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Nonenzymatic assembly of branched polyubiquitin chains for structural and biochemical studies

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Abstract

Polymeric chains of a small protein ubiquitin are involved in regulation of nearly all vital processes in eukaryotic cells. Elucidating the signaling properties of polyubiquitin requires the ability to make these chains *in vitro*. In recent years, chemical and chemical-biology tools have been developed that produce fully natural isopeptide-linked polyUb chains with no need for linkage-specific ubiquitin-conjugating enzymes. These methods produced unbranched chains (in which no more than one lysine per ubiquitin is conjugated to another ubiquitin). Here we report a nonenzymatic method for the assembly of fully natural isopeptide-linked branched polyubiquitin chains. This method is based on the use of mutually orthogonal removable protecting groups (e.g., Boc- and Alloc-) on lysines combined with an Ag-catalyzed condensation reaction between a C-terminal thioester on one ubiquitin and a specific ε-amine on another ubiquitin, and involves genetic incorporation of more than one Lys(Boc) at the desired linkage positions in the ubiquitin sequence. We demonstrate our method by making a fully natural branched tri-ubiquitin containing isopeptide linkages via Lys11 and Lys33, and a ^{15}N-enriched proximal ubiquitin, which enabled monomer-specific structural and dynamical studies by NMR. Furthermore, we assayed disassembly of branched and unbranched tri-ubiquitins as well as control di-ubiquitins by the yeast proteasome-associated deubiquitinase Ubp6. Our results show that Ubp6 can recognize and disassemble a branched polyubiquitin, wherein cleavage preferences for individual linkages are retained. Our spectroscopic and functional data suggest that, at least for the chains studied here, the isopeptide linkages are effectively independent of each other. Together with our method for nonenzymatic assembly of unbranched polyubiquitin, these developments now provide tools for making fully natural polyubiquitin chains of essentially any type of linkage and length.

Keywords

ubiquitin; polyubiquitin; branched chain; isopeptide bond; unnatural amino acids; deubiquitination

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Supplementary data include three supplementary figures.

1. Introduction

Among various post-translational modifications of proteins in the cell perhaps the most fascinating is ubiquitination, i.e. a covalent attachment of a small, 76-a.a. protein ubiquitin (Ub), whose role is to act as a molecular signaling tag. The ubiquitination process involves formation of an isopeptide bond between G76 of Ub and a ε-NH_2 of a lysine of the target protein (could be another Ub) and generally involves a series of enzymatic reactions catalyzed by Ub-activating (E1) and Ub-conjugating (E2) enzymes, and Ub-ligases (E3)[1, 2]. Ub-mediated signaling pathways are involved in regulation of essentially all aspects of eukaryotic cell life[3–6]. The versatility of Ub as a molecular signal stems from its ability to form polymers (polyUb) of various lengths and linkages, in which the protomers – ubiquitins -- are linked to each other through one of the seven lysines in Ub or head to tail (via α-NH_2 of M1)[6, 7]. For example, K48-linked polyUb chains act as a universal signal targeting proteins for degradation by the 26S proteasome, whereas chains linked via K63 act as regulatory, non-proteolytic signals in various processes, including trafficking, DNA repair, and inflammatory response, and K11-linked chains were reported to act as both degradative and non-degradative signals[5, 6]. The biological roles of polyUb chains linked through other lysines are yet unclear. To add to the complexity of Ub-signaling, recent *in vivo* and *in vitro* studies indicate that more than one linkage type can be present in the same polyUb chain[8–13]. Furthermore, these so-called mixed-linkage chains can be unbranched (i.e., no more than one lysine- or methionine-linkage per Ub) or branched (i.e. more than one such linkage per Ub)[8, 12, 13]. This raises an exciting possibility that linkage mixing and branching could enhance the signaling capabilities of the chain or perhaps even bestow polyUb with novel signaling properties not available to homogeneously-linked or unbranched chains.

Despite the significant advances in elucidating the biological/cellular roles of various Ub-signaling pathways in the last decades, understanding of the mechanisms that allow differently linked Ub chains to serve as versatile and yet distinct molecular signals is missing. The current, '*linkage-conformation-signal*' hypothesis is that the Ub-Ub linkage defines the conformational ensemble for a given polyUb chain, which in turn determines the ability of the chain (through conformational selection or induced fit) to adopt the proper conformation required for binding to a specific receptor. Studies of the structural properties and receptor recognition of polyUb require the ability to form these chains *in vitro*. This however has been a major challenge, primarily because lysine-specific E2 enzymes for linkages other than through K11, K63, or K48 are currently not known.

The established biochemical procedures for controlled assembly of polyUb chains from recombinant Ub protomers mostly follow the scheme, designed by Cecile Pickart and co-authors[1, 14]. This method relies on the availability of E2 enzymes to form a desired Lys-linkage and uses chain terminating mutations (i) to control the chain length and (ii) to avoid formation of unwanted linkages due to promiscuity of some E2s. Using genetic incorporation of unnatural amino acids[15] (UAA) with removable protecting groups (e.g., Lys(Boc)) we have recently designed a new strategy that circumvents the need for permanent mutations and allows formation of fully natural polyUb chains using linkage-specific E2 enzymes[16]. However, performing ubiquitination enzymatically is inevitably limited by the availability of linkage-specific E2s and/or substrate-specific E3s.

Enormous progress has been made in recent years in developing chemical or chemical-biology approaches that circumvent the need for Ub-conjugating E2 enzymes to form isopeptide-linked Ub chains[17–25] (reviewed in[26, 27]). In addition, several groups reported assembly of Ub chains using various non-native linkages, including isopeptide-bond replacements or lysine modifications[28–30].

In particular, we focus on developing tools for nonenzymatic assembly of polyUb chains from recombinant Ub monomers that could be implemented in every biochemical laboratory. Using recombinant monomers is particularly critical for NMR and other structural or biophysical studies that require unit-specific or other types of isotopic labeling of Ub chains, which is essentially impractical for chemically synthesized chains because of the high cost of incorporating isotopic labeling into chemical synthesis. Previously, we have demonstrated that by combining UAA incorporation with the use of mutually orthogonal removable protecting groups (Boc- and Alloc-) and Ag-catalyzed condensation reaction between a C-terminal thioester on one Ub and a specific ε-amine on another Ub, it is possible to assemble polyUb chains of essentially any desired length, linkage composition, and isotope-labeling schemes, and in sufficient quantities even for structural studies[25]. Moreover, the resulting chains are fully natural, i.e. they are linked via isopeptide bonds and contain no mutations. Specifically, we successfully made di-Ub chains linked via K11, K27, or K33, as well as K11-linked tri-, and tetra-Ubs, and a mixed-linkage K33 and K11-linked unbranched tri-Ub. By enriching a specific Ub unit in the chain with ^{15}N, we were able to characterize by NMR individual Ub units in some of these chains in order to reveal intra-chain Ub-Ub interactions.

That method relies on incorporation of a single UAA (e.g. Lys(Boc)) at the desired position in the protein sequence, and is inevitably limited to making unbranched chains of either homogeneous or mixed linkages. Making a branched Ub chain requires addressing yet another challenge, i.e. incorporation of UAA at more than one lysine site within the same Ub molecule, which has not been achieved before. In this paper, we describe and successfully demonstrate a method for assembly of fully natural branched polyUb chains that builds upon and extends our previous method for non-enzymatic assembly of unbranched Ub chains. Together, these two methods now allow assembly of essentially *any* polyUb chain, homogeneously-linked or with mixed linkages, branched or unbranched.

