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Designer Reagents for Mass Spectrometry-Based Proteomics: Clickable Cross-Linkers for Elucidation of Protein Structures and Interactions

Chang Ho Sohn[1,7], **Heather D. Agnew**[1,7,8], **J. Eugene Lee**[2], **Michael J. Sweredoski**[3], **Robert L.J. Graham**[3], **Geoffrey T. Smith**[3], **Sonja Hess**[3], **Gregg Czerwieniec**[4], **Joseph A. Loo**[5,6], **James R. Heath**[1], **Raymond J. Deshaies**[2], and **J. L. Beauchamp**[1,*]

[1]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

[2]Division of Biology, California Institute of Technology, Pasadena, CA 91125

[3]The Proteome Exploration Laboratory, Beckman Institute, California Institute of Technology, Pasadena, CA 91125

[4]Molecular Instrumentation Center, University of California, Los Angeles (UCLA), Los Angeles, CA 90095

[5]Department of Chemistry and Biochemistry, University of California, Los Angeles (UCLA), Los Angeles, CA 90095

[6]Department of Biological Chemistry, David Geffen School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA 90095

Abstract

We present novel homobifunctional amine-reactive clickable cross-linkers (CXLs) for investigation of three-dimensional protein structures and protein-protein interactions (PPIs). CXLs afford consolidated advantages not previously available in a simple cross-linker, including (1) their small size and cationic nature at physiological pH, resulting in good water solubility and cell permeability, (2) an alkyne group for bio-orthogonal conjugation to affinity tags *via* the click reaction for enrichment of cross-linked peptides, (3) a nucleophilic displacement reaction involving the 1,2,3-triazole ring formed in the click reaction, yielding a lock-mass reporter ion for only clicked peptides, and (4) higher charge states of cross-linked peptides in the gas-phase for augmented electron transfer dissociation (ETD) yields. Ubiquitin, a lysine-abundant protein, is used as a model system to demonstrate structural studies using CXLs. To validate the sensitivity of our approach, biotin-azide labeling and subsequent enrichment of cross-linked peptides are performed for cross-linked ubiquitin digests mixed with yeast cell lysates. Cross-linked peptides are detected and identified by collision induced dissociation (CID) and ETD with linear quadrupole ion trap (LTQ)-Fourier transform ion cyclotron resonance (FTICR) and LTQ-Orbitrap

[*]To whom correspondence should be addressed at jlbchamp@caltech.edu..
[7]These authors contribute equally to this work.
[8]Current address: Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA 90095 and Integrated Diagnostics, 6162 Bristol Parkway, Culver City, CA 90230.

Supporting Information: Details of the synthesis of the CXL with characterization by mass spectrometry and NMR; cross-linking and CuAAC of model peptides, ubiquitin and HEK 293 cells; biotin-(PEG)3-azide LC-MS elution profiles; instrumental setups for mass spectrometers and the CD spectrometer; the CD spectra of cross-linked ubiquitin; raw mass spectrometric data processing and their xQuest search; ubiquitin cross-linked peptide analysis in comparison with the X-ray crystal structure; and quantum chemical calculation on nitromethane with discussions on ETD of cross-linked peptides. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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mass spectrometers. The application of CXLs to more complex systems (*e.g.*, *in vivo* cross-linking) is illustrated by Western blot detection of Cul1 complexes including known binders, Cand1 and Skp2, in HEK 293 cells, confirming good water solubility and cell-permeability.

Keywords

Chemical Cross-linkers; Cross-linked Peptide; Protein Structure; Protein-Protein Interaction; Mass Spectrometry; Copper-Catalyzed Azide-Alkyne Cycloaddition; Reporter Ion

Introduction

Elucidation of three-dimensional structures of protein complexes and protein-protein interactions (PPIs) is one of the central goals in current biological research. Proteins bind to each other to carry out specific biological functions by forming protein complexes.[1,2] On average, proteins *in vivo* act not alone but rather as part of a protein complex typically composed of 10 protein subunits.[3] Proteolysis signaled by poly-ubiquitination provides a good example of a functional protein complex.[4] The development of new reagents and methods for identification of binding partners and their interfaces is an enabling part of proteomic science.

Chemical cross-linkers have been widely employed in the analysis of three-dimensional protein structures and PPIs.[5,6] For identification of cross-linked proteins, traditional experimental methodologies include affinity-based chromatography and Western blotting. However, no detailed structural information for specific protein interfaces is revealed in these experiments. Full atomistic structures of isolated proteins and their complexes can be obtained from NMR spectroscopy and X-ray crystallography but these methodologies usually require large amounts of sample for analysis. Preparation of diffraction-quality crystals is often the bottleneck in structure determination by X-ray crystallography.

Recently, mass spectrometry (MS)-based analysis has allowed more sensitive detection of binding partners and specific contacting residues.[7–14]*In vitro* cross-linking and enzymatic digestion produce cross-linked peptides containing spatial information between residues reactive to the cross-linker. This topological information constrains relative distances of amino acid residues, thus aiding in the reconstruction of the protein complex structure.

