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Organic Chemistry Applied to Synthetic Proteins: Modifying the Vicinity of the Isopeptide Bond Revealed Differential Behavior of Ubiquitin Chains with Interacting Proteins

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Abstract

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In Every Direction—Chemical synthesis of proteins allowed the synthesis of ubiquitin chains modified in the vicinity of the isopeptide peptide to examine their behavior with deubiquitinases and ubiquitin binding domains. Our results set the ground for the generation of unique probes for studying the interactions of these chains with various ubiquitin-interacting proteins.

Keywords

Ubiquitin Chains; Deubiquitinases; Ubiquitination; Protein synthesis; Peptide ligation

Ubiquitination—the attachment of a ubiquitin (Ub) monomer, composed of 76 amino acids, or of a polyubiquitin (polyUb) chain to a protein target, is involved in a wide range of cellular processes, including protein degradation, trafficking, transcription and the DNA damage response.^[1] Polyubiquitination produces molecular signals that depend on which of the seven lysines in Ub is linked via an isopeptide bond to the C-terminal Gly76 of the consecutive Ub molecules.^[2] In addition, Met1 of Ub can also be used to link the consecutive Ub molecules assembling the so-called linear chains.^[2] Ubiquitination is a reversible posttranslational modification and the reverse reaction i.e. deubiquitination is controlled by a family of enzymes known as deubiquitinases (DUBs), which hydrolyze isopeptide bonds within Ub bioconjugates.^[3]

DUBs are involved in a variety of regulatory processes, such as cell-cycle progression and differentiation, and several DUBs have been implicated in various neurological infectious and neoplastic diseases,^[3b] leading to the emergence of DUBs as potential therapeutic targets. Biochemical analyses of Ub chains revealed different DUBs' specificities towards these chains. Still unclear, however, is how most of these enzymes recognize the different Ub chains in their free or anchored form and what are the molecular bases for their specificities^{[4],[5]}. It appears that various factors are influencing recognition and specificity; among them are the structural topology and dynamics of these chains as well as the peptide sequences near the isopeptide bond that links two Ubs. For example, a single-molecule fluorescence study showed that conformational equilibria of these chains provide another level of regulation of the recognition and specificity of the chains with various DUBs and Ub binding domains (UBDs) and that by interfering with conformational dynamics one could affect chain hydrolysis by DUBs.^[6]

Recent breakthroughs in the chemical and semisynthesis of Ub chains in their native forms offer exquisite control over the atomic structure of any chain^[7] and have opened unprecedented opportunities to shed light on the modes of recognition and processing by the different ubiquitin-interacting proteins such as DUBs. For example, one could investigate the role of the isopeptide bond in the recognition of Ub chains by the different ubiquitin-interacting proteins. Along these lines, we have recently shown that N-methylation^[8] of the isopeptide bond in H2B, ubiquitinated at Lys120 renders it unrecognizable by its specific monoclonal antibody.^[9] This may indicate that the recognition event is mainly mediated by the isopeptide bond and its vicinity. Such understanding of the consequences of

manipulating the isopeptide bond and its nearby residues on chain recognition and processing could also lead to the design of unique probes based on these chains (e.g. highly labile or stable di-Ub^[10]). Here we report on the chemical synthesis and characterization of 14 di-Ub analogues that are site-specifically modified at the nitrogen of the isopeptide bond, Gly76, or at the δ -carbon of the isopeptide Lys residue (Figure 1) and their behavior with ubiquitin-interacting proteins. Our study revealed differential stability and affinities of these analogues with various DUBs and UBDs.

In order to investigate how small changes near the isopeptide bond could affect the recognition of ubiquitin-interacting proteins, we envisioned the chemical preparation of Ub chains with specific modifications at the nitrogen of the isopeptide bond, α -carbon of Gly76 of the distal Ub, or the δ -carbon of the isopeptide Lys residue (Figure 1). In pursuing the preparation of these analogues, three different strategies were adopted that enabled their highly efficient synthesis for biochemical and structural analyses with DUBs and UBDs. To prepare the modified analogues at the δ -carbon, we utilized our previously developed synthesis of di-Ub chains^[7c] mediated by δ -mercaptolysine^[11] to enable isopeptide chemical ligation (ICL) and generated the thiol modified K48- or K63-linked di-Ub, **1**, **2**, respectively. These analogues were either desulfurized to obtain the native di-Ub (**3**, **4**) or alkylated at the thiol handle with iodoacetamide, methyl vinyl sulfone, or maleimide to give the analogues **5–10** (Scheme 1). Product isolation and refolding allowed us to study the effect of these chemical modifications on the DUBs' activity.

