mass spectrometer coupled with an EASY-nLC 1000 liquid chromatograph (Thermo Fisher Scientific). Raw files were processed by PinPoint software version 1.3 (Thermo Fisher Scientific). The transition list and standard curves of the ubiquitin peptides are shown in Table 3-1 and Figure 3-23, respectively.

5.15. Chemical Conjugation of Ubiquitin to Substrate Proteins

A ubiquitin G76C mutant was reduced with 5 mM 2-mercaptoethanol and then buffer exchanged into 50 mM sodium phosphate buffer pH 7.5 using a PD-10 desalting column (GE). Reduced ubiquitin mutants were mixed with 20-fold molar excess of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Tokyo Chemical Industry) and then incubated for 1-3 hours at room temperature with vigorous shaking. The reaction solution was purified with ligation buffer (20 mM Tris-HCl, 50 mM NaCl and 1 mM EDTA, pH 7.0) using PD-10 desalting column. The cysteine mutant of the respective substrate protein was also reduced, buffer exchanged into ligation buffer and mixed with 3-fold molar excess of the activated ubiquitin G76C-DTNB for 1 hour.

5.16. Isolation of Chemically Ubiquitylated Proteins

Chemically ubiquitylated proteins were isolated by hydrophobic interaction chromatography (HIC) chromatography using a HiTrap Phenyl HP column (GE Healthcare). The proteins were dissolved in high salt buffer (2 M potassium phosphate, pH 7.0), applied onto the column, washed with more than two column volumes and eluted by a salt gradient (2 to 0 M potassium phosphate, pH 7 in five column volumes). The purity of the eluted protein was examined by SDS-PAGE and MALDI-TOF mass spectrometry (Bruker) analysis.

5.17. NMR Spectroscopy

All NMR spectra were acquired at 298 K or 310 K on an Avance 600 NMR spectrometer equipped with a 5 mm $^{15}\text{N}/^{13}\text{C}/^1\text{H}$ z-gradient triple resonance cryoprobe (Bruker Biospin).

Resonance assignments for ^1H - ^{15}N cross peaks are based on the previous studies (Bai et al., 2008; Mustafi et al., 2013). To probe the folding of native and heat-treated (poly-)ubiquitylated FKBP12, ^1H - ^{15}N SOFAST-HMQC (Schanda and Brutscher, 2005) spectra were acquired. For the T_1 relaxation experiment, a series of spectra with relaxation delays of 10, 20, 40, 180, 300, 500 and 1000 milliseconds were measured. For the T_2 relaxation experiment, a series of spectra with relaxation delays of 10, 30, 50, 70, 90, 110 and 150 milliseconds were acquired. In the ^1H - ^{15}N NOE, T_1 and T_2 relaxation measurements, the recycle delay was set to 3-5 seconds to assure adequate longitudinal relaxation between acquisitions. Data processing was performed in NMRPipe (Delaglio et al., 1995) and CCPN (Vranken et al., 2005).

5.18. NMR Relaxation Analysis

In the T_1 and T_2 relaxation experiments, the signal intensities $I(t)$ of each cross-peak with different relaxation delays t were fitted to the equation $I(t) = I(0) \exp\left(-\frac{t}{T_{\text{relaxation}}}\right)$ to obtain the relaxation constant $T_{\text{relaxation}}$. Fitting was performed using the fitting program GLOVE (Sugase et al., 2013). ^1H - ^{15}N hnNOE values were calculated by the equation $(\text{hnNOE value}) = \frac{I_{\text{sat}}}{I_{\text{eq}}}$ where I_{sat} and I_{eq} were the cross-peak intensities with and without proton saturation. The respective spectral density functions $J(\omega)$ are obtained from the experimental relaxation parameters by the following equations:

$$J(0.87 \omega_{\text{H}}) = \frac{4\gamma_{\text{N}}(\text{NOE} - 1)}{5d^2\gamma_{\text{H}}T_1} \quad (\text{Eq. 1})$$

$$J(\omega_{\text{N}}) = \frac{\frac{1}{T_1} - \frac{7d^2}{4}J(\omega_{\text{H}})}{\frac{3}{4}d^2 + c^2} \quad (\text{Eq. 2})$$

$$J(0) = \frac{\frac{1}{T_2} - \left(\frac{3}{8}d^2 + \frac{c^2}{2}\right)J(\omega_{\text{N}}) - \frac{13d^2}{8}J(\omega_{\text{H}})}{\frac{d^2}{2} + \frac{2c^2}{3}} \quad (\text{Eq. 3})$$