For investigation of *in vivo* PPIs, protein complex immunoprecipitation (*i.e.*, co-IP or “pull-down”) is often performed to recover strongly interacting partners, such as an enzyme bound to its inhibitor. Co-IPs require the use of several antibodies to validate putative binding partners. Alternatively, affinity tags can be infused into genes of target proteins to permit efficient purification from cell lysates.[15] However, many of the important signaling pathways are believed to be relayed *via* weak and transient interactions[16,17] that occur at the periphery of strongly-bound core protein complexes,[2] and the co-IP strategy often fails to identify these weak binding partners. Chemical cross-linking has been performed to freeze weak interactions by forming covalent bonds, and then sample analysis is usually combined with other targeted protein purification techniques.[18,19] Some previous reports have shown promising results by taking an *in vivo* chemical cross-linking strategy for investigation of PPIs.[19,20]

Nonetheless, the formidable complexity of protein interaction networks greatly hinders identification of PPIs using cross-linking strategies at the systems level. The complexity of protein samples steeply increases upon cross-linking, roughly proportional to half of the square of the numbers of enzymatically cleaved peptides in the cell.[12] This leads to two

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practical problems associated with computational and experimental challenges. A protein cross-link searching program accounting for PPIs in mammalian cells on a genome-wide scale is not available, and current computational platforms are limited to just a few proteins. Computational resources required for this type of unrestricted searching algorithm are substantial. A recent study by the Aebersold group tackled this problem by introducing a new searching algorithm, xQuest, which reduces the search space by an upstream candidate-peptide search and by isotope-coded cross-linkers.[21] They successfully showed that cross-linked peptides can be identified from a total *E. coli* lysate with an unrestricted database search.

For selective and sensitive detection of cross-linked peptides, functionalized cross-linking reagents are required. Various designs including biotinylated[22–24], isotope-coded[25–28], fluorophore labeled[29–31], mass-tag labeled[32], amidinating[33] and chromophore labeled[34] cross-linking reagents have been reported. However, the addition of functional groups can often cause the cross-linker to become very bulky or less cell-permeable, and thus not very effective for *in vivo* cross-linking of cytoplasmic proteins.[20] To reduce the total size of the cross-linker, separation of the cross-linking step from conjugation of affinity tags is one effective strategy.

New cross-linking and enrichment strategies for separation of the cross-linking reaction from enrichment steps have recently been developed based on bio-orthogonal chemistries such as the azide-alkyne "click" cycloaddition[35–37] and Staudinger ligation[38] using alkyne[39,40] or azide[41,42] tagged cross-linkers. Azides and alkynes are not naturally found in proteins, peptides, nucleic acids, or glycans. The orthogonality of azides and alkynes to biological processes (*i.e.*, competing reactions) is a significant advantage of this approach, and holds great promise for protein cross-linking studies. Moreover, the "click" cycloaddition can be performed under aqueous conditions, allowing the enrichment of cross-linked products by conjugation of an appropriate affinity tag.

Here we report a novel clickable cross-linker (CXL) that addresses many of the current challenges described above (Scheme 1).

Whereas some of their functionality has been incorporated in previously described cross-linkers, CXLs offer consolidated advantages that include (1) small size, (2) good cell permeability, (3) water solubility, (4) ease of synthesis, (5) alkyne group for bio-orthogonal conjugation to a biotin-azide affinity label via copper-catalyzed azide-alkyne cycloaddition (CuAAC, or click reaction) for enrichment of cross-linked peptides by avidin-biotin chromatography, (6) a highly selective gas-phase nucleophilic displacement reaction by the 1,2,3-triazole ring formed in the click reaction, yielding a lock-mass reporter ion at *m/z* 525.3 for fast screening of cross-linked peptides, and (7) highly charged cross-linked peptide ions in the gas-phase for augmented electron transfer dissociation (ETD) yields.

Ubiquitin, a protein rich in lysine, was chosen as our model system for three-dimensional protein structure study using a hexynyl CXL. To demonstrate the non-denaturing feature of CXLs in solution, secondary structures of cross-linked ubiquitin were monitored using circular dichroism (CD) spectroscopy. Cross-linked peptides labeled with biotin-azide by CuAAC were enriched by strong cation exchange (SCX) fractionation and avidin affinity chromatography. Cross-linked peptides from ubiquitin were analyzed by collisional induced dissociation (CID) and ETD using linear quadrupole ion trap (LTQ)-Fourier transform ion cyclotron resonance (FTICR) and LTQ-Orbitrap XL mass spectrometers. The application of CXL in more complex systems was also tested by *in vivo* cross-linking of Cul1, a E3 ubiquitin ligase, in HEK 293 cells, confirming good water solubility and cell-permeability.

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Experimental Section

Briefly, the CXL was prepared *via* 1) deprotection of 2-(hex-5-ynyl)isoindoline-1,3-dione, 2) *N*-alkylation of 6-amino-hex-1-yne using methyl bromoacetate, 3) deprotection of methyl esters in KOH and 4) activation of carboxylic acids using *N*-hydroxysuccinimide (Supporting Information, Scheme S1). Details of the synthetic scheme, mass spectrometric analysis, and other experimental methodologies can be found in Supporting Information.

Results and Discussion

There are three structural types of cross-linked peptides: interpeptide, intrapeptide, and "dead-end" cross-linked peptides (Scheme 1; See ref. 8 for the nomenclature). Interpeptide cross-linked peptides (*i.e.*, 1) intermolecular cross-linking between proteins or 2) intramolecular cross-linking between two residues within a protein, and enzymatically cleaved) only exist sub-stoichiometrically compared to other intrapeptide cross-linked peptides, dead-end cross-linked peptides having one hydrolyzed cross-linker end, and unmodified linear peptides (Scheme 1). Among these species, only interpeptide cross-linked peptides provide direct information on PPIs. This complexity of a product mixture from a cross-linking reaction dictates that the clickable cross-linker (CXL) that we describe needs to be evaluated in model systems prior to using it in the biological environment of a cell.

Model Peptide Cross-Linking

ESI-MS and CID spectra of the intra- and interpeptide cross-linked model peptide, Ac-AAKAAAAAKAR and Ac-AAAAKAAAAAR are shown in Figure 1. The two lysine residues in the model peptide, Ac-AAKAAAAAKAR are loop-linked by CXL (*m/z* 609.9, Figure 1a). Protonation sites are expected to be the arginine side chain and the central tertiary amine in the cross-linker. The 1,2,3-triazole product from conjugation of biotin-$(PEG)_3$-azide via CuAAC corresponds to the doubly-charged ion at *m/z* 832.1 in Figure 1b. No precursor ion (*m/z* 609.9) is observed, indicating quantitative conversion *via* CuAAC (Figure 1b). CID of the biotin-$(PEG)_3$-azide conjugated peptide dication yields two backbone fragments along with the reporter ion at *m/z* 525.3. This product ion is generated by a nucleophilic attack forming a six-membered ring as depicted in Scheme 2.[43] The singly-charged peptide ion after loss of the reporter ion is observed at *m/z* 1138.9.

