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Preparing to Read the Ubiquitin Code: Characterization of Ubiquitin trimers by Top-down Mass Spectrometry

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Abstract

The profound effects of ubiquitination on the movement and processing of cellular proteins depend exquisitely on the structures of mono and polyubiquitin modifications. Unconjugated polyubiquitins also have a variety of intracellular functions. Structures and functions are not well correlated yet, because the structures of polyubiquitins and polyubiquitin modifications of proteins are difficult to decipher. We are moving towards a robust strategy to provide that structural information. In this report electron transfer dissociation mass spectra of six synthetic ubiquitin trimers (multiply branched proteins with molecular masses exceeding 25,600 Da) are examined using an Orbitrap Fusion Lumos instrument to determine how top-down mass spectrometry can characterize the chain topology and linkage sites in a single, facile workflow. The efficacy of this method relies on the formation, detection, and interpretation of extensive fragmentation.

Keywords

polyubiquitins; electron transfer dissociation; top-down analysis; branched proteins; workflow

INTRODUCTION

Ubiquitin (Ub) is a small protein that has been conserved in eukaryotes all along the evolutionary chain and is critical for cell survival.[1,2,3] It is found free in cells, as a monomer and as polymers of various topologies, and both of these have been observed covalently conjugated to other proteins. Unanchored polyubiquitins[4,5] and protein (poly)ubiquitination have been linked to many cellular processes, including endocytosis, [6,7] protein degradation,[8] and DNA repair.[9] Hence reliable and facile strategies are needed for characterization of both free and conjugated polyubiquitins. This manuscript describes a top-down method to characterize the size and connectivity of unanchored trimeric ubiquitins. It builds on our previous work [10,11] differentiating isomeric Ub dimers and moves towards the objective of a general approach to characterize polyubiquitins free and conjugated to target proteins.

Ubiquitin chains can be formed in various topologies. In trimers these can be generally grouped as unbranched and branched (Fig. 1). In this study the proximal (P) Ub is defined as

the Ub with the free C-terminus; distal (D) Ubs are characterized as terminal in an unbranched or branched chain; and endo (E) Ubs exist only in unbranched chains (Fig. 1). Each Ub in a chain can be linked with another Ub moiety at seven different lysines (K6, K11, K27, K29, K33, K48, K63) and also at the N-terminal methionine. This last attachment does not generate a branched polypeptide and is not part of the present study. Characterization of the lengths and linkage types present in polyubiquitin chains has been a topic of great interest for the last three decades.[5,12–15]

Several mass spectrometry-based methods have been implemented for polyubiquitin characterization. Bottom-up proteomics relies on proteolysis using trypsin, which cleaves Ub chains at R74, leaving a GG tag at the linkage site on a peptide from the polyubiquitin chain or from a target protein.[16–21] This strategy has been used very successfully in identifying ubiquitinated proteins, however it has the drawback that the tag (GG) does not retain information on the length or connectivity of polyubiquitin modifications. Two strategies have been evaluated to shorten the branches in polyubiquitins and still retain a connectivity backbone: time-limited acid cleavage and limited trypsinolysis.[10,11,15,20] Both of these middle-out approaches have been demonstrated successfully on tri-ubiquitins, the focus of the present work. In time-limited studies a large mixture of peptides is created, reflecting many potential cleavage sites. This makes proteomic searches more difficult and lowers the relative abundances of the peptides of interest. Top-down mass spectrometric methods have also been evaluated for polyubiquitins. Molecular masses can be determined, however investigators using collisionally induced dissociation and photo-dissociation reported difficulty in obtaining sufficient bond cleavage (and thus structural information) across the entire ubiquitin polymer.[5,23]

The objective of the current study is to obtain and interpret high quality ETD mass spectra of a set of tri-ubiquitins (Fig. 1) to discern the topology as branched or unbranched and to identify linkage sites. An Orbitrap Fusion Lumos mass spectrometer is shown to provide and record extensive fragmentation in these highly redundant branched structures. However, it is also necessary to be able to interpret the spectrum. We report one successful approach utilizing the top-down software ProSight Lite[24] to expedite manual interpretation. Middle-out acid cleavage analysis is also used in two cases to clarify linkage sites.

MATERIALS AND METHODS

Synthesis of Ubiquitin Trimers

Ub-³³SUb-³³SUb and [Ub]₂-^{11,33}SUb were assembled chemically through silver-mediated ligation of an activated Ub to the selectively deprotected lysine of the other Ub.[25,26] [Ub]₂-^{6,48}SUb (E1, Ubch7, and NleL), [Ub]₂-^{11,63}SUb (E1, Ube2s, MMS2, and Ubc13),[27] Ub-⁴⁸SUb-⁴⁸SUb (E1 and E2-25K),[28] and Ub-⁶³SUb-⁴⁸SUb (E1, MMS2, and Ubc13, E2-25K)[29] were generated enzymatically.[28,30] These structures are presented in Fig. 1. Molecular masses differ (See Supplemental Table 1) because of residue replacements in some Ub sequences required for controlled synthesis.