in which NOE is the ^1H - ^{15}N hnNOE value, T_1 and T_2 are relaxation constants. ω_{N} and ω_{H} are the Larmor frequencies of the ^{15}N and ^1H nuclei, respectively; γ_{N} and γ_{H} are the gyromagnetic ratios of the ^{15}N and ^1H nuclei. $d = \frac{\mu_0 h \gamma_{\text{N}} \gamma_{\text{H}}}{8\pi^2 r_{\text{NH}}^3}$ and $c = \frac{\omega_{\text{N}}}{\sqrt{3}(\sigma_{\text{parallel}} - \sigma_{\text{perpendicular}})}$. μ_0 is the permeability of the vacuum, r_{NH} is the length of the amide bond vector, h is the Plank constant, and σ_{parallel} and $\sigma_{\text{perpendicular}}$ are the parallel and perpendicular components of the assumed axially symmetric chemical shift tensor.

Chapter 6. Thesis Conclusions

With the continuing rise in Alzheimer's, Parkinson's and other neurodegenerative diseases, there is much interest among the general public in the pathogenesis of such disorders. Nevertheless, it has remained unclear why abnormal protein aggregates are observed in the patient brains since Arnold Pick first identified these protein aggregates in 1882. Furthermore, it is well known that such protein inclusion bodies often contain ubiquitin, a polypeptide with high physical and chemical stability; however, the reason and the mechanism for the formation of ubiquitin-positive aggregates have been unknown.

To date, a myriad of researches have revealed not only physiological functions of ubiquitin, but also its physically and chemically unique properties. However, no direct evidence to link ubiquitin with intracellular aggregate formation observed in diseases has been obtained. Therefore, it is possible to hypothesize that some important properties of ubiquitin may be overlooked and unobserved. In particular, various forms of ubiquitin in the modification such as mono- and polyubiquitylation have been well characterized; in contrast, the role of ubiquitin polymers with a wide range of chain-lengths remains unclear. Furthermore, the physicochemical properties of ubiquitin in the polymeric and substrate-conjugated form have not been studied precisely.

To elucidate the chain-length-dependent and substrate-conjugation-induced physicochemical properties, large-scale and efficient methods to prepare samples have been established in this thesis (Chapter 2 and Chapter 4). The method to prepare large amounts of and highly purified ubiquitin chains with a certain chain-length (Chapter 2) is appropriate for biological and physicochemical assays and for structural studies such as X-ray diffraction experiments and NMR measurements because the crystallization of K48-linked tetra-, hexa and octa-ubiquitin confirms the quantity and quality of their ubiquitin chains. In addition, this established protocol would be applied to chain-length separation of other ubiquitin-like modifier polymers such as poly-SUMO chains, and to isolation of protein- or nucleotide-based polymeric molecules because this method is based on separation of target molecules by their electrostatic differences.

Based on this established method, further biological and physicochemical analyses of ubiquitin chains with various chain-lengths have been performed (Chapter 3 and Chapter 4). Unexpectedly, although the ubiquitin monomer shows extraordinarily high folding stability and solubility, these properties are decreased simply by its covalent linkage with another

ubiquitin or another protein. Notably, as the polyubiquitin chain length is increased, the polyubiquitin fibrils are formed at more physiological conditions. Importantly, the fibrillar aggregate formation of polyubiquitin chains is independent of the linkage type. Moreover, ubiquitylated proteins (*i.e.*, other substrate proteins conjugated with ubiquitin chains) also form fibrillar aggregates regardless of the kind of substrate proteins. These results suggest that any proteins conjugated with ubiquitin chains are thermodynamically destabilized and that they would form fibrillar aggregates not only under physiological *in vitro* conditions, but also in living cells. Actually, intracellular aggregates of ubiquitylated proteins have been observed and their aggregation propensity depends on the chain-length, similarly to the *in vitro* observations. Furthermore, the intracellular aggregates of ubiquitylated proteins are recognized by the autophagic receptor proteins and selectively targeted for degradation by macroautophagy.

Interestingly, the folding destabilization by ubiquitylation is detected not only in ubiquitin chains, but also in substrate proteins that are conjugated with ubiquitin chains (Chapter 4). Tryptophan fluorescence analysis reveals that a substrate protein is destabilized thermodynamically due to ubiquitylation. In addition, ubiquitylation changes a physicochemical property of its substrate protein: the structural reversibility, which is closely related with protein aggregate formation. Although the detail mechanism how ubiquitylation causes the folding destabilization remains obscure, structural dynamics analysis provides insight to the ubiquitylation-induced physicochemical properties of substrate proteins: global structural fluctuations of substrate proteins are brought about, which may result in their folding destabilization.