Intermolecular cross-linking of Ac-AAAAKAAAAAR was performed to investigate the fragmentation pattern of interpeptide cross-linked peptides by CID. Figure 1d shows the ESI-MS spectrum of interpeptide cross-linked Ac-AAAAKAAAAAR. Because there is no specific interaction between Ac-AAAAKAAAAAR peptides, only diffusive encounters lead to formation of the interpeptide cross-linked dimer (Figure 1d, *m/z* 716.1, 3+). The dead-end cross-linked peptide (Figure 1d, *m/z* 590.5, 2+) is the most abundant peptide species, formed when the unreacted *N*-hydroxysuccinimide (NHS) group is hydrolyzed after the reagent reacts with a single lysine side chain. After click conjugation of biotin-$(PEG)_3$-azide, both dead-end and interpeptide cross-linked peptides were observed at *m/z* 812.5 (2+) and 864.0 (3+), respectively, with quantitative conversion (data not shown). The CID spectrum of the doubly-charged dead-end peptide (*m/z* 812.5) is shown in Figure 1e. The prominent reporter ion at *m/z* 525.3 is observed with other b and y ions. The singly-charged complement ion to the reporter ion is observed at *m/z* 1099.8. The CID spectrum of the triply-charged cross-linked peptide (*m/z* 864.0) is shown in Figure 1f, yielding abundant b- and y-type ions with the reporter ion at *m/z* 525.3. The resulting b- and y-type ions generated by CID of cross-linked peptides thus allow for unambiguous sequencing of cross-linked peptide chains. It is noteworthy that the fragmentation process yielding the reporter ion occurs in competition with formation of b and y ions, yielding rich sequence information.

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Ubiquitin Cross-linking

Cross-linking reactions should be efficient without perturbing the structure of the target protein. As a control, we monitored the cross-linking of ubiquitin by CXL using circular dichroism (CD).[4445] CD spectra of ubiquitin acquired in the presence of 0.25 or 1 mM CXL (t = 0) and after a duration of 30 min are shown in Figure S1 (Supporting Information), and indicate that the secondary structure of ubiquitin is essentially unchanged by the addition of CXL (t = 0) or its cross-linking reaction (t = 30 min). A tryptic digest of cross-linked ubiquitin was then obtained for MS analysis.

Ubiquitin cross-linked peptides, and their conjugates with biotin-$(PEG)_3$-azide, were analyzed by nanoLC-LTQ-FTICR and LTQ-Orbitrap XL mass spectrometers using CID and ETD experiments. The resulting LC/MS and MS/MS spectra were searched against xQuest. The different types of cross-links (Scheme 1) found before the click reaction are summarized in Tables S1 and S2, Supporting Information. Notably, six cross-linked peptides were identified by CXL (K6–K48, K6–K48, K11–K33, K29–K33, K48–K48 and K48–K63) while other ubiquitin cross-linking studies performed using a more commonly employed cross-linker, disuccinimidyl suberate (DSS), and the other comparable functionalized cross-linker, CLIP (click-enabled linker for interacting proteins) have reported three cross-links (N-terminal-K6, K6–K11 and K48–K63 for DSS; K33–K48, K48–K48 and K48–63 for CLIP).[39,46]

As an example, the CID spectrum of the 5+ charged ^{30}IQD^{33}K^EGIPPDQQ^{42}R–^{7}TLTG^{11}K^TITLEVEPSDTIENV^{27}K ion is shown in Figure 2a. Abundant b- and y-type ions are generated and show good sequence coverage for each peptide chain. The diagnostic lock-mass reporter ion appears at *m/z* 525.3, and it does not overlap with other backbone fragments. Pre-filtering of CID scans using the reporter ion significantly reduces the number of candidate CID spectra. For analyses of crude mixtures of ubiquitin cross-linked peptides after CuAAC, we typically observed approximately a 90% reduction in the number of MS/MS spectra and the cross-linked peptide hits from the reduced data set were identical for all experiments performed in this study.

It should be also noticed that the formation of the reporter ion occurs in competition with backbone fragmentations (Figure 2a), suggesting that the energetics of reporter ion formation are similar to those of backbone fragment ions.[43] In previous studies performed with other functionalized cross-linkers, the diagnostic reporter ions were often dominant in MS/MS spectra suppressing other backbone fragmentations, and thus additional MS3 experiments or UV photodissociation were required for sequencing.[23,42,47] In comparison, MS2 spectra of CXL cross-linked peptides provide sufficient information simultaneously for sequencing and pre-filtering of cross-linked peptides.

ETD of the 5+ charged peptide ion produces many c and z ions (Figure 2b). It is noteworthy that the charge-reduced molecular ion species in Figure 2b are less abundant than in the ETD spectra of the previously-reported cross-linker by Chowdhury et al.[39] where a nascent hydrogen atom generated by electron transfer may become immobilized by the nitro group of their cross-linker, interfering with ETD backbone fragmentation (See Supporting Information for further discussion).[48] As anticipated, CuAAC with biotin-$(PEG)_3$-azide increases the charge states of cross-linked peptides (Tables S1 and S2) due to the high proton affinities of the tertiary amine and 1,2,3-triazole ring.