Trimer Analysis by LC-MS/MS with ESI

Intact trimers were diluted in solvent A (97.5% water, 2.5% ACN and 0.1% formic acid) to 0.03 mg/mL. The chromatography was performed using an Ultimate 3000 ultra-high performance liquid chromatograph (ThermoFisher Scientific, San Jose, CA) interfaced to an Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher Scientific). Three μ L of intact sample were injected, concentrated, and desalted on a PepSwift Monolith trap (200 μ m $\times$ 5 mm) for 5 mins before separation on a ProSwift RP-4H column (100 μ m $\times$ 25 cm) (ThermoFisher Scientific) with a gradient of 20% to 40% solvent B (75% ACN, 25% water, 0.1% formic acid) over 15 mins. The potential for in-source fragmentation was set to 10V. Precursor and fragment ion masses were acquired with a resolution of 120,000. Fragmentation was triggered in data dependent mode by electron transfer supported by chemical ionization (ET_hcD) with a 6 msec ETD reaction time and supplemental activation at 10% normalized HCD.

Interpreting the Spectra

Precursor and fragmentation ions were deconvoluted using Xtract 3.0 (Thermo Scientific). Fragment ions from the top m/z precursor ions selected in data dependent mode were combined and then matched against the sequence of monoubiquitin using ProSight Lite (<http://prosightlite.northwestern.edu/>) with a 3 ppm mass tolerance. In our strategy the monoubiquitin sequence is used as a template to assess the fragmentation patterns of each of the Ub moieties present in the trimer. ProSight Lite allows for custom mass additions to any amino acid in the template sequence. Masses equivalent to one or two Ub moieties were added, and changes in fragmentation patterns assigned by ProSight Lite were used to assign the topology of each trimer as branched or unbranched chain. This is discussed in detail in the Results and Discussion section. Finally, linkage sites were assigned by inspection of fragmentation patterns assigned to the monoubiquitin template. ProSight Lite also identifies ions as c and z formed primarily by ETD, and b and y formed primarily by HCD.

Microwave-Assisted Acid Cleavage

Ubiquitin trimers were diluted to 0.15 mg/mL in 12.5% acetic acid and digested for 60 sec at 140°C using 300 W of power in a CEM Discover microwave (Matthews, NC). These conditions have been previously determined to produce partial cleavage of polyubiquitins at Asp residues.[10,11] Digested trimers were lyophilized and resuspended in solvent A (97.5% water, 2.5% ACN and 0.1% formic acid) at 0.1 mg/mL for chromatography performed using the LC-MS/MS system specified above. Five μ L of each digested sample were injected, concentrated, and desalted on a Zorbax C8 trap (0.5 $\times$ 3 mm, Agilent Technologies) for 5 mins before being separated on a Zorbax C8 column (3.5 μ m, 150 mm $\times$ 75 μ m, Agilent Technologies, Santa Clara, CA) with a 500 nL/min flow rate and a gradient of 30% to 37% solvent B (solvent B: 75% ACN, 25% water, 0.1% formic acid) over 45 mins. Spectra were acquired and processed as described above.

RESULTS AND DISCUSSION

Six isomeric Ub trimers were synthesized for this study (Fig.1) and used to develop and test our top-down strategy. High-resolution mass spectrometry was used to identify each isomer's

intact mass within 5 ppm (Supplemental Table 1). The workflow in Fig. 2 is specifically designed for Ub trimers. Thus the intact mass determination in **Step 1** is vital. In analysis of a cell lysate it is envisioned that polyubiquitins would be enriched by immuno or affinity precipitation, trimers would be recognized in the chromatographic eluent by their masses, and their tandem mass spectra would be subjected to the interpretation developed here for tri-ubiquitins.

Once the sample is recognized as a Ub trimer, extensive fragmentation is required to determine all other features of the chain. In **Step 2** all the fragment ions are matched using ProSight Lite to the sequence of the proximal Ub (Fig. 3). High fragmentation density will support the tentative assignment as an Ub. In Step 2 a linkage site on the proximal Ub is elucidated. Because the only free C-terminus is in the proximal Ub, any y/z-ions characterized on the monoubiquitin template must be formed from the proximal Ub. This series of y/z fragment ions will be terminated on the template when a mass addition occurs in the sequence (Fig. 3). This can be the addition of two Ub masses in an unbranched chain, or a single Ub mass in the case of branching.