2. Materials and Methods

2.1. Assembly of $[Ub]_2$–^{11,33}Ub

2.1.1. Preparation and ^{15}N enrichment of Ub K11Boc,K33Boc—Ub with the (unnatural) amino acid Lys(Boc) at both positions 11 and 33 was expressed in BL21(DE3) *E. coli* cells. The plasmid pTXB1, carrying ampicillin resistance, contained the *E. coli* codon-optimized Ub gene with TAG codon at positions 11 and 33, fused to an intein tag with a chitin binding domain (CBD). Incorporation of the Lys(Boc) at the TAG sites was accomplished with the plasmid pSUP-PylT-PylS containing the cellular machinery for recognition of the TAG codon and incorporation of Lys(Boc). The latter plasmid carried chloramphenicol resistance, allowing for double transformation into the BL21(DE3) chemically competent cells[16]. ^{15}N-enriched Ub K11Boc,K33Boc was produced in autoinducing minimal media[31] supplied with Lys(Boc) (final concentration 4 mM) in powder form[16] and containing $^{15}NH_4Cl$ as the sole source of nitrogen. Ub protein was cleaved from the intein tag with 50 mM dithiothreitol at 25 °C. Two elutions at 48 hours and 120 hours were collected. The protein was purified via size exclusion chromatography, buffer exchanged into dH_2O, and lyophilized. The presence of two Lys(Boc) groups was confirmed by ESI-MS.

2.1.2. Thioesterification of Ub—E1 Ub-activating enzyme was used to add a sodium 2-mercaptoethane-sulfonate (MESNA)-derived thioester group to the C-terminus of unlabeled wild-type ubiquitin as described previously[25]. Two 5 mg Ub reactions were performed in a 1 mL solution of 20 mM sodium phosphate buffer (pH 8.0) containing 10 mM ATP, 10 mM $MgCl_2$, 100 mM MESNA, and 250 nM E1 enzyme. The reaction was incubated at 37 °C for

16 hours. Acid-sensitive E1 enzyme was precipitated from the reaction with two drops of glacial acetic acid. Thioesterification was confirmed by ESI-MS.

2.1.3. Alloc protection of Ub monomers—Two 5 mg portions of lyophilized thioesterified Ub (Ub-COSR) and 4.2 mg Ub K11Boc,K33Boc were each dissolved in 450 μL dimethyl sulfoxide (DMSO) and allowed to react with 17 μL *N,N*-diisopropylethylamine (DIEA) and 75 μL freshly prepared 40 mg/mL *N*-(Allyloxycarbonyloxy) succinimide (Alloc-OSu). Solutions were allowed to react at room temperature on an orbital shaker for two hours, followed by cold ether precipitation of the protein. Complete Alloc protection was confirmed by ESI-MS.

2.1.4. Removal of Boc groups—The Alloc-protected Ub K11Boc,K33Boc was dissolved on ice in 500 μL 60% TFA and placed on a shaker for four hours at 4 °C. Protein was precipitated with cold ether. Removal of both Boc protection groups was verified by ESI-MS.

2.1.5. Ligation—10 mg of Alloc-protected Ub-COSR and 5 mg TFA-treated Ub K11Boc,K33Boc were combined in a volume of 300 μL DMSO. To this, 13.2 μL DIEA, 7.5 μL each of freshly prepared 390 mg/mL *N*-hydroxysuccinimide (H-OSu) and 57 mg/mL silver nitrate were added. The reaction was incubated in the dark on an orbital shaker for 30 hours. The now gel-like solution was split into three tubes and reduced to a rosy orange powder by four rounds of cold ether precipitation. Formation of tri-Ub was confirmed by SDS-PAGE.

2.1.6. Global Alloc deprotection—All Alloc groups were removed from the Ub ligation mixture with 50 mol % ruthenium catalyst, chloro-pentamethylcyclopentadienyl-cyclooctadiene-ruthenium(II) ([Cp*Ru(cod)Cl]) and 50 equivalents of thiophenol. The reaction assumed a slight excess of over nine amines per Ub monomer, and 16 mg of overall protein. The protein was dissolved in 1680 μL of DMSO, to which were added 960 μL dH_2O, 465 μL freshly prepared 20 mM [Cp*Ru(cod)Cl] in DMSO, and 95 μL thiophenol. The reaction was incubated at 50 °C for two hours in a thermal cycler, before being allowed to sit at 25 °C overnight. The solution was creamy orange with a black crust precipitate at the bottom. The supernatant was aliquoted into over 20 tubes and then reduced to a flaky brown precipitate after being subjected to over 20 rounds of cold ether precipitation.

2.1.7. Renaturation and purification—Each pellet was dissolved in 100 μL 6 M guanidine hydrochloride (GdnHCl), 20 mM sodium phosphate (pH 6.8) with periodic gentle agitation for 30 minutes, and centrifuged to remove precipitate. Supernatant was combined in a 50 mL conical tube and slowly diluted with 25 mL 20 mM sodium phosphate, 130 mM NaCl (pH 6.8). This solution was dialyzed against 2 L of the same buffer at room temperature overnight in 3K MWCO dialysis tubing. The dialysate was concentrated to a volume of 1.2 mL in a 3K MWCO Amicon concentrator and purified through size exclusion. The fractions corresponding to trimer were verified in purity via SDS-PAGE, concentrated and buffer exchanged into 20 mM sodium phosphate buffer (pH 6.8). Molecular weight of the purified branched tri-Ub was verified via ESI-MS.

2.2. Chain disassembly assays with Ubp6

Ubp6-catalyzed disassembly of various polyUb chains was assessed via SDS-PAGE analysis. Assays were performed in 50 μL phosphate-buffered saline (pH 7.4) using 25 μM of the polyUb chain and a sub-stoichiometric amount (8 μM) of Ubp6. The reactions were incubated at 30 °C for 21 hours. Time points were taken by quenching 4 μL reaction in 7 μL reducing SDS-PAGE loading buffer, then storing at −20 °C.

2.3. Enzymatic assembly of $[Ub]_2$–^{11,63}Ub

Tri-Ub branched at K11 and K63 was prepared using controlled-length chain assembly with chain-terminating K-to-R mutations and linkage-specific E2 enzymes[14]. K63-linked Ub_2 was first constructed by reacting Ub K11R,K63R (distal Ub) and ^{15}N Ub D77 (proximal Ub) in the presence of K63-specific E2 enzyme complex of Ubc13 and Mms2. After cation purification, the Ub_2 was reacted with Ub K11R,K63R (distal Ub) in the presence of K11-specific E2 enzyme Ube2S to form a K11 isopeptide linkage on the proximal Ub.

2.4. NMR experiments

All NMR measurements were performed on an Avance III 600 MHz spectrometer (Bruker Biospin) equipped with TCI cryoprobe using standard or in-house pulse sequences[32]; sample temperature was set to 23 °C. All protein NMR samples were in pH 6.8, 20 mM sodium phosphate buffer containing 5% D_2O and 0.02% (w/v) NaN_3. Combined amide chemical shift perturbations (CSPs) were determined from differences in peak positions in ^{1}H-^{15}N NMR spectra using the equation $\Delta\delta = [(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are differences in the ^{1}H and ^{15}N chemical shifts, respectively, for a given residue. For ^{15}N T_1 experiment, errors in intensity measurements were estimated using median noise calculations within Sparky[33]. Errors in ^{15}N T_1 relaxation times were determined using 500 Monte Carlo trials in the in-house program, RELAXFIT.

2.5. Mass spectrometry

For all proteins, high-resolution mass spectra of m/z 250–2500 were acquired with a JEOL AccuTOF-CS mass spectrometer in electrospray (ESI-MS) positive mode using flow injection. To determine the molecular weight, spectra were deconvoluted using MagTran software with the maximum charge set to 35.