The ubiquitin lysine residues cross-linked by CXL are mapped onto the X-ray crystal structure in Figure 3 (PDB ID: 1UBQ). The observed lysine pairs cross-linked by CXL are all located within 20 Å. This is consistent with previous reports[39,46,49] using other cross-linkers that display a range of chain lengths similar to CXL. For example, in

the ^{30}IQD^{33}K^EGIPPDQQ^{42}R–^{7}TLTG^{11}K^TITLEVEPSDTIENV^{27}K cross-linked peptide, the distance between the alpha carbons in each lysine residue (K11 and K33) is 12.9 Å. The maximum length of the cross-linker in the all-trans conformation is ~6.6 Å which is shorter by 6.3 Å. However, the distance between NZ atoms in the side chains of lysine residues is only 7.2 Å. Considering the flexibility of the lysine side chain and thermal motions in proteins, the observation of cross-linking between K11 and K33 residues is reasonable. The homodimer of the ^{43}LIFAG^{48}K^QLEDG^{54}R peptide is also detected as in the previous report, indicating the formation of native ubiquitin homodimers in solution.[39]

For sensitive detection of cross-linked peptides, SCX was performed for separation and removal of the abundant linear peptide species.[21,33] The marginal peptide fractionation was achieved by applying a salt gradient for highly-charged cross-linked peptides (Figures 4b–e). Additionally, the residual non-polar impurities from CuAAC (*e.g.*, biotin-(PEG)$_3$-azide and ligands) were effectively removed by SCX. Further discussions on this subject with detailed analyses of LC-MS elution profiles can be found in the Supporting Information.

Avidin Affinity Chromatograpy

The general design of CXLs and their capacity for bio-orthogonal incorporation of an affinity tag *via* CuAAC were validated by a biotin-avidin enrichment strategy. Here, monomeric avidin affinity chromatography was employed for enrichment of cross-linked peptides. A highly complex peptide sample prepared by mixing equal amounts of yeast cell lysates and ubiquitin cross-linked peptides (*w/w*) was subjected to avidin affinity chromatography. Figures 4f and g depict LC-MS TIC chromatograms of the samples from SCX clean-up (f), and avidin enrichment (g). Yeast peptides were mostly eliminated during the avidin capture step, and only a few were detected after enrichment. Unmodified ubiquitin peptides that present no biotin tags were also mostly removed. The two identified interpeptide cross-linked peptides ^{30}IQD^{33}K^EGIPPDQQ^{42}R–^{7}TLTG^{11}K^TITLEVEPSDTIENV^{27}K, and the homodimer of ^{43}LIFAG^{48}K^QLEDG^{54}R reproduce those that were detected in the absence of yeast cell lysate (Table S2). Interestingly, one additional interpeptide cross-linked peptide, ^{28}A^{29}K^IQD^{33}K–^{30}IQD^{33}K^EGIPPDQQ^{42}R was identified only by avidin affinity chromatography (Table S2). This demonstrates that the click reaction for attachment of an affinity tag is efficient, and that SCX combined with avidin affinity chromatography is effective for enrichment of cross-linked peptides even in an extremely complex environment.

Low abundance cross-linked peptides may be difficult to detect following successive chemical reactions and sample treatments (Table S2). Compared to the missing peptides, three interpeptide cross-linked peptides detected after avidin enrichment have more closely positioned lysine pairs. This result can be rationalized by the relationship between the spatial orientation of the lysine pairs (*i.e.*, opportunity for cross-linking) and the resulting copy number of the interpeptide cross-linked peptides, and is also consistent with the crystal structure. Therefore, SCX fractionation alone is sufficient for separation of cross-linked peptides in low complexity samples (*e.g.*, *in vitro* cross-linking of protein complexes that include dozens of known proteins) without any sacrifice in sensitivity. For high complexity samples, however, further enrichment by clicking an affinity tag is still necessary.

In Vivo Cross-linking of HEK 293

To test the cell permeability and water solubility of CXL, *in vivo* cross-linking of HEK 293 cells, followed by Western blot analysis of Cul1 were performed. For control experiments, we also employed a common cross-linker DSS, whose structure is composed of simple homobifunctional NHS-activated esters jointed by a six methylene chain. Cul1 is a ubiquitin

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ligase that attaches a ubiquitin chain to target substrates for proteasome-catalyzed degradation.[50,51] Cul1 is a prototype of the cullin ligase family, and forms modular ligase complexes with other binding partners. During the design of CXL, we considered two features, the cationic nature of CXL in physiological pH, and the small size for efficient penetration of cross-linkers through cell membranes, which is a widely-employed strategy in the synthesis of drug-delivery carriers using cationic polymers.[52]

We found that treatment of samples with CXL up to 1 mM did not induce any significant cell toxicity and visual change such as might be caused by the entanglement of cell debris. We postulated that if cytoplasmic Cul1 protein were cross-linked by CXL, then this would confirm the cell-permeability and water solubility of the CXL. Figure 5a depicts the Western blot analysis of cross-linked Cul1 samples acquired from *in vivo* cross-linking of HEK 293 cell lysates. From the observation of the higher molecular-weight bands, it is clear that Cul1 was cross-linked by CXL in the 0.5 to 1.0 mM range. CXL is, therefore, soluble in PBS and cell-permeable, showing applicability within the cellular environment for *in vivo* cross-linking that is comparable to commonly employed DSS.

To confirm whether known Cul1 binding partners are cross-linked by CXL and DSS, we repeated Western blot analyses of Cul1 pull-down samples against Cul1, Cand1, and Skp2 (Figures 5b–d, respectively) that form SCF complexes. Cul1 pull-down samples may contain both cross-linked and noncovalently attached proteins. If a specific protein is cross-linked to Cul1 and purified by Co-IP, there should be at least one common high molecular-weight band across all lanes blotted against known binders. We found that three bands highlighted by red lines in Figure 5 were matched in each blot, indicating that specific cross-linking occurred with known binders.