Initially, we chose two enzymes from the USP family; Isopeptidase T (IsoT)^[12] and Ubiquitin Specific Protease 2 (USP2),^[13] which are known to cleave free ubiquitin chains with preference towards the K48 and K63-linked chains. To determine the hydrolysis percentage, our di-Ub analogues were incubated separately with each of the two enzymes and analyzed by HPLC and ESI-MS (Supporting Information, SI). Surprisingly, despite the use of various alkyl groups of different sizes, all the analogues were cleaved in a similar percentage as the unmodified chains (Figure 2). These results may reflect the conformational flexibility of the isopeptide linker in the di-Ub chains. This could also be a result of the fact that the two DUBs examined here, IsoT and USP2, are not linkage specific, hence they are less sensitive to changes in the nearby residues of the isopeptide bond. Our results also indicate that modifying the thiol handle after ICL does not necessarily generate di-Ub chains that are highly stable against all DUBs. However, one may consider modifying the thiol with various probes to follow di-Ub cleavage, chains' dynamics and for other studies. Such strategy is currently under investigation.

Next, we focused on studying the effect of N-methylation of the isopeptide on the Ub chain conformation, stability to DUBs, and binding to a UBD. We had recently reported that N-methylation of the isopeptide bond in ubiquitinated peptides and proteins makes these conjugates stable against DUBs.^[8] However, this had not been examined with Ub chains. The strategy for the synthesis of N-methylated di-Ub (Scheme 2) relies on two-fragments that will be linked via native chemical ligation (NCL) followed by desulfurization.^[14] Accordingly, we succeeded in synthesizing building block **12**, containing the C-terminal part of the distal and proximal Ub linked via an N-methylated isopeptide bond and each presenting N-terminal Cys for NCL with Ub(1-45)-thioester.

After cleavage and purification by HPLC, this peptide was ligated with the complementary part of Ub fragment, Ub(1-45)-thioester. The subsequent desulfurization of the two Cys residues to their parent Ala gave the desired product **13**, in 24% yield (for the two steps). Notably, this strategy has never been applied to the synthesis of Ub chains and can also be used in future studies related to the synthesis and studies of other Ub chains with the

advantage of relying only on peptide synthesis and NCL, without the need for the special amino acid δ -mercaptolysine.

Interestingly, when examining analogue **13** with both IsoT and USP2, although it exhibited higher stability in the presence of IsoT or USP2 than the unmodified chain, **3**, (see SI), DUBs were still able to cleave it. These results are in sharp contrast to the N-methylated H2B-Ub, which exhibited remarkable stability even in cell lysate, and to the known behavior of N-methylation of backbone amide bond in peptides.^[15] This provides another example of the complexity of recognition and processing by DUBs of their substrates. It also indicates that N-methylation of the isopeptide bond in Ub bioconjugates cannot be used as a general strategy for resisting DUB activities as it depends on both the type of substrate and its specific DUB.

To complete the scanning of the isopeptide bond's vicinity and to study the effect of the modification at the C-terminus of the distal Ub, we modified Gly76 with other amino acids such as Ala, Cys, and Cys-(acetamide) (Scheme 3). The syntheses of these analogues were carried out using SPPS and NCL.^{[16],[17]} Here, Fmoc-Lys-(Dde)-OH was used as the orthogonally protected Lys to enable selective removal of the Lys protecting group for the formation of the isopeptide bond. After the synthesis of Ub with the Lys-(Dde) using Fmoc-SPPS, the ϵ -amine of the Lys residue was unmasked using hydrazine hydrate. Subsequently, the free amine was coupled with Boc-Cys(Trt)-OH to enable ligation with the distal Ub(1-75)-thioester. After peptide cleavage and purification, this fragment was ligated with the Ub(1-75)-thioester to afford di-Ub **16** in 40% yield. This di-Ub was either desulfurized to afford di-Ub **17** with the G76A mutation, or alkylated with iodoacetamide to furnish di-Ub **18**.