2.5.1 Tryptic digestion of $[Ub]_2$–^{11,33}Ub, ^{15}N-labeled on the proximal Ub—The purified $[Ub]_2$–^{11,33}Ub (40 μM in PBS pH 7.4 buffer) was digested with 0.3 μg trypsin (Promega) at 37 °C overnight. The digest was then acidified with 1 μL formic acid and stored at −20 °C until analysis. For LC-MS/MS analysis, 2 μL digest was injected and trapped into an Agilent Zorbax C18 trapping column (0.3 × 5 mm), desalted for 10 min before eluted onto an Agilent Zorbax C18 nano column (0.075 × 150 mm) with a gradient of 5–35% B in 40 min (Solvent A: 97.5% water, 2.5% ACN, 0.1% formic acid, solvent B: 2.5% water, 97.5% ACN, 0.1% formic acid). The LTQ Orbitrap was set to analyze 1 full scan MS with R=30,000 at m/z 400 followed by 5 data dependent MS/MS of the most abundant ions using the ion trap. The resulting data were searched against a human protein database using in-house mascot server to locate tryptic peptides of ^{14}N Ub. Existence of ^{15}N-enriched Ub was verified by pairs of unmodified tryptic peptides corresponding to the molecular ion of ^{14}N and ^{15}N peptides; ratios of ^{14}N/^{15}N peptide are appropriately 2/1 (data not shown). This verifies that for each molecule of ^{15}N Ub, there are 2 molecules of ^{14}N Ub.

3. Results

Here we describe a method to assemble all-natural branched ubiquitin chains via Ag-mediated condensation reactions that allow isopeptide linkages to form between available ε-amines of specific lysines on one Ub and the activated C-termini of other Ub monomers. This procedure is an extension of our work published recently[25] that uses UAA incorporation and orthogonal removable lysine-protecting groups. Specifically, we will illustrate this method for the construction of a branched tri-Ub chain comprising both K11 and K33 isopeptide linkages on one Ub (Scheme 1). To distinguish the architecture of branched chains from unbranched mixed-linkage chains comprising the same linkages, we

will use recently proposed nomenclature[34] designating the branched chain as $[Ub]_2$–^{11,33}Ub and the unbranched chain as Ub–^{33}Ub–^{11}Ub, where the proximal Ub is shown on the right, the distal Ub is on the left, and the superscripts indicate the lysines involved in the linkages and, in the case of unbranched chain, also the linkage order. Note that in the branched chain, the proximal Ub is the one with the free C-terminus and the ε-amines of K11 and K33 covalently attached via isopeptide bonds to the C-termini of two other (distal) Ubs. Critical to the characterization of these polyUb chains with high-resolution structural methods, our scheme permits the isotopic labeling of either the distal Ubs collectively or the proximal Ub. Selective isotopic labeling of specific Ub units in polyUb chains is essential to avoid signal overlap in NMR spectra due to the homo-polymeric nature of polyUb[25].

3.1 Preparation and characterization of K11Boc,K33Boc Ub monomer

The most critical aspect of our method lies in the successful preparation of the proximal Ub with the genetic incorporation of Lys(Boc) amino acid at the specific lysine positions targeted for the isopeptide linkages. Previously, we demonstrated successful incorporation of a single Lys(Boc) at a specific lysine through encoding with an (amber) TAG stop codon. The genetic code of *E. coli* strain is expanded by adding an orthogonal pyrrolysyl-tRNA-synthetase/tRNAPyl pair that specifically recognizes the TAG codon and incorporates Lys(Boc) at that position in the emerging peptide[16, 35]. Here we introduced two TAG stop codons, at residues 11 and 33 in Ub via site-directed mutagenesis. Using procedures previously described[16, 25], we produced ~5 mg of pure Ub containing both K11Boc and K33Boc mutations (Ub K11Boc,K33Boc). To enable NMR characterization of this protein, we enriched it with ^{15}N by overexpressing it in cells growing in minimal media containing $^{15}NH_4Cl$ as the sole source of nitrogen. By SDS-PAGE, expression of Ub K11Boc,K33Boc appeared comparable to that of Ub containing a single Lys(Boc) (Supplementary Figure S1), but the overall yield of the purified Ub was 70–75% lower. Mass spectrometric analysis of the purified Ub K11Boc,K33Boc confirmed that the protein indeed contained two Boc protecting groups (Figure 1B).

To further characterize the Ub K11Boc,K33Boc monomer, we collected a ^{1}H-^{15}N NMR spectrum of this protein and compared it with the spectrum of Ub (Figure 2B). The similarity of the two spectra indicates that Ub K11Boc,K33Boc is indeed folded and retains a spectral “fingerprint” pattern characteristic of monomeric Ub (monoUb). Importantly, the backbone amide resonances of K11 and K33 are absent in the spectrum of Ub K11Boc,K33Boc, indicating the successful incorporation of unlabeled Lys(Boc) amino acid at those positions. Furthermore, we quantified the shifts in the position of the NMR signals (otherwise known as chemical shift perturbations, or CSPs) on a per residue basis (Figure 3B). Most spectral perturbations are localized to those residues surrounding K11 and K33 in the 3D structure of Ub (not shown).

3.2 Assembly of branched K11,K33-linked tri-Ub chain

Preparation of the distal Ub component(s) was achieved via overexpression and purification of monoUb from *E. coli* cells. To ready the monoUb for chemical ligation, its C-terminus was modified with a thioester moiety by reacting with Ub-activating E1 enzyme and the MESNA compound[20, 25]. To prevent all other available amines on both monoUb and Ub K11Boc,K33Boc from reacting in the chemical ligation process, these two proteins were reacted with Alloc-OSu, which specifically modifies the amines with the orthogonal protecting group Alloc[25]. Subsequent treatment of Ub K11Boc,K33Boc with TFA then selectively removed the Boc protecting group from the K11 and K33 side chains, rendering these particular ε-amines available for the ligation reaction, while keeping all other amines protected with the Alloc group (Scheme 1).

To generate the branched tri-Ub chain, a mixture of both monoUb and TFA-treated Ub K11Boc,K33Boc in a 2:1 stoichiometric ratio was reacted in the presence of H-OSu and $AgNO_3$. The Ag-mediated condensation reaction resulted in formation of isopeptide bonds between the activated C-termini of the distal Ubs and the exposed ε-amine groups of K11 and K33. Successful formation of a tri-Ub product was monitored via SDS-PAGE (Figure 1A). The Alloc protection groups were then chemically removed, and the protein was renatured as previously described[25]. Branched tri-Ub was separated from incompletely reacted dimer and unreacted monomer by using size exclusion chromatography (Figure 1C,D). A final yield of 0.5 – 1 mg of purified tri-Ub was achieved from a total input of 14 mg of Ub units (4 mg of Ub K11Boc,K33Boc and ~10 mg of monoUb). The purity of the branched tri-Ub product ($[Ub]_2$–^{11,33}Ub) was assessed via SDS-PAGE analysis of this and other tri-Ub chains, including a mixed-linkage unbranched tri-Ub chain containing K11 and K33 linkages (Ub–^{33}Ub–^{11}Ub) (made as detailed in[25]) and another branched tri-Ub (enzymatically-assembled $[Ub]_2$–^{11,63}Ub) linked via K11 and K63 (Figure 1E). The observed molecular weight of $[Ub]_2$–^{11,33}Ub from mass-spectrometric analysis was 25,755 Da (expected 25,756 Da) (Figure 1F). This number is consistent with a tri-Ub species comprising two ^{14}N-Ubs (each 8,564 Da) and a near-uniformly ^{15}N-enriched Ub (~8,660 Da) with two water molecules (2×18 Da) released upon isopeptide formation at K11 and K33 side chains. The existence of both K11 and K33 linkages on the ^{15}N-enriched proximal Ub of $[Ub]_2$–^{11,33}Ub was confirmed via tryptic digestion and subsequent LC-MS/MS analysis (Supplementary Figure S2).