Application to Complex Systems

The encouraging results reported in this work from both *in vitro* and *in vivo* cross-linking experiments hold promise for future application in systems-wide cross-linking experiments. The reporter ion observed in the CID spectra can play a critical role in reducing the number of the MS/MS spectra that are subjected to database searching. To provide more rich topological information, cross-linkers with various chain lengths can be prepared by employing methyl 3-bromopropionate, methyl 4-iodobutyrate and compounds with even longer methylene (or ethylene glycol) chains for *N*-alkylation of 6-amino-hex-1-yne with the current synthetic scheme (Scheme S1, Supporting Information). Finally, isotope-coded cleavable affinity tags with an azide functional group[53] can also be prepared and coupled to cross-linked peptides *via* CuAAC to accommodate isotope signatures, allowing for unlimited database search in systems level cross-linking studies using xQuest.[21]

Conclusions

A highly-versatile clickable cross-linker (CXL) has been developed for selective and sensitive identification of cross-linked peptides from complex mixtures. The CXL is designed to have good solubility, cell permeability, and an alkyne functional group for attachment of an affinity tag via CuAAC. Several advantages of CXLs have been validated using model peptides, a model protein (ubiquitin), and a biological system (HEK 293 cells). The application of CXL for the analysis of complex systems is demonstrated by successful fractionation and enrichment of cross-linked peptides, and MS/MS scan filtering using the lock-mass reporter ion. We are currently pursuing the application of CXLs in more complex systems.

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Supplementary Material

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References

(1). Hartwell LH, Hopfield JJ, Leibler S, Murray AW. Nature. 1999; 402:C47. [PubMed: 10591225]

(2). Pereira-Leal JB, Levy ED, Teichmann SA. Philosophical Transactions of the Royal Society B: Biological Sciences. 2006; 361:507.

(3). Sharan R, Suthram S, Kelley RM, Kuhn T, McCuine S, Uetz P, Sittler T, Karp RM, Ideker T. Proc. Natl. Acad. Sci. U. S. A. 2005; 102:1974. [PubMed: 15687504]

(4). King RW, Deshaies RJ, Peters J-M, Kirschner MW. Science. 1996; 274:1652. [PubMed: 8939846]

(5). Wong, SS. Chemistry of Protein Conjugation and Cross-Linking. CRC Press; 1991.

(6). Phizicky EM, Fields S. Microbiol. Rev. 1995; 59:94. [PubMed: 7708014]

(7). Back JW, de Jong L, Muijsers AO, de Koster CG. J. Mol. Biol. 2003; 331:303. [PubMed: 12888339]

(8). Sinz AJ. Mass Spectrom. 2003; 38:1225.

(9). Sinz A. Mass Spectrom. Rev. 2006; 25:663. [PubMed: 16477643]

(10). Gingras AC, Gstaiger M, Raught B, Aebersold R. Nat. Rev. Mol. Cell Biol. 2007; 8:645. [PubMed: 17593931]

(11). Lee YJ. Mol. BioSyst. 2008; 4:816. [PubMed: 18633483]

(12). Leitner A, Walzthoeni T, Kahraman A, Herzog F, Rinner O, Beck M, Aebersold R. Mol. Cell. Proteomics. 2010; 9:1634. [PubMed: 20360032]

(13). Petrotchenko EV, Borchers CH. Mass Spectrom. Rev. 2010; 29:862. [PubMed: 20730915]

(14). Sinz A. Anal. Bioanal. Chem. 2010; 397:3433. [PubMed: 20076950]

(15). Collins MO, Choudhary JS. Curr. Opin. Biotechnol. 2008; 19:324. [PubMed: 18598764]

(16). Acuner Ozbabacan SE, Engin HB, Gursoy A, Keskin O. Protein Eng. Des. Sel. 2011

(17). Rudolph J. Nature Rev.Cancer. 2007; 7:202. [PubMed: 17287826]

(18). Tagwerker C, Flick K, Cui M, Guerrero C, Dou YM, Auer B, Baldi P, Huang L, Kaiser P. Mol. Cell. Proteomics. 2006; 5:737. [PubMed: 16432255]

(19). Guerrero C, Milenkovic T, Przulj N, Kaiser P, Huang L. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:13333. [PubMed: 18757749]

(20). Zhang H, Tang X, Munske GR, Tolic N, Anderson GA, Bruce JE. Mol. Cell. Proteomics. 2009; 8:409. [PubMed: 18936057]

(21). Rinner O, Seebacher J, Walzthoeni T, Mueller L, Beck M, Schmidt A, Mueller M, Aebersold R. Nat. Methods. 2008; 5:315. [PubMed: 18327264]

(22). Trester-Zedlitz M, Kamada K, Burley SK, Fenyo D, Chait BT, Muir TW. J. Am. Chem. Soc. 2003; 125:2416. [PubMed: 12603129]

(23). Tang XT, Munske GR, Siems WF, Bruce JE. Anal. Chem. 2005; 77:311. [PubMed: 15623310]

(24). Kang S, Mou LY, Lanman J, Velu S, Brouillette WJ, Prevelige PE. Rapid Commun. Mass Spectrom. 2009; 23:1719. [PubMed: 19412923]

(25). Chu FX, Mahrus S, Craik CS, Burlingame AL. J. Am. Chem. Soc. 2006; 128:10362. [PubMed: 16895390]

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(26). Muller DR, Schindler P, Towbin H, Wirth U, Voshol H, Hoving S, Steinmetz MO. Anal. Chem. 2001; 73:1927. [PubMed: 11354472]

(27). Collins CJ, Schilling B, Young ML, Dollinger G, Guy RK. Bioorg. Med. Chem. Lett. 2003; 13:4023. [PubMed: 14592499]

(28). Petrotchenko EV, Olkhovik VK, Borchers CH. Mol. Cell. Proteomics. 2005; 4:1167. [PubMed: 15901824]