To determine the effect of these modifications on DUB activity, we tested the di-Ub analogues with IsoT and USP2 as described above. While di-Ub **16** and **18** were completely stable, di-Ub **17** exhibited some degree of cleavage (~20%) after 30 min incubation. Previous studies by Hodgins et al.^[18] showed that the Ub Gly76Ala mutation results in irreversible conjugation to proteins or to itself when forming Ub chains, causing a change in the Ub conjugate equilibrium. On the other hand, Muir's group has reported that H2B-Ub(G76A) was cleaved by UCH-L3 in a similar manner to the native substrate.^[17] Our results show that K48-linked di-Ub chain, with the G76A mutation, also is not completely stable with IsoT (20% vs 49% after 30 min) and USP2 (13% vs 66% after 30 min), however it was cleaved to a lesser extent compared to the unmodified chains (See SI, Figures S38+S39).

It is important to note that the two DUBs that we have examined so far are not linkage specific. It is likely that the recognition of the Ub surfaces is the driving force for DUB recognition and cleavage of the chains. Next, we questioned whether the linkage-specific DUB, OTUB1,^[19] which is known to cleave only K48-linked chains, would recognize some of the modified analogues. For this, we examined three representative analogues, **5**, **13**, and **16** that were compared to enzymatically-assembled wild-type K48-linked di-Ub.^[20] Interestingly, in all of the modified cases OTUB1 failed to cleave any of these chains (see SI, Figure S42), which indicates the importance of the linkage and its nearby residues in the recognition and cleavage of these chains.

To examine the effect of modifying the isopeptide bond on the recognition of ubiquitin-interacting proteins, we decided to measure the binding affinity of representative analogues, **13**, **16**, and **18** as well as unmodified K48- and K63-linked di-Ub **3** and **4**. Specifically we were interested in studying the di-Ub analogues' affinities for Ubiquitin-Associated 2 domain (UBA2) of the human homologue of protein Rad23 (hHR23a), which binds

preferentially to K48-linked chains over K63-linked chains.^[21] For this, we synthesized UBA2 that is biotinylated at its N-terminus in order to immobilize it onto a NeutrAvidin coated-chip. Binding affinity was measured by surface plasmon resonance (SPR). First, the biotin-UBA2 was bound to the NeutrAvidin coated-chip, followed by flowing each of the stable di-Ub analogues (**13**, **16**, and **18**) as well as the native analogues (**3** and **4**) as controls at various concentrations (0.5–22 μ M), (Figure 3). The binding affinities of the native analogues matched very well with the reported values that were determined by NMR titrations.^[21a] In addition, the N-methylated di-Ub **13** exhibited similar binding affinity, which indicated that the N-methylation had not interfered with the binding of UBA2. Surprisingly, the stable analogues **16** and **18** exhibited 10-fold higher affinities when compared to the native analogue. It is not clear yet why analogues **16** and **18** exhibited higher affinities towards UBA2. One possibility might be that restricting the conformation of the isopeptide-surrounding area by modifying Gly76 could induce favorable binding compared to the unmodified isopeptide.

To shed further light on the binding of analogues **13** and **16** to UBA2, we used NMR to examine and compare the residue-specific patterns of interactions of these analogues and enzymatically-assembled wild-type K48-linked di-Ub^[20] with ¹⁵N-labelled UBA2. Changes in the backbone amide chemical shifts of UBA2 upon titration with di-Ub were monitored via ¹H-¹⁵N SOFAST-HMQC experiments, quantitated as chemical shift perturbations (CSPs), and mapped onto the protein surface of UBA2 (Figure 4). With all three di-Ub analogues, a significant number of signal attenuations were observed in UBA2 during the course of the titration experiment, indicative of slow exchange, hence tight binding. The overall signal attenuation profiles and signal trajectories for individual residues were similar across all three titrations, suggesting that UBA2 interacts with each di-Ub analogue in a similar manner, despite that ligand saturation was not reached with analogues **13** and **16** by the titration endpoint. The similarity of NMR signal trajectories indicates that UBA2 makes similar physical and chemical contacts with these di-Ub analogues as with the wild-type K48-linked di-Ub, thus suggesting identical binding modes (see SI, Figure S45). Importantly, the UBA2 residues that exhibited significant CSPs or signal attenuations map to the same UBA2 surfaces that were previously identified to interact specifically with K48-linked di-Ub.^[21] In fact, UBA2 was shown to bind K48-linked di-Ub in a sandwich-like manner, where each side of UBA2 forms a specific interface with the corresponding Ub unit and the isopeptide linker (Figure 4D). Thus, our results suggest that despite the isopeptide bond modifications, both K48-linked di-Ub analogues (**13** and **16**) bind to UBA2 specifically and in a similar binding mode as native K48-linked di-Ub. As UBA2 is known to bind across the Ub-Ub linker, these findings suggest that UBA2 is able to recognize the K48 linkage in each of the di-Ub analogues.