3.3 NMR characterization of the branched K11,K33-linked tri-Ub

To examine $[Ub]_2$–^{11,33}Ub by NMR, a ^{1}H-^{15}N TROSY spectrum was collected of the ^{15}N-enriched proximal Ub in this chain and overlaid with the corresponding spectrum of monoUb (Figure 2A). The overall similarity between the spectra indicates that the proximal Ub in the branched tri-Ub exhibits spectral properties similar to those of monoUb. The differences between the two spectra (quantified as CSPs) are illustrated in Figure 3A. Strikingly, the CSP pattern is nearly identical to the CSP pattern of monomeric Ub K11Boc,K33Boc *versus* wild type Ub, shown in Figure 3B. In other words, the effects of the K11- and K33-isopeptide linkages on the backbone amide resonances of the proximal Ub in the branched tri-Ub are quite similar to the effects of the Boc protection groups on K11 and K33 in the Ub K11Boc,K33Boc monomer. Thus, we conclude that the Boc moieties on K11 and K33 serve as isopeptide mimics. The data then suggest that the breadth of CSPs observed in the proximal Ub of $[Ub]_2$–^{11,33}Ub is primarily a consequence of the chemical modifications to K11 and K33 resulting from the isopeptide linkage formation, and the additional effect of non-covalent interactions (if they exist) between the proximal Ub and the two distal Ubs is minimal.

To investigate the effects of the individual isopeptide linkages on the spectral properties of the proximal Ub in $[Ub]_2$–^{11,33}Ub, we compared its spectrum with the spectra of proximal Ubs in K11-linked Ub_2 (Figure 3C) and in K33-linked Ub_2 (Figure 3E). Strikingly, the CSPs shown in Figure 3C are nearly identical to the CSPs of the proximal Ub in K33-linked Ub_2 *versus* monoUb (Figure 3D). This is because the comparison with K11-linked Ub_2 (Figure 3C) essentially has "subtracted" the contributions of the K11 isopeptide linkage and revealed the effects of the K33 isopeptide linkage on the spectrum of the branched tri-Ub. Similarly, the CSPs between the spectra of the proximal Ub in $[Ub]_2$–^{11,33}Ub and the proximal Ub of K33-linked Ub_2 (Figure 3E), are nearly identical to those of the proximal Ub in K11-linked Ub_2 *versus* monoUb (Figure 3F). This means that when we have accounted for the K33 isopeptide linkage effects, we observe CSPs resulting from the K11 isopeptide linkage, and *vice versa*. Importantly, this suggests that the effects of the individual K11 and K33 isopeptide linkages can be considered independent of each other. Thus, the CSPs in

Figure 3A appear to be a sum of the perturbations arising from the K33 isopeptide linkage (Figure 3D) and the K11 isopeptide linkage (Figure 3F). This suggest that if any interactions exist between the two distal Ubs in $[Ub]_2$–^{11,33}Ub, they have little effect on the proximal Ub. Revealing contacts between the distal units by NMR would require the ability to isotopically label them individually, which is beyond the current methodology.

As a first attempt to characterize the structure and dynamics of the $[Ub]_2$–^{11,33}Ub at atomic level resolution, we measured ^{15}N longitudinal relaxation time (T_1) for each backbone amide of the proximal Ub (Figure 4). The ^{15}N T_1 relaxation time reflects the overall tumbling rate of a protein, and is correlated with its molecular weight[36]. As its size increases, protein tumbles more slowly in solution, and this increases the T_1. For the proximal Ub in $[Ub]_2$–^{11,33}Ub, the average ^{15}N T_1 for residues in secondary structure elements is 0.962 ± 0.044 s. This number is consistent with a species of a molecular weight between 24 and 28 kDa[36]. This range is in excellent agreement with the reported molecular weight (25.5 kDa) of the branched tri-Ub from mass spectrometric analysis. For comparison, this ^{15}N T_1 for the branched tri-Ub is significantly higher than that of a (smaller in size) Ub_2 species (Figure 4). On a residue-specific level, the overall pattern of ^{15}N T_1 relaxation times for the proximal Ub in $[Ub]_2$–^{11,33}Ub is very similar to the ^{15}N T_1 pattern observed in the proximal Ub of K11-linked Ub_2. This indicates that the local backbone dynamics within the proximal Ub units of both Ub_2 and the branched tri-Ub are largely identical.

3.4 Disassembly of branched tri-Ub chains

To assess the recognition properties of the branched tri-Ub, we performed a disassembly assay using yeast Ubp6 (USP14 in humans), a proteasome-associated deubiquitinating enzyme (DUB). Ubp6, together with other DUBs, regulates and maintains the amount of free Ub in the cell. We monitored the time course of the deubiquitination reaction by SDS-PAGE analysis (Figure 5). As controls, we monitored disassembly of the Ub_2 chains comprising different linkages, particularly K11, K33, and K63 (Figure 5D–F). K11-linked and K63-linked Ub_2 are mostly reduced to Ub monomer after 21 hours, whereas a significant amount of K33-linked Ub_2 relative to Ub monomer is still present at this endpoint. The varying disassembly rates indicate that these chains are differentially processed by Ubp6.

The Ubp6-catalyzed disassembly of $[Ub]_2$–^{11,33}Ub was compared with that of the unbranched mixed-linkage chain, Ub–^{33}Ub–^{11}Ub (Figure 5A, 5B). It is clear that the apparent rate of cleavage for $[Ub]_2$–^{11,33}Ub nearly matches that of the mixed-linkage chain comprising the same, K11 and K33 linkages. This strongly suggests that Ubp6 is able to independently identify and cleave the K11 and K33 linkages in the branched $[Ub]_2$–^{11,33}Ub chain, and that branching of K11 and K33 linkages does not present any new recognition motifs for Ubp6. We observed that even at the final time point (21 hours), a significant amount of Ub_2 was still present. From a comparison with the Ub_2 disassembly results, we speculate that most of the dimer at this time point is K33-linked Ub_2, since nearly all K11-linked Ub_2 is reduced to monomer by this time (Figure 5E).

To compare the rate of disassembly of $[Ub]_2$–^{11,33}Ub with another branched tri-Ub, we performed a Ubp6 disassembly assay on a branched tri-Ub containing K11 and K63 linkages ($[Ub]_2$–^{11,63}Ub) (Figure 5C). Interestingly, only Ub monomer was present at the final time point for that chain. This contrasts with the slower rate of cleavage for branched $[Ub]_2$–^{11,33}Ub. It is interesting to note that the apparent rate of cleavage for $[Ub]_2$–^{11,63}Ub is consistent with the rates of cleavage for K11-linked Ub_2 and K63-linked Ub_2. Both of these Ub_2s are effectively reduced to monomer after 21 hours, just as $[Ub]_2$–^{11,63}Ub. In summary, our disassembly data suggest that Ubp6 can distinguish the different isopeptide linkages in the two particular branched tri-Ubs assayed here. Moreover, from the gels in Figure 5 it

appears that the two linkages in a branched tri-Ub are cleaved independently from each other.