(29). Wine RN, Dial JM, Tomer KB, Borchers CH. Anal. Chem. 2002; 74:1939. [PubMed: 12033289]

(30). Sinz A, Wang K. Biochemistry. 2001; 40:7903. [PubMed: 11425319]

(31). Sinz A, Wang K. Anal. Biochem. 2004; 331:27. [PubMed: 15245993]

(32). Back JW, Hartog AF, Dekker HL, Muijsers AO, de Koning LJ, de Jong L. J. Am. Soc. Mass Spectrom. 2001; 12:222. [PubMed: 11212007]

(33). Lauber MA, Reilly JP. Anal. Chem. 2010; 82:7736. [PubMed: 20795639]

(34). Gardner MW, Vasicek LA, Shabbir S, Anslyn EV, Brodbelt JS. Anal. Chem. 2008; 80:4807. [PubMed: 18517224]

(35). Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. Angew. Chem.-Int. Edit. 2002; 41:2596.

(36). Tornoe CW, Christensen C, Meldal M. J. Org. Chem. 2002; 67:3057. [PubMed: 11975567]

(37). Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA, Bertozzi CR. Proc. Natl. Acad. Sci. U. S. A. 2007; 104:16793. [PubMed: 17942682]

(38). Saxon E, Bertozzi CR. Science. 2000; 287:2007. [PubMed: 10720325]

(39). Chowdhury SM, Du X, Tolić N, Wu S, Moore RJ, Mayer MU, Smith RD, Adkins JN. Anal. Chem. 2009; 81:5524. [PubMed: 19496583]

(40). Trnka MJ, Burlingame AL. Mol. Cell. Proteomics. 2010; 9:2306. [PubMed: 20813910]

(41). Nessen MA, Kramer G, Back J, Baskin JM, Smeenk LEJ, de Koning LJ, van Maarseveen JH, de Jong L, Bertozzi CR, Hiemstra H, de Koster CG. J. Proteome Res. 2009; 8:3702. [PubMed: 19402736]

(42). Vellucci D, Kao A, Kaake RM, Rychnovsky SD, Huang L. J. Am. Soc. Mass Spectrom. 2010; 21:1432. [PubMed: 20472459]

(43). Sohn CH, Lee JE, Sweredoski MJ, Graham RLJ, Smith GT, Hess S, Czerwieniec G, Loo JA, Deshaies RJ, Beauchamp JL. J. Am. Chem. Soc. 2012; 134:2672. [PubMed: 22225568]

(44). Greenfield NJ. Nat. Protocols. 2007; 1:2876.

(45). Jenson J, Goldstein G, Breslow E. Biochim. Biophys. Acta. 1980; 624:378. [PubMed: 6251899]

(46). Kruppa GH, Schoeniger J, Young MM. Rapid Commun. Mass Spectrom. 2003; 17:155. [PubMed: 12512095]

(47). Yang L, Tang X, Weisbrod CR, Munske GR, Eng JK, von Haller PD, Kaiser NK, Bruce JE. Anal. Chem. 2010; 82:3556. [PubMed: 20373789]

(48). Sohn CH, Chung CK, Yin S, Ramachandran P, Loo JA, Beauchamp JL. J. Am. Chem. Soc. 2009; 131:5444. [PubMed: 19331417]

(49). Novak P, Young MM, Schoeniger JS, Kruppa GH. Eur. J. Mass Spectrom. 2003; 9:623.

(50). Deshaies RJ. Annu. Rev. Cell Dev. Biol. 1999; 15:435. [PubMed: 10611969]

(51). Lee JE, Sweredoski MJ, Graham RLJ, Kolawa NJ, Smith GT, Hess S, Deshaies RJ. Mol. Cell. Proteomics. 2010; 10 M110.006460.

(52). Nam YS, Kang HS, Park JY, Park TG, Han SH, Chang IS. Biomaterials. 2003; 24:2053. [PubMed: 12628825]

(53). Szychowski J, Mahdavi A, Hodas JJL, Bagert JD, Ngo JT, Landgraf P, Dieterich DC, Schuman EM, Tirrell DA. J. Am. Chem. Soc. 2010; 132:18351. [PubMed: 21141861]

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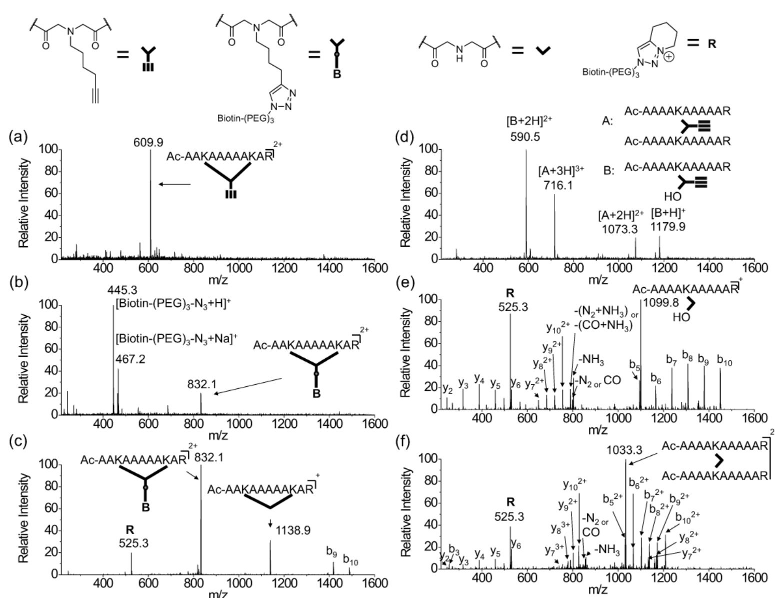

Figure 1.