In summary, we have presented three different approaches for the preparation of 14 different analogues of Ub chains modified in the vicinity of the isopeptide bond. Examination of these analogues revealed differential behavior with DUBs and UBDs. In particular, DUBs that are linkage specific exhibited greater sensitivity to these modifications while DUBs that are active against various chains were tolerant to many of these modifications. Notably, modifications of Gly76 generated the most stable analogues with increased binding affinity towards UBA2. Our results lay the groundwork for creating a new generation of probes based on Ub chains for various studies and should enable further understanding on the mode of recognition and processing of their native substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. a) Glickman MH, Ciechanover A. *Physiol Rev.* 2002; 82:373. [PubMed: 11917093] b) Chen ZJ, Sun LJ. *Mol Cell.* 2009; 33:275. [PubMed: 19217402]
2. Eddins MJ, Varadan R, Fushman D, Pickart CM, Wolberger C. *J Mol Biol.* 2007; 367:204. [PubMed: 17240395]
3. a) Reyes-Turcu FE, Wilkinson KD. *Chem Rev.* 2009; 109:1495. [PubMed: 19243136] b) Love KR, Catic A, Schlieker C, Ploegh HL. *Nat Chem Biol.* 2007; 3:697. [PubMed: 17948018]
4. Komander D, Clague MJ, Urbe S. *Nat Rev Mol Cell Biol.* 2009; 10:550. [PubMed: 19626045]
5. The only known structure of DUB with its substrate is the AMSH enzyme complexed with K63-linked di-Ub, for more details see: Sato Y, Yoshikawa A, Yamagata A, Mimura H, Yamashita M, Ookata K, Nureki O, Iwai K, Komada M, Fukai S. *Nature.* 2008; 455:358. [PubMed: 18758443]
6. Ye Y, Blaser G, Horrocks MH, Ruedas-Rama MJ, Ibrahim S, Zhukov AA, Orte A, Klenerman D, Jackson SE, Komander D. *Nature.* 2012; 492:266. [PubMed: 23201676]
7. a) Spasser L, Brik A. *Angew Chem Int Ed Engl.* 2012; 51:6840. [PubMed: 22696461] b) Strieter ER, Korasick DA. *ACS Chem Biol.* 2012; 7:52. [PubMed: 22196026] c) Kumar KS, Spasser L, Erlich LA, Bavikar SN, Brik A. *Angew Chem Int Ed Engl.* 2010; 49:9126. [PubMed: 20815002] d) Kumar KS, Bavikar SN, Spasser L, Moyal T, Ohayon S, Brik A. *Angew Chem Int Ed Engl.* 2011; 50:6137. [PubMed: 21591043] e) El Oualid F, Merckx R, Ekkebus R, Hameed DS, Smit JJ, de Jong A, Hilkmann H, Sixma TK, Ovaa H. *Angew Chem Int Ed Engl.* 2010; 49:10149. [PubMed: 21117055] f) Castaneda C, Liu J, Chaturvedi A, Nowicka U, Cropp TA, Fushman D. *J Am Chem Soc.* 2011; 133:17855. [PubMed: 21962295] g) Virdee S, Ye Y, Nguyen DP, Komander D, Chin JW. *Nat Chem Biol.* 2010; 6:750. [PubMed: 20802491] h) Bavikar SN, Spasser L, Haj-Yahya M, Karthikeyan SV, Moyal T, Kumar KS, Brik A. *Angew Chem Int Ed Engl.* 2012; 51:758. [PubMed: 22131237]
8. Haj-Yahya M, Eltareer N, Ohayon S, Shema E, Kotler E, Oren M, Brik A. *Angew Chem Int Ed Engl.* 2012; 51:11535. [PubMed: 23065695]
9. Minsky N, Shema E, Field Y, Schuster M, Segal E, Oren M. *Nat Cell Biol.* 2008; 10:483. [PubMed: 18344985]
10. a) Eger S, Scheffner M, Marx A, Rubini M. *J Am Chem Soc.* 2010; 132:16337. [PubMed: 21033666] b) Weikart ND, Sommer S, Mootz HD. *Chem Commun.* 2012; 48:296.
11. Kumar KS, Haj-Yahya M, Olschewski D, Lashuel HA, Brik A. *Angew Chem Int Ed Engl.* 2009; 48:8090. [PubMed: 19780082]
12. Wilkinson KD, Tashayev VL, O'Connor LB, Larsen CN, Kasperek E, Pickart CM. *Biochemistry.* 1995; 34:14535. [PubMed: 7578059]
13. Renatus M, Parrado SG, D'Arcy A, Eidhoff U, Gerhartz B, Hassiepen U, Pierrat B, Riedl R, Vincenz D, Worpenberg S, Kroemer M. *Structure.* 2006; 14:1293. [PubMed: 16905103]
14. Dawson PE. *Isr J Chem.* 2011; 51:862.
15. Chatterjee J, Gilon C, Hoffman A, Kessler H. *Accounts Chem Res.* 2008; 41:1331.
16. Kumar KS, Spasser L, Ohayon S, Erlich LA, Brik A. *Bioconjug Chem.* 2011; 22:137. [PubMed: 21235224]
17. Similar approach was previously reported for the synthesis of H2B-Ub, for more details see McGinty RK, Kohn M, Chatterjee C, Chiang KP, Pratt MR, Muir TW. *ACS Chem Biol.* 2009; 4:958. [PubMed: 19799466]
18. Hodgins RR, Ellison KS, Ellison MJ. *J Biol Chem.* 1992; 267:8807. [PubMed: 1315740]