4. Discussion

In this work, we have demonstrated the successful assembly of an all-natural branched tri-ubiquitin chain ($[Ub]_2$–^{11,33}Ub), verified via both NMR and tryptic digestion combined with LC-MS/MS analyses. Importantly, because K33-linkage Ub-conjugating E2 enzymes are not available, our method provides a unique opportunity to make this chain, and the ability to specifically isotopically label the proximal Ub allowed us to study it by NMR, which would be impractical when using total chemical synthesis. Our method is an extension of the previous work, in which we demonstrated the ability to build all-natural homogeneous- or mixed-linkage polyUb chains of any desired length and isotopic labeling scheme[25]. That work relied upon successful incorporation of a *single* unnatural amino acid (Lys(Boc)) at a specific site targeted for the isopeptide linkage. For branched Ub chains, our method necessitates successful incorporation of *multiple* Lys(Boc) amino acids at the same time into a single Ub. Here, for the first time, we have demonstrated that at least two UAAs can be incorporated into Ub. This is a notable achievement because of the very nature of how UAAs are incorporated in proteins expressed in *E. coli* cells. In most cases (here as well), UAAs are introduced into proteins by recoding a stop codon sequence (e.g. the amber codon, TAG) to be recognized by an orthogonal tRNA-synthetase/tRNA pair harboring the desired UAA. Thus, the orthogonal tRNA machinery competes with the natural release mechanism that recognizes the stop codon and terminates translation. As a result, the efficiency of incorporation of multiple UAAs into a single protein is dramatically reduced. In fact, we observed a significant drop in yield for Ub containing two Lys(Boc) amino acids (1 mg/L culture) versus Ub containing one Lys(Boc) amino acid (5 mg/L culture). However, recent advances in this field[37, 38] may circumvent the competition between UAA incorporation and the release machinery in cells, allowing effective incorporation of multiple UAA in a single Ub without a significant decrease in protein yield. These advances could facilitate the assembly and structural and biochemical studies of polyUb chains branched at more than two lysines.

One limitation of the branched chains assembled using our method with regard to monomer-specific analysis (e.g., by NMR) is that it allows specific isotopic labeling of the proximal Ub, but not selective labeling of only one of the distal Ubs. The reason is that our method does not distinguish the Lys(Boc) side chains from each other nor the distal Ubs attached to them (Scheme 1). For example, in the case of $[Ub]_2$–^{11,33}Ub assembly, the TFA treatment of Ub K11Boc,K33Boc deprotects the ε-amines of both K11 and K33 indiscriminately. To achieve specific labeling of the distal Ubs, one of the lysines will have to be selectively deprotected at a time and then reacted with a selectively labeled distal Ub. This would require incorporation of different lysine variants (bearing different protecting groups) at specific positions in the sequence, which would necessitate further development of tools for expansion of *E.coli* genetic code.[39]

From our NMR studies of the proximal Ub in $[Ub]_2$–^{11,33}Ub, we can make several important observations regarding branched polyUb chains. First, the CSPs in the proximal Ub of $[Ub]_2$–^{11,33}Ub nearly match the CSPs of the Ub K11Boc,K33Boc variant vs. monoUb. This observation suggests that the majority of the CSPs seen in the proximal Ub of this branched tri-Ub stem not from Ub/Ub interactions but rather from the chemical modification of the K11 and K33 side chains with isopeptide linkages. We demonstrated previously that the Boc moiety on Lys side chains acts as an isopeptide mimic[25]. Second, the CSPs in the proximal Ub of $[Ub]_2$–^{11,33}Ub appear to be essentially a sum of the CSPs observed previously in the proximal Ubs of K11-linked Ub_2 and K33-linked Ub_2. This is an

important observation as it suggests that the K11 and K33 linkages in the branched tri-Ub act almost independently of each other (at least in their effect on the proximal Ub). Interestingly, we observed similar behavior for the CSPs in the proximal Ub of another branched tri-Ub comprising K11 and K63 linkages (Figure S3). The CSPs of the proximal Ub in $[Ub]_2$–^{11,63}Ub versus monoUb, nearly match those of the proximal Ub in K11-linked Ub_2 versus monoUb, with the exception of the CSPs present for residues 62–65. Because the K63-linkage only affects residues adjacent to (or in the vicinity of) K63 in the proximal Ub[40], we can consider the CSPs in the proximal Ub of $[Ub]_2$–^{11,63}Ub to be a linear superposition of CSPs arising from the K11 and K63 isopeptide linkages.

The functional studies of both branched tri-Ubs via Ubp6-catalyzed disassembly certainly suggest that each branched tri-Ub exhibits properties of their respective isopeptide linkages, rather than a new set of properties that could result from linkage branching. In other words, at least for the two branched tri-Ubs assayed here, their recognition by Ubp6 and the rate of cleavage is dependent only on the lysine-specific properties of the individual isopeptide linkages present in each chain. Taken together, our functional and NMR studies suggest, that at least for both $[Ub]_2$–^{11,33}Ub and $[Ub]_2$–^{11,63}Ub, the isopeptide linkages are effectively independent of each other, i.e. the linkages appear to be recognized and cleaved independently. It is important to note that the observed linkage independence in these branched tri-Ubs is not necessarily present in all other branched polyUb chains, especially ones whose lysines are spatially closer to each other than the K11–K33 or K11–K63 pairs. However, we can infer that in $[Ub]_2$–^{11,33}Ub and $[Ub]_2$–^{11,63}Ub, the locations of the lysine residues permit the attachment of Ub without introducing any structural perturbations on the proximal Ub or introducing any new interactions between the proximal Ub and the distal Ub units.

That Ubp6 can recognize and disassemble branched tri-Ubs is an important finding because it suggests that the proteasomal machinery has a mechanism for recognizing and treating such chains. It remains to be seen if this holds for other branched chains, including the so-called “forked” chains, branched on two closely positioned lysines (K6 & K11, K27 & K29, K29 & K33), which have been reported to resist disassembly by purified proteasomes[12, 41]. The ability to assemble fully natural branched polyUb chains without the need for linkage-specific E2 enzymes provides tools for addressing these and other questions in order to elucidate and understand the mechanisms involved in Ub-mediated signaling pathways.

5. Conclusions

In conclusion, we developed a method for nonenzymatic assembly of all-natural branched polyUb chains. We demonstrated this method by making, for the first time, a branched tri-Ub chain linked via K11 and K33 and isotopically enriched on a specific Ub unit. Based on our spectroscopic and functional data, the two linkages in the branched trimer appear to act independently of each other. Because our method does not rely on the existence of linkage-specific E2 enzymes, branched chains of virtually any linkage composition can be constructed. This includes chains containing linkages such as K33, for which a specific E2 is unavailable. Together with our method for nonenzymatic assembly of unbranched polyUb chains, these developments now provide tools for making fully natural polyUb chains of essentially any type of linkage and length. As recent advances in the detection of more complex Ub chains have indicated the existence and involvement of mixed-linkage or branched polyUb chains in cellular signaling pathways, we anticipate that the method presented here will facilitate structural and biochemical studies of these branched Ub chains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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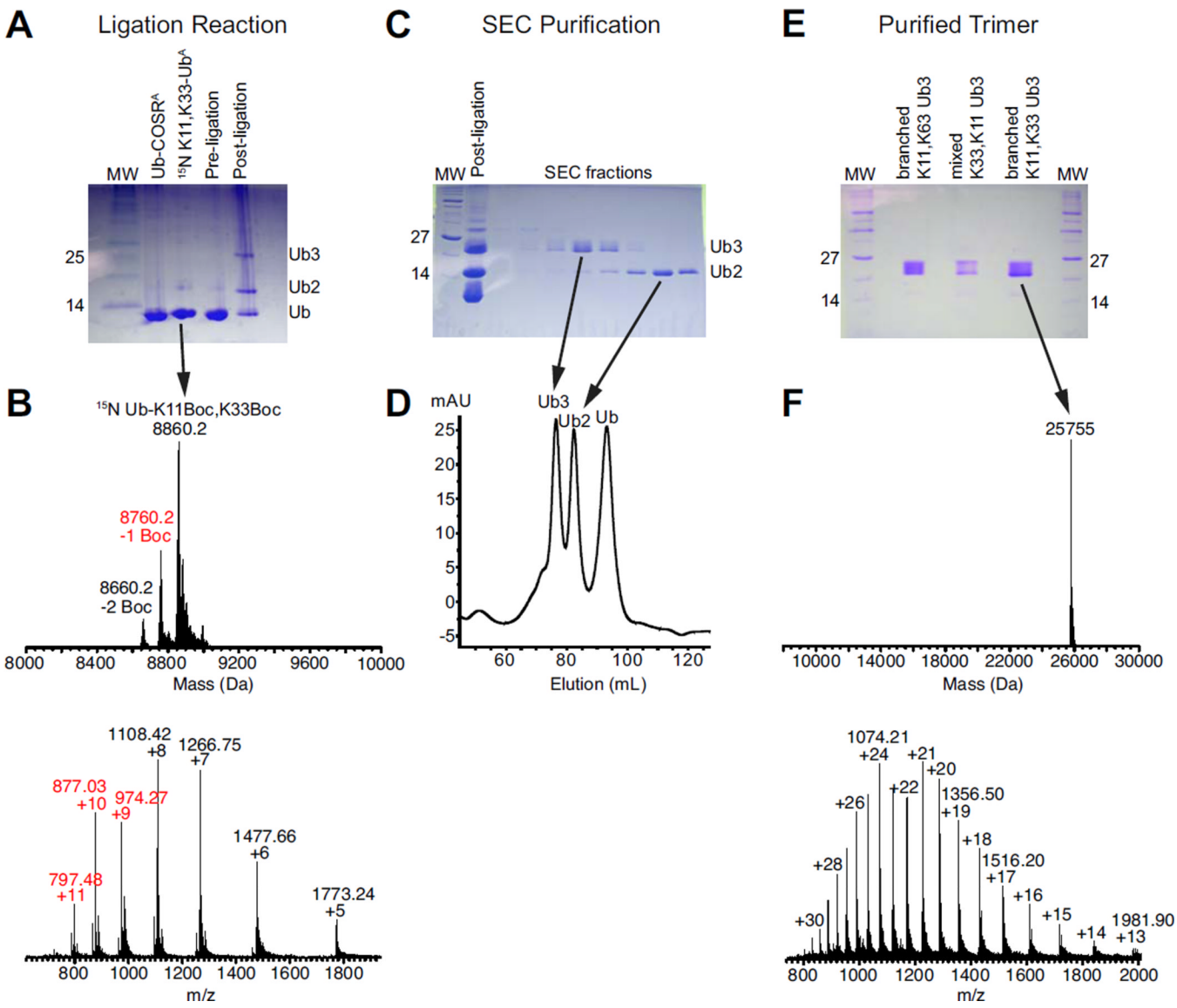


