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

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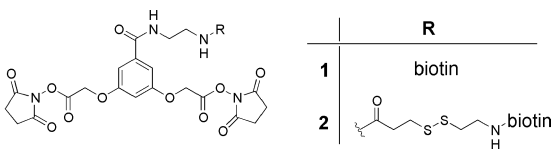
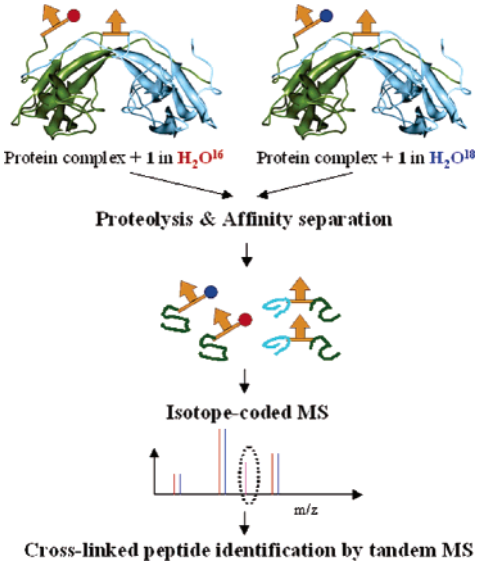
Protein bait affinity studies on the yeast proteome have begun to reveal the nature and complexity of cellular protein−protein interactions.[1] However, our knowledge of the actual recognition interfaces among these macromolecules is sparse.[2] To gain mechanistic insights into various cellular processes, 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-linker-modified 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 cross-linkers, **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 well-documented cross-linkers, disuccinimidyl suberate (DSS) and bis-(sulfosuccinimidyl) suberate (BS^3).[5] Cross-linker **1** and **2** were tested with a synthetic peptide and a well-characterized dimeric "fold-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).

Scheme 1



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 H_2O^{18} buffer. Hydrolysis of NHS groups of **1** in each buffer thus resulted in O^{16} or O^{18} incorporation into hydrolyzed, nonproductive cross-links. In contrast, oxygen is not incorporated into cross-linked or nonreactive protein residues. Protein samples cross-linked in H_2O^{16} and H_2O^{18} 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 O^{16} and O^{18} 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 Δmass doublet isotope pattern

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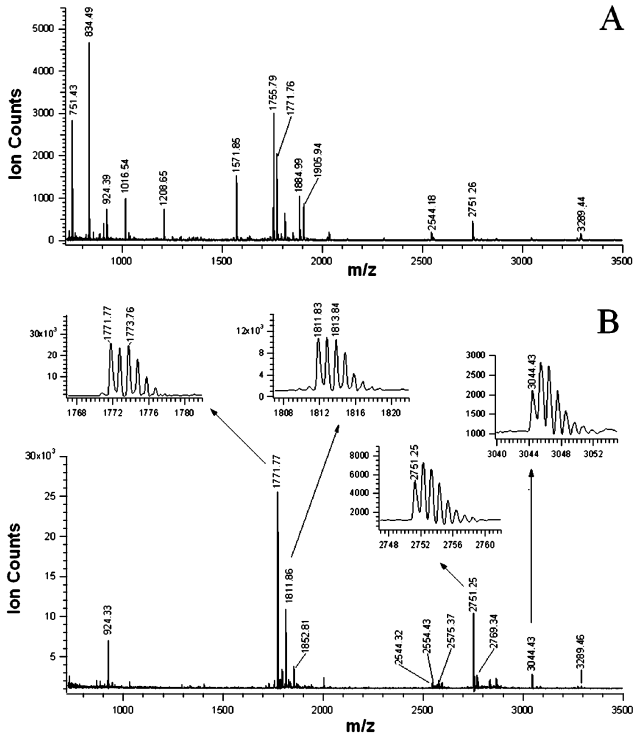

Figure 1. MALDI TOF-MS spectrum of the Lys-C digestion mixture of **1**-treated ecotin before (A) and after (B) affinity separation.

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 cross-linked species at *m*/*z* 3044.43 consists of an intermonomer cross-link 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 cross-linked 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 structure[8] (Figure 2) and that of another cross-linking study.[6] Use of O^{16} and O^{18} 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 isotope-coded and affinity-tagged cross-linking strategy will be of general use for the study of more complex protein assemblies where separation and rapid recognition of cross-linked peptides are highly desirable.

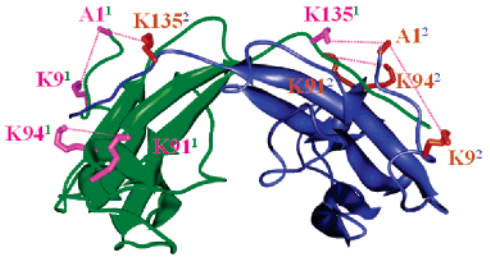

Figure 2. Crystal structure of ecotin. The cross-linked residues are connected with dotted lines and annotated with color-coded superscripts to indicate their chain origin.

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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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(7) See Supporting Information for low-energy CID tandem MS spectrum of this cross-linked species.

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