ESI-MS and MS/MS by CID spectra of the cross-linked model peptide.

The ESI-MS spectra of model peptides, Ac-AAKAAAAAKAR and Ac-AAAAKAAAAAR after cross-linking (a and d), and CuAAC reactions (b). The CID spectra of the intrapeptide cross-linked and clicked Ac-AAKAAAAAKAR (c), dead-end and clicked (e), and interpeptide cross-linked and clicked (f) Ac-AAAAKAAAAAR peptides. The reporter ion at *m/z* 525.3 is observed along with backbone fragments in the CID spectra (c, e, and f). Note that in Figure 1b, CuAAC with biotin-$(PEG)_3$-azide proceeded almost quantitatively, showing no cross-linked precursor peptide ion at *m/z* 609.

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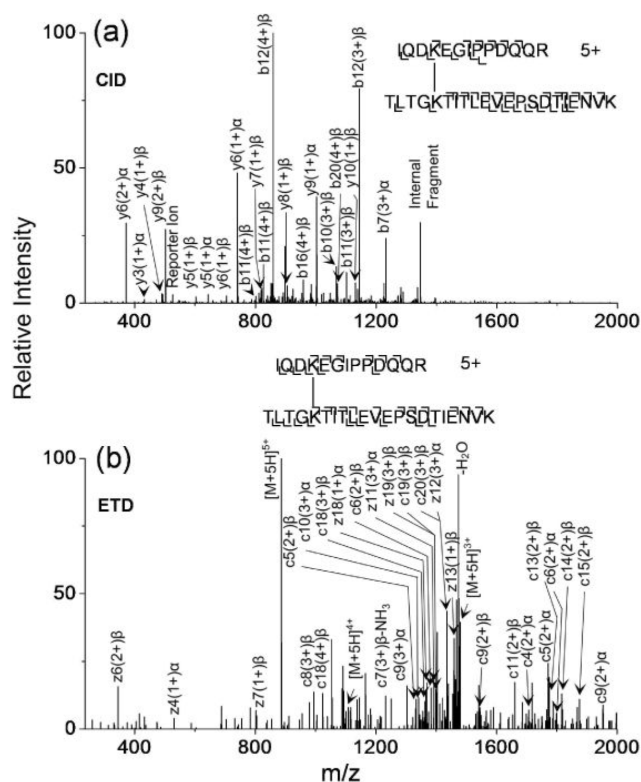

Figure 2.

CID and ETD of the 5+ charged cross-linked peptide.

CID (a) and ETD (b) of the 5+ charged cross-linked peptide, IQD^KEGIPPDQQR-TLTG^KTITLEVEPSDTIENVK where ^K is the cross-linked residue. (a) Although the molecular weight (4431 Da) of the cross-linked peptide is relatively large for fragmentation by CID, good coverage of b- and y-type ions is observed. The reporter ion at *m/z* 525.3 is also observed with acceptable yield. (b) The high charge state of the cross-linked peptide yields abundant c- and z-type ions in the ETD spectrum. This shows the potential for the general use of CID and ETD for efficient sequencing of cross-linked peptides.

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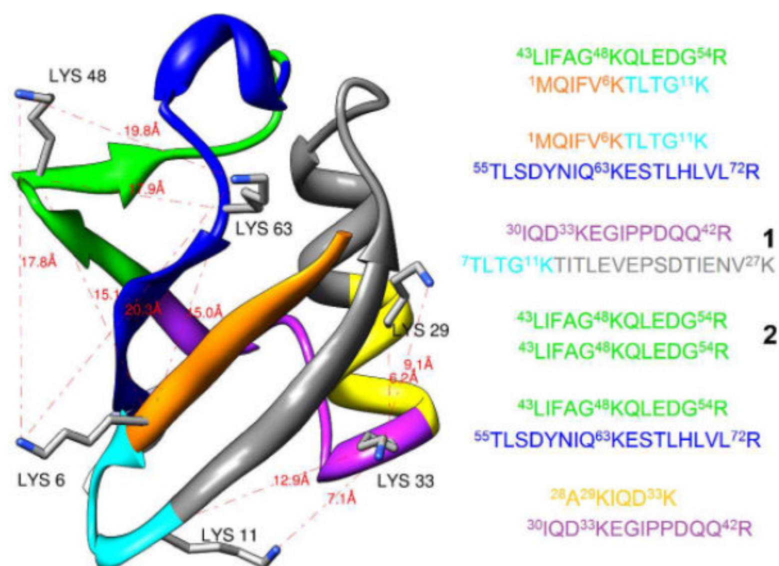

Figure 3.

Cross-linked peptides from ubiquitin

Ubiquitin X-ray crystal structure (PDB: 1UBQ), presented in ribbon diagram and color coded to highlight the peptide chains that are cross-linked by CXL. Lysine residues are shown explicitly, and C_α–C_α and NZ-NZ distances (in Å) for the experimentally observed cross-linked peptides are shown in red. Two labeled peptides (K11–K33 and a homodimer of K48) are denoted as **1** and **2** and used for labeling in Figure 4.

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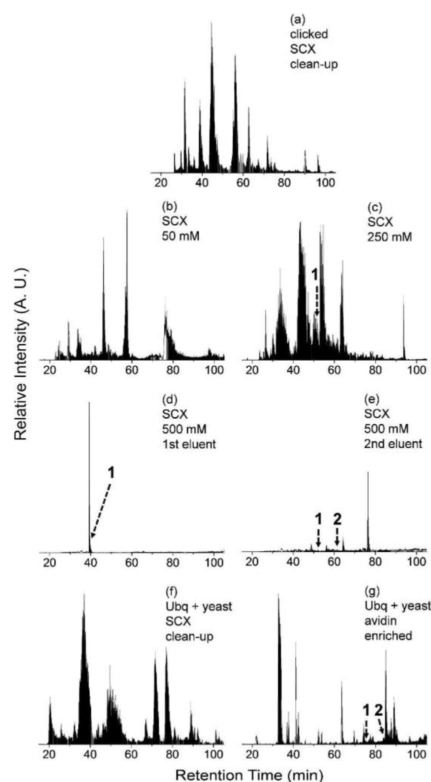

Figure 4.