Figure 1.

Assembly of $[Ub]_2$–^{11,33}Ub. (A) SDS-PAGE of the ligation reaction. Ub-COSR^A is a fully Alloc-protected Ub containing a C-terminal thioester. ^{15}N K11,K33 Ub^A is Ub that is Allocprotected except for K11 and K33, where the genetically-incorporated Boc protecting groups have been removed via TFA treatment. After 16 hours, ligation was complete and shows a tri-Ub band. (B) ESI-MS analysis of the ^{15}N Ub K11Boc,K33Boc protein prior to Alloc protection or TFA treatment. The observed molecular weight is 8,860 Da, consistent with a ^{15}N-enriched Ub containing two ^{14}N-labeled Lys(Boc) amino acids. While the MS data also indicate the presence of a Ub species containing a single Boc group, this protein would terminate at the dimer stage during a ligation reaction. (C,D) Size-exclusion chromatography of fully Alloc-deprotected and renatured ligation product, indicating separation of tri-Ub from incompletely reacted Ub_2 and Ub. (E) $[Ub]_2$–^{11,33}Ub is >95% by SDS-PAGE. (F) ESI-MS analysis of purified $[Ub]_2$–^{11,33}Ub, indicating a molecular weight of 25,755 Da, consistent with a tri-Ub species containing one ^{15}N Ub, two ^{14}N Ub units, and the loss of two H_2O molecules upon isopeptide bonds formation.

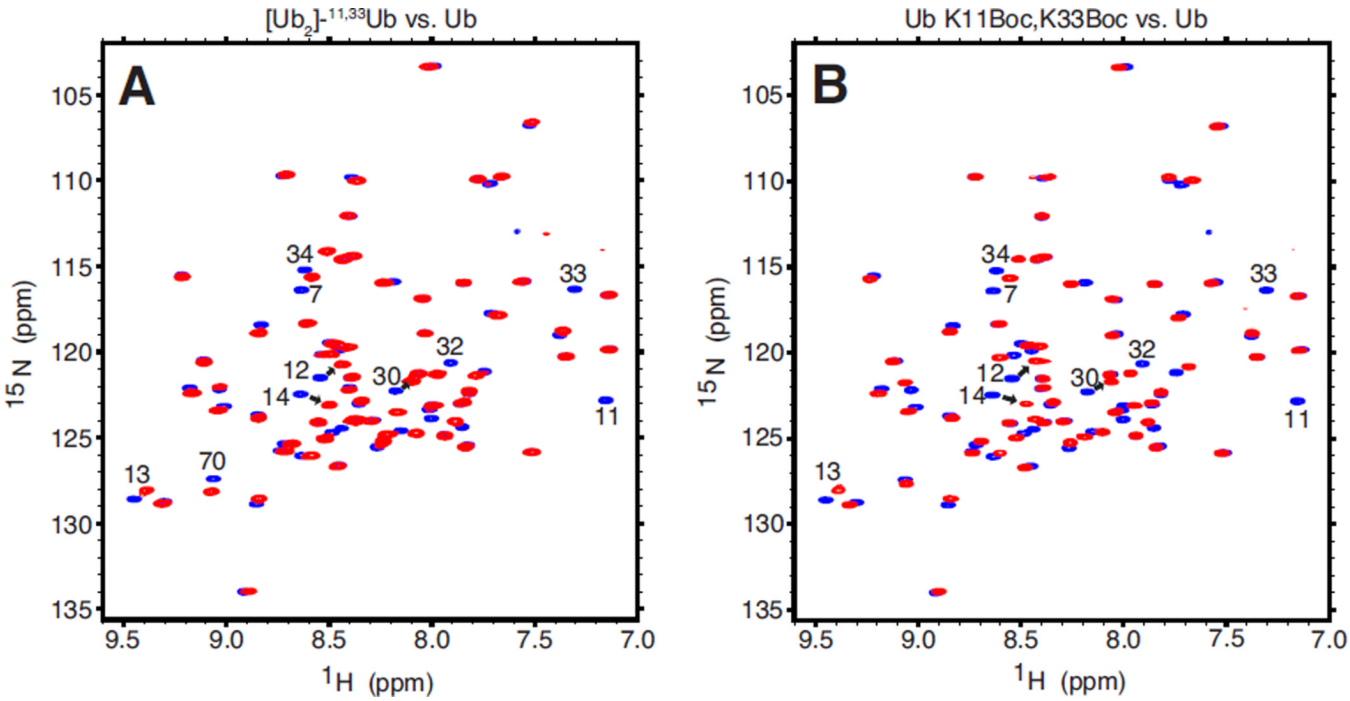


Figure 2.

NMR characterization of $[Ub]_2$–^{11,33}Ub and Ub K11Boc,K33Boc. (A) Overlay of ^{1}H-^{15}N TROSY spectra of the proximal Ub in $[Ub]_2$–^{11,33}Ub (red) and of monomeric wild type Ub (blue). (B) Overlay of ^{1}H-^{15}N TROSY spectra of Ub K11Boc,K33Boc monomer (red) and of wild type Ub (blue). Note the absence of (red) NMR signals for residues 11 and 33 in A and B due to the incorporation of unlabeled Lys(Boc) at these positions.

