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Isotope-Coded and Affinity-Tagged Cross-Linking (ICATXL): An Efficient Strategy to Probe Protein Interaction Surfaces

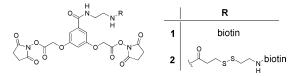
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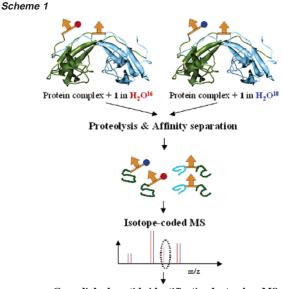
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Protein bait affinity studies on the yeast proteome have begun to reveal the nature and complexity of cellular protein—protein interactions.¹ However, our knowledge of the actual recognition interfaces among these macromolecules is sparse.² To gain mechanistic insights into various cellular processes, it is important to devise strategies that enable the mapping of protein—protein interaction surfaces even at low resolution. We and others have recently shown that valuable spatial restraints can be obtained from chemical cross-linking of protein assemblies through use of tandem mass spectrometry to identify the specific cross-linked amino acid participants. Such investigations provide information on protein folding, connectivity of secondary structures, as well as interaction surfaces.³

Proteolytic digestion of the cross-linked protein complexes and structural analysis of the cross-linked species by tandem mass spectrometry have advantages in speed, sensitivity, and capability of handling large protein assemblies. Nevertheless, detection and accurate assignment of the cross-linked peptides is often challenging, complicated by the heterogeneity of the peptide mixture and low stoichiometry of the cross-linked products sought. Incorporation of affinity tags into cross-linkers can reduce the complexity of the analytes and optimize the detection of low abundant cross-linked species by permitting specific enrichment of species modified with the affinity tag.⁴ Amine-reactive cross-linkers are often used in protein cross-linking studies, due to the favorable distribution of lysine residues on protein surfaces in solution. However, formation of the initial products with an ϵ -aminolysyl function is subsequently dominated by hydrolysis of the second reactive functionality, requiring a method for distinction of legitimate cross-links from these half-cross-linking reaction products, that is, the cross-linkermodified peptides. Herein, we present a novel cross-linking strategy termed ICATXL (isotope-coded and affinity-tagged cross-linking) that relies on the use of affinity-tagged cross-linkers and isotope coding on the cross-linker-modified species (Scheme 1). This strategy permits sensitive and facile analysis of cross-linked protein samples.



We first synthesized two homofunctional, affinity-tagged crosslinkers, **1** and **2** (see Supporting Information). Biotin was selected as the affinity tag for its high affinity and small size. Cross-linker **2** has a thiol-cleavable site to facilitate the recovery of cross-linked peptides after an avidin affinity purification step. The distance between the two reactive carbonyls is similar to that of the welldocumented cross-linkers, disuccinimidyl suberate (DSS) and bis-(sulfosuccinimidyl) suberate (BS³).⁵ Cross-linker **1** and **2** were tested with a synthetic peptide and a well-characterized dimeric "fold-



Cross-linked peptide identification by tandem MS

specific" protease inhibitor, ecotin.⁶ As expected, both cross-linkers reacted with N-terminal α -amino and lysine ϵ -amino groups and gave similar cross-linking and cross-linker modification results (see Supporting Information).

A novel isotope-coded strategy was developed to differentiate cross-linked peptides from cross-linker-modified peptides (Scheme 1). The protease inhibitor was cross-linked with 1 in both H_2O^{16} and H₂O¹⁸ buffer. Hydrolysis of NHS groups of 1 in each buffer thus resulted in O¹⁶ or O¹⁸ incorporation into hydrolyzed, nonproductive cross-links. In contrast, oxygen is not incorporated into cross-linked or nonreactive protein residues. Protein samples crosslinked in H₂O¹⁶ and H₂O¹⁸ were then combined, dialyzed, and proteolyzed. Cross-linked peptides and cross-linker-modified peptides were purified and enriched using monomeric avidin purification. Since peptides from hydrolyzed, nonproductive cross-links were derived equally from O16 and O18 incorporation, they appear as characteristic "doublets" in the mass spectra. Hence, further structural analysis is simplified by the fact that these are readily distinguished from actual cross-linked peptides. Figure 1A shows the Lys-C digestion mixture of 1-treated ecotin. Analysis of the individual peaks revealed the presence of unmodified proteolytic peptides, cross-linker-modified peptides, and cross-linked peptides. After affinity separation on monomeric avidin, cross-linked peptides are no longer "suppressed" by the more abundant unmodified peptides and are thus detected with higher sensitivity. For example, the cross-linked species at m/z 3044.43 is significantly discernible after affinity purification (compare Figure 1A and 1B). As expected, all abundant peptides contain the biotin moiety from 1 (Table 1). Cross-linker-modified peptides from the half-cross-linking reaction are characterized by a unique 2 Da Δ mass doublet isotope pattern