19. a) Wang T, Yin L, Cooper EM, Lai MY, Dickey S, Pickart CM, Fushman D, Wilkinson KD, Cohen RE, Wolberger C. *J Mol Biol.* 2009; 386:1011. [PubMed: 19211026] b) Edelmann MJ, Iphofer A, Akutsu M, Altun M, di Gleria K, Kramer HB, Fiebiger E, Dhe-Paganon S, Kessler BM. *Biochem J.* 2009; 418:379. [PubMed: 18954305]
20. Castaneda CA, Liu J, Kashyap TR, Singh RK, Fushman D, Cropp TA. *Chem Commun.* 2011; 47:2026.
21. a) Varadan R, Assfalg M, Raasi S, Pickart C, Fushman D. *Mol Cell.* 2005; 18:687. [PubMed: 15949443] b) Raasi S, Varadan R, Fushman D, Pickart CM. *Nat Struct Mol Biol.* 2005; 12:708. [PubMed: 16007098]

Figure 1.
General scheme on points of interest within the Ub chains to be modified and tested.

Figure 2. Enzymatic hydrolysis of di-Ub analogues (1–10) with IsoT and USP2: A) Lys48 linked di-Ub; B) Lys63 linked di-Ub. The percent of hydrolysis was determined from the HPLC data for each reaction using identical reaction conditions (30 min). Error bars correspond to the standard deviation of three measurements.

Figure 3. Surface plasmon resonance (SPR) study of the native diUb-K48 (**3**), native diUb-K63 (**4**), analogues (**13**, **16** and **18**), and their calculated K_d values.

Figure 4.

NMR mapping of the binding interactions of K48-linked di-Ub analogues with the UBA2 domain of hHR23a. (A–C) Shown are amide chemical shift perturbations (CSPs) in backbone amides of the UBA2 domain at the titration endpoints with enzymatically-assembled K48-di-Ub (native) (A), K48-di-Ub **13** (B), and K48-di-Ub **16** (C). CSPs were calculated as $\Delta\delta = (\Delta\delta_H^2 + \Delta\delta_N^2/25)^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the changes in the chemical shifts of ^1H and ^{15}N , respectively. Those residues that experienced significant attenuation during the course of the titration are marked by gray bars. A number of resonances remained attenuated at the titration endpoint (residues missing black bars in parts B–C) as a consequence of not having attained full saturation with ligand, in contrast to part A. (D) Residues that experienced CSP > 0.2 ppm and/or strong signal attenuation are colored red on the surface of the UBA2 domain. The UBA2 domain coordinates are from the UBA2:K48-di-Ub complex (PDB ID 1ZO6): the front-side of UBA2 (left) contacts the distal Ub (including the C-terminal linker) in K48-linked di-Ub, whereas the back-side of UBA2 (right) interacts with the proximal Ub.

Scheme 1.

Synthesis of native (**3**, **4**) and thiol alkylated (**1**, **2**, **5–10**) di-Ub chains. TCEP- Tris (2-carboxyethyl) phosphine hydrochloride

Scheme 2.

Synthesis of N-methylated K48-linked di-Ub chain. DBU-Diazabicycloundecene, *o*-NBSCl-2-Nitrobenzenesulfonyl chloride, Me-*p*-NBS- Methyl 4-nitrobenzenesulfonate.

Scheme 3.
Synthesis of di-Ub chain with Gly76 modifications.