Almost no information can be gathered about the linkage or topology using b/c ions, because all three N-termini of a native tri-ubiquitin chain can produce the same b/c-ions. In this study unmutated distal Ubs will produce a redundant and indistinguishable set of b/c-ions (Fig. 3b and e) whereas the synthetic chains with mutations on the distal Ubs have b/c-ions cut off at the first mutation. The absence of such redundant fragmentation is reflected in Fig. 3a, c, d, and f. All sequence variants are highlighted in Fig. 1. However, even with mutations, the b/c-ions that are present do serve to confirm the sample as a ubiquitin.

Once we know that the sequence is related to that of Ub we can ask what the topology of the chain is (**Step 3**). To answer this question we must look at the structural difference between the branched and the unbranched trimers. An important difference is shown in Fig. 1. The unbranched trimer contains an “endo” Ub, a ubiquitin which carries isopeptide bonds at both the C-terminus and on one of its lysines, whereas the branched isomer does not. Characteristic fragment ions can prove the presence of an endo Ub, thus an unbranched topology, or by their absence a branched topology (Step 3, Fig. 4). In this step a new template is established using ProSight Lite in which the mass of a proximal moiety is added at G76. (The mass of water is subtracted in the attachments.) ProSight Lite is then used to map fragment ions against the modified template. Ions that are unique to an endo Ub are formed by y and/or z cleavages between K63 and the C-terminus, and carry the mass of the proximal mono-Ub attached to the C-terminus. In an unbranched trimer the third or distal Ub moiety will be attached to the endo Ub at lysine residues up to and including K63 (Fig. 4). If y/z ions formed by amide bond cleavage between K63 and G76 (Boxed in Fig. 4) and carrying the mass of the proximal Ub are observed, the trimer is unbranched. (Fig. 1) If y/z sequence ions are not observed, then the trimer is unlikely to have unbranched topology.

After the topology of the trimer is characterized as unbranched or branched, the linkage locations are verified in **Step 4** by inspection of the fragmentation pattern on a topologically correct trimeric template. A simple approach, which avoids tedious iterative addition of one or two Ub masses at each lysine in the proximal and then endo moieties, is to add the

appropriate mass to the N-termini (M1) and trace amide bond cleavage to the point where the fragmentation stops (Fig. 5 and 6). Since the topology is known, the mass addition indicated from Step 2 on the proximal Ub will be one (branched) or two Ub (unbranched). For example, in the case of the Ub-⁴⁸Ub-⁴⁸Ub unbranched chain, y/z fragmentation in the proximal moiety is observed to occur up to K48, indicating that the mass of di-Ub should be added to K48 (Fig. 5a). The absence of contradictory ions can be confirmed. An analogous approach is then applied to the endo Ub in the Ub-⁴⁸Ub-⁴⁸Ub example. After adding the mass of a mono-Ub to the C-terminus of the endo template, formation of ions assigned as y/z is seen not to occur beyond K48. This indicates that the endo Ub is also modified at K48. For confirmation, if the distal mono-Ub mass is added to the N-terminus no change is observed in this y/z fragmentation pattern. Despite the large masses of the modifications, this strategy is supported by ProSight Lite in a manner similar to the way that the mass increment of a classical GG tag is handled by conventional bottom up software.

If Step 3 indicates that the chain is branched, the linkage locations can be determined through a similar process. Now the proximal Ub is modified by two distal Ub moieties. Using the branched synthetic standard [Ub]₂-^{11,33}Ub as an example, Step 2 will already have shown a Ub addition at K33 (Fig. 3e) due to the absence of y/z ions formed before E34. In a generalized approach, which should confirm this observation and find the remaining linkage, a template was constructed in which the mass of a mono-Ub is added to the N-terminus, just as in the unbranched chain determination, and the mass of another Ub is added to K63 (i.e. the closest linkage site to the C-terminus) (Fig. 6b). The fragmentation pattern can then be used to determine where the b/c ions and y/z ions start and end. Again the fragmentation pattern shown in Fig. 6b is consistent with the assignment of one linkage at K33, because in this example formation of y/z ions is only observed after K33. Revealing b/c-ions are observed only after K11 in the sequence of the proximal ubiquitin, and the fragmentation pattern revealed by the new template localizes the second Ub addition at the amino terminus, at either K6 or K11. Using this strategy the structure of the [Ub]₂-^{6,48}Ub trimer was unambiguously defined (Fig. 7), while in the [Ub]₂-^{11,63}Ub trimer the top-down strategy localized the linkage site toward the N-terminus, but cannot distinguish K6 and K11. In the two ambiguous cases reported here, middle-out analysis was used as a supplementary technique to assign the position of attachment (see below). In the event that bond cleavage is observed between K6 and K11, as in [Ub]₂-^{6,48}Ub, the strategy proposed here will be entirely applicable.