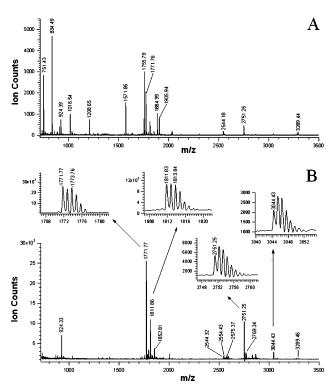


Figure 1. MALDI TOF-MS spectrum of the Lys-C digestion mixture of 1-treated ecotin before (A) and after (B) affinity separation.

Table 1. Affinity-Separated Peptides Observed by MALDI TOF-MS

obsd mass $[M + H]^+$	calcd mass $[M + H]^+$	intensity (%)	reacted residues ^a	sequence assignment
924.33 ^b	924.41	22	K94, 1 mod.	93-95
1771.77 ^b	1771.80	100	N-term, 1 mod.	N-term-9
1811.86^{b}	1811.86	38	K135, 1 mod.	132-142
1852.81^{b}	1852.86	10	K18, 1 mod.	10-21
2544.32^{b}	2544.27	5	K135, 1 mod.	127-142
2554.43	2554.21	3	K95, 1 mod.	95-112
2575.37	2575.16	7	K91-K94	77-92, 93-95
2751.25^{b}	2751.31	40	N-term-K9	N-term-18
2769.33	2769.32	8	K9, 1 mod.	N-term-18
3044.43^{b}	3044.48	13	N-term-K135	N-term-9, 132-142
3289.46	3289.48	2	N-term, K9, 1 mod.	N-term-18

^{*a*} **1** mod. refers to modification introduced by cross-linker **1**, and N-term refers to the N-terminal α -amino group of the protein. ^{*b*} Identity of the peptides was confirmed by tandem MS.

(e.g., peptides at m/z 1771.77 and 1811.83 (Figure 1B)). In contrast, the enriched cross-linked peptides have a normal isotope pattern (e.g., peptides at m/z 2751.25 and 3044.43 (Figure 1B)). The crosslinked species at m/z 3044.43 consists of an intermonomer crosslink between the N-terminal α -amino group of one monomer chain and Lys135 of the other monomer chain (Figure 2). The sequence of these two cross-linked peptides and the identity of the crosslinked residues were conclusively established by tandem MS.7 Not all cross-linking products of nearby lysines were observed, presumably due to the discrepancy in reactivity of primary amines and in detection of peptides. The assignment of the cross-linked residues agrees with its crystal structure8 (Figure 2) and that of another crosslinking study.⁶ Use of O¹⁶ and O¹⁸ for isotope coding is amenable to introduction of a liquid chromatography separation step since isotopes of oxygen do not affect retention time (data not shown) as those of hydrogen do.9 Therefore, we anticipated that our isotopecoded and affinity-tagged cross-linking strategy will be of general

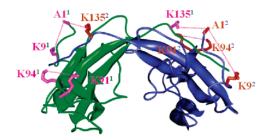


Figure 2. Crystal structure of ecotin. The cross-linked residues are connected with dotted lines and annotated with color-coded superscripts to indicate their chain origin.

use for the study of more complex protein assemblies where separation and rapid recognition of cross-linked peptides are highly desirable.

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Supporting Information Available: Complete ref 1a and 1b, experimental procedures, and detailed characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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