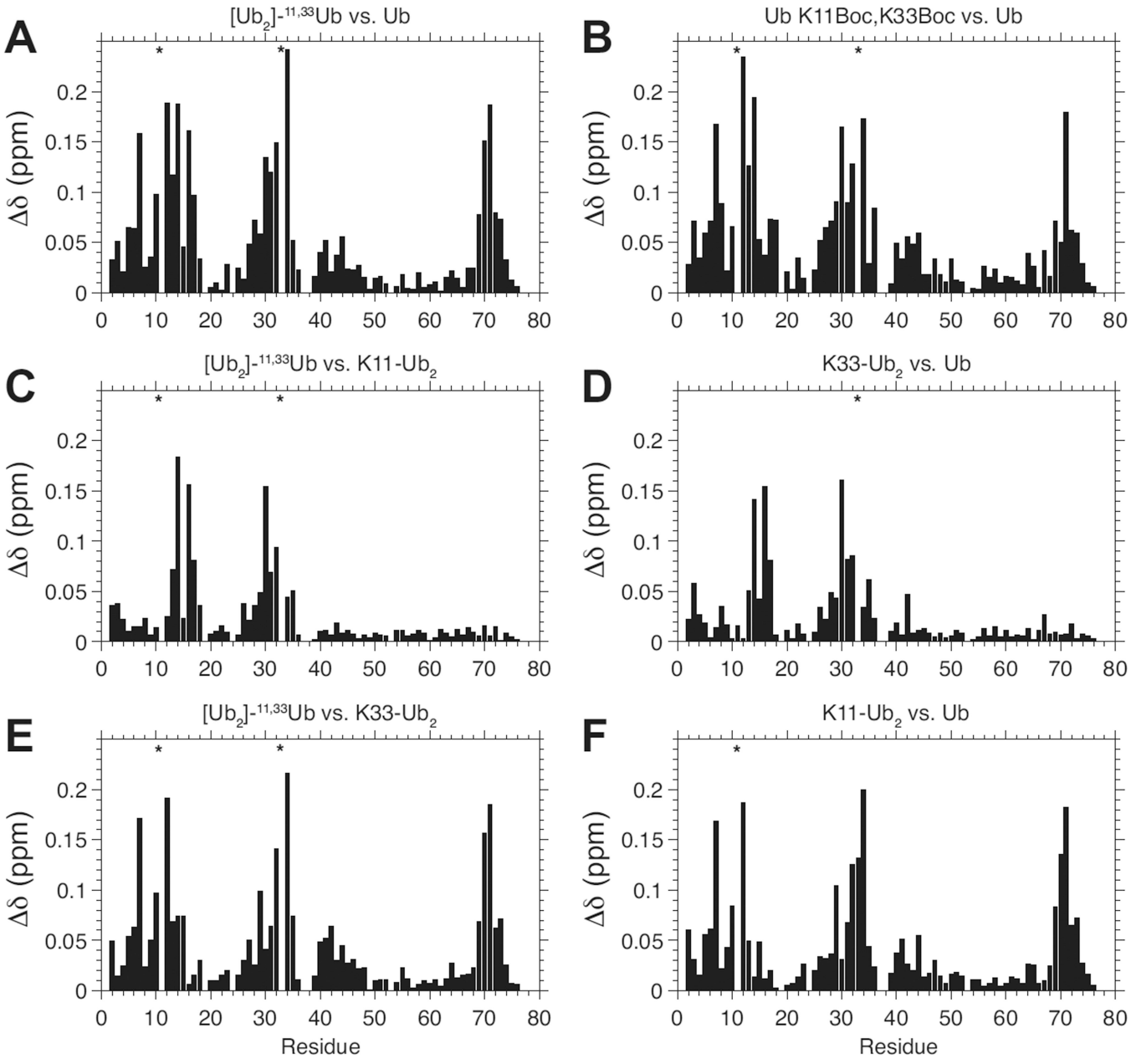


Figure 3.

Spectral differences, quantified as amide chemical shift perturbations (CSPs, Δδ), of the proximal Ub in $[Ub]_2$–^{11,33}Ub *versus* (A) monoUb, (C) the proximal Ub in K11-linked Ub_2, and (E) the proximal Ub in K33-linked Ub_2. (B) CSPs of Ub K11Boc,K33Boc monomer versus Ub. (D,F) CSPs of the proximal Ub in K33-linked Ub_2 (D) and proximal Ub in K11-linked Ub_2 (F) *versus* Ub. Absent NMR signals due to the incorporation of unlabeled Lys(Boc) are marked with asterisks.

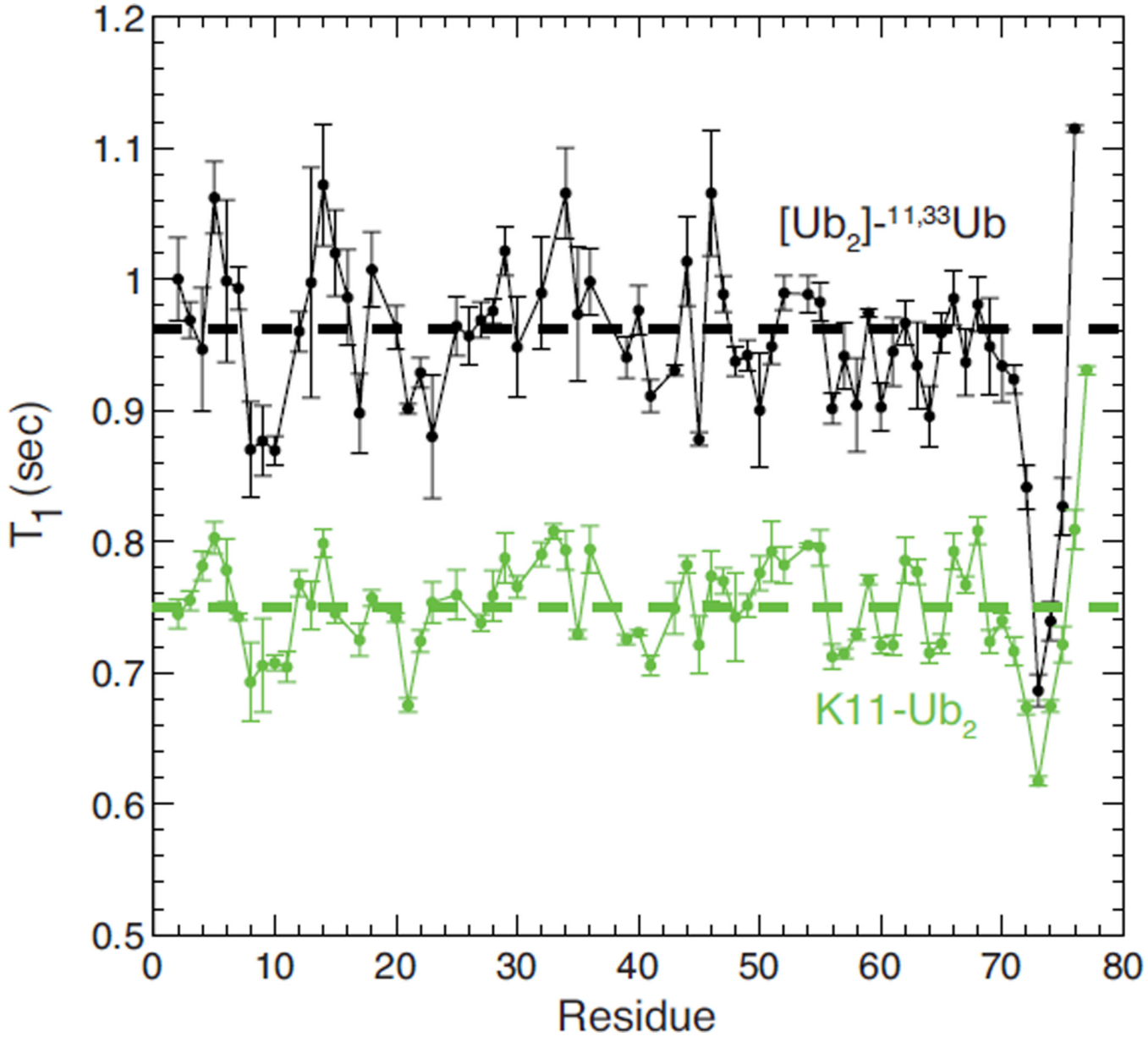


Figure 4.