After all linkage sites are confirmed, a final image can be put together (**Step 5**). Fragmentation density should be the highest when mapped against this final correct structure. Thus Step 5 provides final confirmation of the correct assignment. Final images are shown in Fig. 7 for the six isomeric trimers studied.

To resolve the sites of attachment in the branched trimers [Ub]₂-^{11,33}Ub and [Ub]₂-^{11,63}Ub in the present work, microwave-supported acid cleavage[9] was used with the intention to recover a partially cleaved product in which the two distal Ubs are cleaved at D52 and the backbone is retained to include the two linkage sites (Supplemental Fig. 2) This truncated product allowed easier determination of linkage sites between K6 and K11 because the truncated distal Ubs no longer produce b/c ions that duplicate those formed from the

proximal moiety. Thus the b/c ions seen in Fig. 8 are unique to the proximal moiety and distinguish attachment at K6 from K11. Both unbranched and branched trimers can be analyzed by acid cleavage, however less advantage is offered for the unbranched structures.

CONCLUSION

The successful strategy presented here for analysis of unbranched and branched trimers of Ub relies on high bond coverage achieved here by electron transfer reactions supplemented by high energy collisional dissociation that proceed efficiently at high concentrations of reactants. These reactions, which fragment bonds across the entire polymer, are now available in state-of-the-art mass spectrometers. Another component of this workflow is the algorithm made available by the Kelleher laboratory,[24] which obviates extensive manual interpretation of the complex mass spectra. It is also important that the analyses are carried out on the chromatographic time scale, and are thus potentially applicable to polyubiquitin mixtures enriched from cell lysates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Fig. 2.

a. 1 M Q I F V K T L T G K T I T L E V E P S D T I E N 25 26 V K A K I Q D K E G I P P D Q Q R L I F A G K Q L 50 51 E D G R T L S D Y N I Q K E S T L H L V L R L R G 75 76 G	b. 1 M Q I F V K T L T G K T I T L E V E P S D T I E N 25 26 V K A K I Q D K E G I P P D Q Q R L I F A G K Q L 50 51 E D G R T L S D Y N I Q K E S T L H L V L R L R G 75 76 G
c. 1 M Q I F V K T L T G K T I T L E V E P S D T I E N 25 26 V K A K I Q D K E G I P P D Q Q R L I F A G K Q L 50 51 E D G R T L S D Y N I Q K E S T L H L V L R L R G 75 76 G	d. 1 M Q I F V K T L T G K T I T L E V E P S D T I E N 25 26 V K A K I Q D K E G I P P D Q Q R L I F A G K Q L 50 51 E D G R T L S D Y N I Q K E S T L H L V L R L R G 75 76 G
e. 1 M Q I F V K T L T G K T I T L E V E P S D T I E N 25 26 V K A K I Q D K E G I P P D Q Q R L I F A G K Q L 50 51 E D G R T L S D Y N I Q K E S T L H L V L R L R G 75 76 G	f. 1 M Q I F V K T L T G K T I T L E V E P S D T I E N 25 26 V K A K I Q D K E G I P P D Q Q R L I F A G K Q L 50 51 E D G R T L S D Y N I Q K E S T L H L V L R L R G 75 76 G

Fig. 3.

Fig. 4.

Endo		Endo		Endo	
1	QIFVKTLTGKTTITLEVEPSDTIEN	25	1	QIFVKTLTGKTTITLEVEPSDTIEN	25
26	VKAKIQDKEGIPPDQQLIFAGKQL	50	26	VKAKIQDKEGIPPDQQLIFAGKQL	50
51	EDGRTLSDYNIQKESITLHVLRLRLG	75	51	EDGRTLSDYNIQKESITLHVLRLRLG	75
76	G		76	G	
Proximal		Proximal		Proximal	
1	QIFVKTLTGKTTITLEVEPSDTIEN	25	1	QIFVKTLTGKTTITLEVEPSDTIEN	25
26	VKAKIQDKEGIPPDQQLIFAGKQL	50	26	VKAKIQDKEGIPPDQQLIFAGKQL	50
51	EDGRTLSDYNIQKESITLHVLRLRLG	75	51	EDGRTLSDYNIQKESITLHVLRLRLG	75
76	G		76	G	

Fig. 5.

Fig. 6.

Fig. 7.