In summary, the observations in this thesis propose a novel pathway to eliminate intracellular proteins, in which proteins are polyubiquitylated and then form aggregates, which subsequently undergo degradation by autophagy. In addition, because the folding destabilization and fibrillar aggregation propensity depends on the chain length of ubiquitin chains, ubiquitylation may function like a timekeeper; namely, modification of substrate proteins by longer ubiquitin chains will lead to more efficient autophagic degradation of their protein aggregates. It has been reported that impairment of autophagy directly results in abnormal accumulation of ubiquitin-positive inclusion bodies in cells; therefore, ubiquitylation is closely related with autophagic protein degradation and the deactivation of this degradation system would lead to intractable diseases such as cancer and various neurodegenerative diseases. Thus, the polyubiquitin fibril formation and ubiquitylation-

induced folding destabilization provide novel insights into the enigmatic aggregation of intracellular ubiquitylated proteins: pathological hallmarks of human sporadic proteinopathies. Future studies should aim to elucidate the underlying mechanism in the physicochemical properties induced by ubiquitylation and how their properties would be related with neurodegenerative diseases.

Chapter 7. Appendix

7-1. NMR Processing

In this thesis, processing of NMR data is performed using NMRPipe (Delaglio et al., 1995). This software is a UNIX-based system and can be run not only on Linux systems, but also on Mac OS and Windows OS with Microsoft Services for UNIX. Data of the spectrum can be converted to an NMRPipe-format file. In this study, all data have been measured on a Bruker NMR spectrometer; therefore, a raw data file (*ser*) is converted by *bruk2pipe* program in NMRPipe. Then, the converted data is Fourier transformed by using NMRPipe. The script for this process depends on the kind of measurements and a typical processing script for a 2D spectrum is shown in Figure 7-1. The obtained spectra are basically analyzed with CCPN (Vranken et al., 2005) and with nmrDraw in NMRPipe. For opening in other analysis software such as sparky and NMRView, the NMRPipe-format spectrum can be converted to an ucsf or nv format file using *pipe2ucsf* or *pipe2xyz* program in NMRPipe.

```
nmrPipe -in test.fid \
| nmrPipe -fn POLY \
| nmrPipe -fn SP -off 0.5 -end 0.98 -pow 2 -c 0.5 \
| nmrPipe -fn ZF -auto \
| nmrPipe -fn FT -verb \
| nmrPipe -fn PS -p0 0 -p1 0 -di \
| nmrPipe -fn EXT -left -sw \
| nmrPipe -fn TP \
| nmrPipe -fn SP -off 0.5 -end 0.98 -pow 1 -c 1.0 \
| nmrPipe -fn ZF -auto \
| nmrPipe -fn FT -verb \
| nmrPipe -fn PS -p0 -135 -p1 180 -di \
| nmrPipe -fn TP \
| nmrPipe -fn POLY -auto \
-verb -ov -out test.ft2
```

Figure 7-1. The processing script for ^1H - ^{15}N HSQC spectra.

7-2. Abbreviations

Table 7-1. **List of abbreviations.**

Abbreviations	Meaning
A	Alanine
Aggrephagy	Selective autophagic degradation of aggregated proteins
ALS	Amyotrophic lateral sclerosis
Atg	Autophagy-related gene
ATP	Adenosine triphosphate
Autophagy	Macroautophagy
C	Cysteine
C-terminal (C-terminus)	Carboxy-terminal (C-terminus)
CD	Circular dichroism
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
<i>E. coli</i>	<i>Escherichia coli</i>
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
G	Glycine
HMQC	Hetero-nuclear multiple quantum coherence
HSQC	Hetero-nuclear single quantum coherence
I	Isoleucine
Ile	Isoleucine
K	Lysine
M	Methionine
MALDI	Matrix assisted laser desorption/ionization
MEF	Mouse embryonic fibroblast
MS	Mass spectroscopy
N-terminal (N-terminus)	Amino-terminal (Amino-terminus)
NMR	Nuclear magnetic resonance
PAGE	Poly-acrylamide gel electrophoresis
PDB	Protein data bank

PRM	Parallel reaction monitoring
S	Serine
SAXS	Small angle X-ray scattering
SDS	Sodium dodecyl sulfate
SUMO	Small ubiquitin-like modifier
Sumoylation	Post-translational modification by SUMO
TCEP	Tris(2-carboxylethyl)phosphine
ThT	Thioflavin-T
TOF	Time of flight
Ub	Ubiquitin
UBD	Ubiquitin binding domain
Ubiquitylation	Post-translational modification by ubiquitin
Ubl	Ubiquitin like modifier
Ub ^{VV}	C-terminal Val-Val mutant of ubiquitin

Chapter 8. Bibliography

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