^{15}N T_1 relaxation time measured for backbone amides in the proximal Ub of $[Ub]_2$–^{11,33}Ub (black) and in the proximal Ub in K11-linked Ub_2 (green). The dashed horizontal lines represent the average levels of ^{15}N T_1 for residues in secondary structure elements.

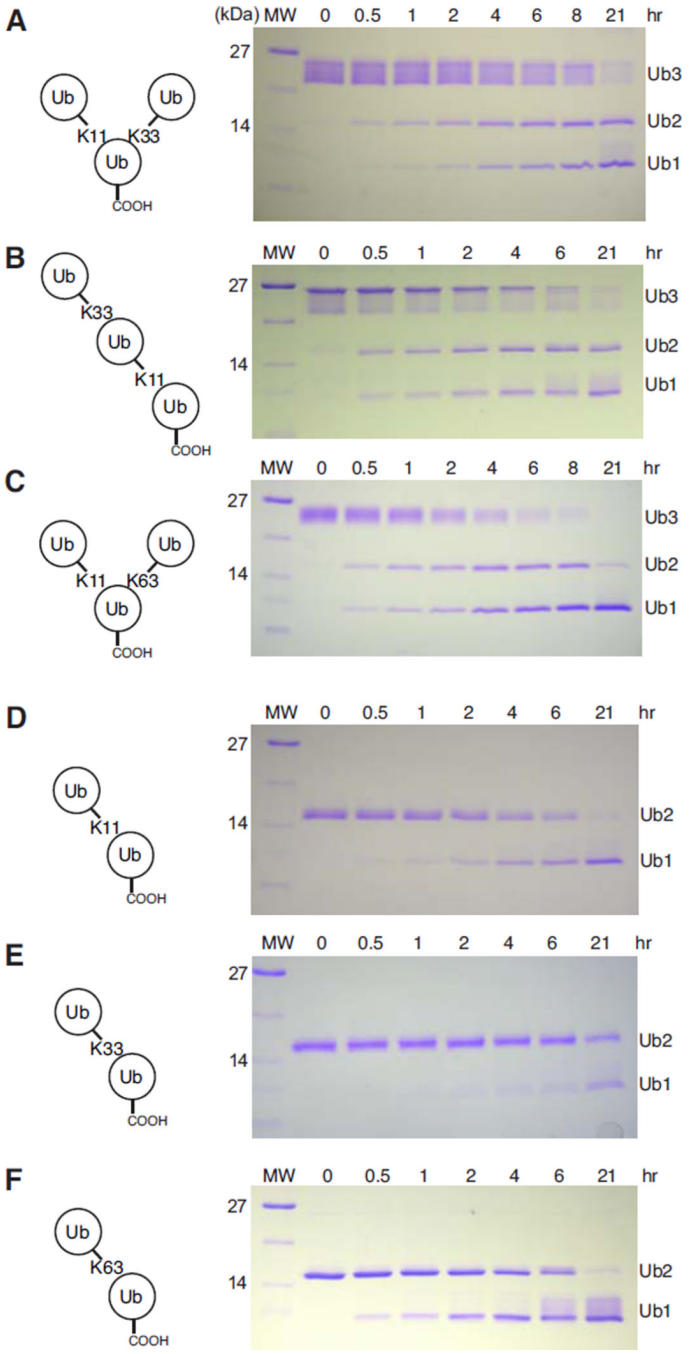
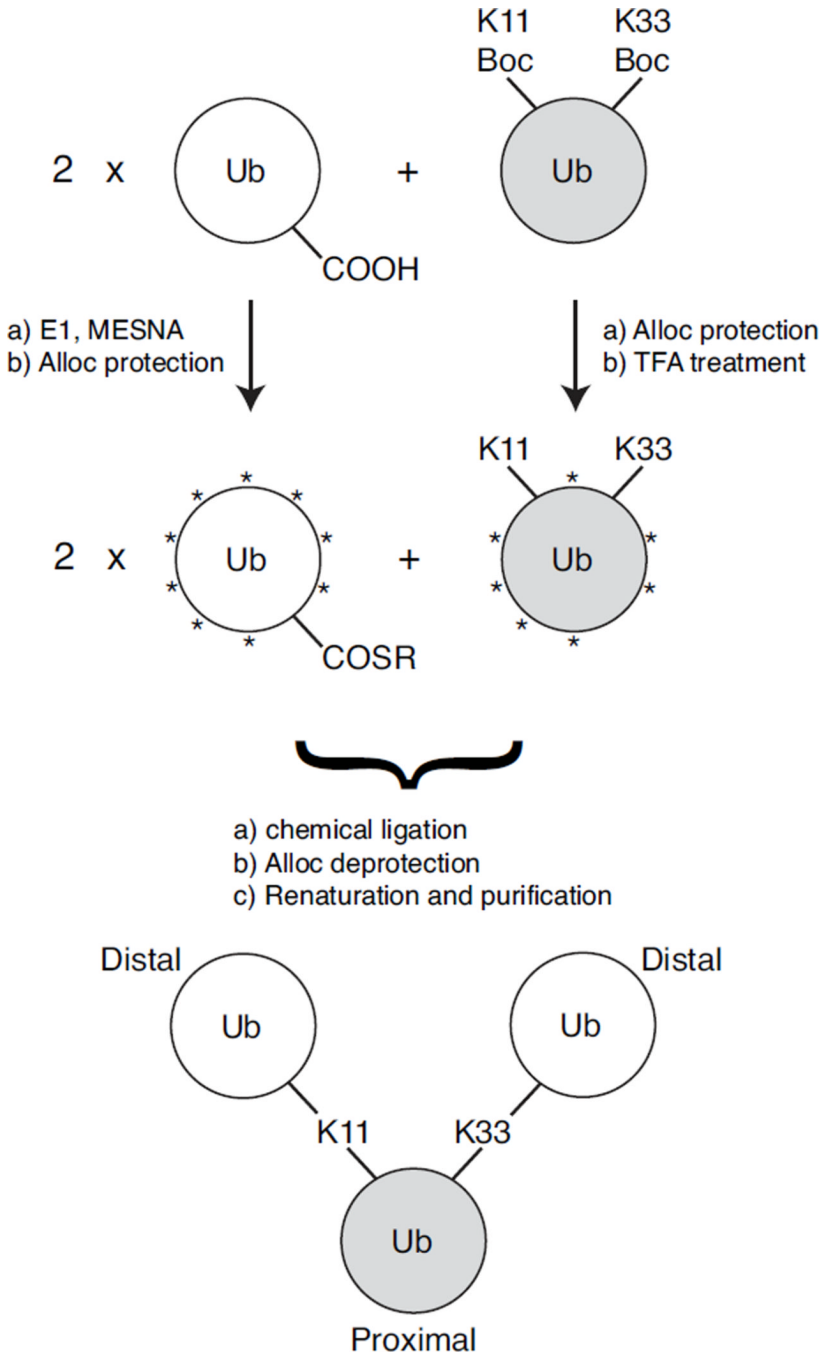


Figure 5.
Disassembly of polyubiquitin chains by a proteasome-associated deubiquitinase Ubp6. A cartoon representation of each polyUb species is depicted on the left side of the corresponding gel.



Scheme 1.

Non-enzymatic assembly of K11,K33 branched tri-Ub ($[Ub]_2$–$^{11,33}Ub$). Gray shading indicates Ub unit that is ^{15}N-enriched. Asterisks (*) represent the Alloc protecting group.