LC-MS total ion current (TIC) chromatograms of cross-linked peptides.

LC-MS TIC chromatograms of (a) cross-linked ubiquitin digest after CuAAC with biotin-$(PEG)_3$-azide and SCX clean-up (without fractionation); (b) 50, (c) 250, (d) 1st 500, and (e) 2nd 500 mM ammonium acetate SCX eluents from cross-linked and clicked ubiquitin digests; (f) 1:1 mixture (by weight) of cross-linked ubiquitin and yeast cell lysate after CuAAC with biotin-$(PEG)_3$-azide and SCX clean-up (without fractionation); and (g) 1:1 mixture (by weight) of ubiquitin (cross-linked and clicked) and yeast cell lysate peptides after monomeric avidin enrichment. By applying SCX, highly-charged cross-linked peptides were fractionated in high salt elutions (250 and 500 mM buffers, c–d). Avidin capture reduced the complexity of yeast lysate samples (from f to g) and resulted in the same identification of cross-linked peptides to those observed in the SCX fractionated samples (c–d). Two cross-linked peptides (**1** for K11–K33 and **2** for a homodimer of K48) are marked. All identified cross-linked peptides in each fraction are summarized in Table S2 (Supporting Information).

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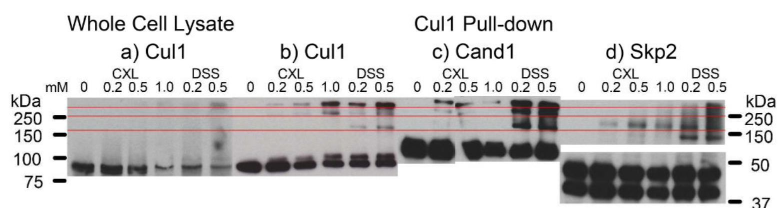

Figure 5.

In vivo Cross-linking of HEK 293 Cells

Western blot analyses of *in vivo* cross-linked HEK 293 cell lysates against a) Cul1, and of Cul1 pull-down by avidin against b) Cul1 and known Cul1 binders: c) Cand1, and d) Skp2. The CXL (0 to 1.0 mM) and DSS (0.2 and 0.5 mM) were applied to the cross-linking of Cul1. The uncross-linked $(his)_6$-biotin signal peptide tag (HB)-labeled and neddylated Cul1 were observed at the slightly higher molecular-weight band than that of endogenous Cul1. The observation of higher molecular-weight bands, one located between 150 and 250 kDa, and two located over 250 kDa in 0.5 and 1.0 mM CXL, and 0.2 and 0.5 mM DSS lanes in a) indicate successful cross-linking of cytoplasmic Cul1 protein complexes, and also imply specific cross-linking of known binders, Cand1 and Skp2 in c) and d). Three red lines highlight the higher molecular-weight bands of cross-linked Cul1 complexes with Cand1 and Skp2.

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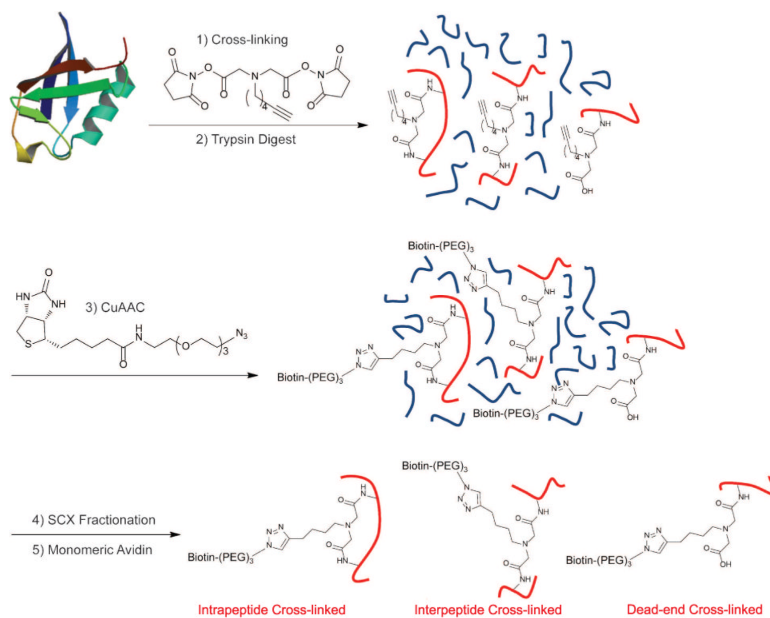

Scheme 1.

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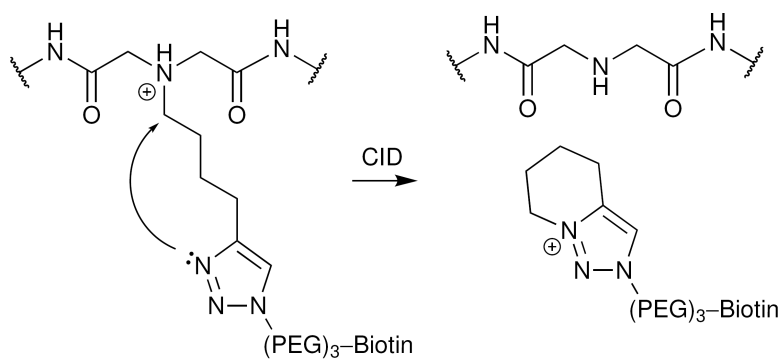

Scheme 2.