


Research Article

Asymmetric Chemical Cross-Linking Enables Isotope Tagging of Interacting Proteins (iTIP)

Harsha P Gunawardena*

Abstract

An isotope labeled cross-linker (asymmetric d4-DTSSP) was developed to streamline the efforts needed for the detection of cross-linked peptides. The cross-linking and mass spectrometry strategy we call Isotope Tagging of Interacting Proteins (iTIP) has improved the specificity of detecting cross-linked peptides and the correct identification of the interacting peptide sequences via the incorporation of isotopic signatures that are readily observed in the MS/MS spectra. All tryptic peptides derived from the cross-linking reactions of a protein complex are subjected to ETD-MS² which results in the facile cleavage of the cross-linker at the disulfide bond and the release of inter-linked polypeptide chains that are detected as a pair of peaks (doublets) in the MS² spectrum. The constituent peptide halves that are tagged by the heavy/light ends of the cross-linker are easily mass-selected from all other fragment ions, and each polypeptide half is then subjected to CID- or HCD-MS³ for identification. The MS³ spectra are subjected to conventional database search strategies available for the sequencing of linear or non-cross-linked peptides. The confident identification of each polypeptide is further aided by the presence of a stable isotope labeled fragment ions that localizes the cross-linked site on the polypeptide sequence.

Keywords: CID: Collision induced dissociation; ETD: Electron transfer dissociation; ECD: Electron capture dissociation; DTSSP-3,3: Dithiobis[sulfosuccinimidylpropionate]; DSP: Dithiobis[succinimidylpropionate]; h4: Four hydrogen atoms; d4: Four deuterium atoms; H: Heavy end of the cross-linker; L: Light end of the cross linker; α : Short polypeptide; β : Long polypeptide; SS: Disulfide bond; Doublets: Pair of peaks with a constant mass off-set

Introduction

A majority of gas-phase dissociation reactions of protonated cross-linkers lack the sensitivity and specificity required to fragment the cross-linker at a desired location while giving rise to diagnostic fragment ions; only a few reports have attempted to address the issue of selectivity [1-8]. Reid and co-workers described a cross-linker containing a sulfonium ion that showed specific fragmentation of the C-S bond during collision induced dissociation (CID) [9]. Brodbelt and co-workers reported the preferential cleavage of N-N hydrazone bond of a bis hydrazone cross-linked peptides by electron transfer dissociation (ETD) [10]. In both reports, despite the fact that dominant fragment channels arose from a specific cross-linker cleavage, the inability to readily distinguish fragment ions unique to the cross-linker from backbone fragment ions makes cross-linked peptide diagnosis challenging. Bouchers and co-workers have reported an isotope coded CID cleavable cross-linker

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[7]. Although their cross-linker has diagnostic fragment ions, the biotin moiety introduced to their cross-linker adds substantial mass to the resulting interlinked peptides. Here, we have synthesized deuterium labeled 3,3'-Dithiobis[sulfo succinimidylpropionate] (asymmetric d4-DTSSP) a cross-linker with a relatively smaller mass that can be selectively fragmented at a specific location, while allowing the dissociated cross-linked fragment ions to be distinguished easily from other backbone fragments. The stable isotopes are incorporated into a commercially available DTSSP via asymmetric positioning of four deuterium labels on two of the four methylene carbons, giving rise to a mass asymmetry across the two-fold axis of the S-S bond. Further, asymmetric d4-DTSSP is structurally analogous to the commercially available DTSSP reported elsewhere [11]. However, the unique asymmetric isotope tagging signature of our cross-linker can be detected via the selective ETD of the disulfide bond [12] of the cross-linker. The asymmetric nature of the cross-linker can give a doublet of reporter ions with a nominal mass off-set of 4 Da per unit charge for each inter-linked peptide halves, due to the equal likelihood of orienting the light and heavy ends of the cross-linker during the reaction. The doublets that are observed are due to fully protonated h4-tag or deuterated d4-tag are products ions associated with the disulfide cross-linker and hence diagnostic of each interacting peptide pair. The small mass off-set makes these fragment ions predictable, easy to visualize, and easily distinguishable from other ETD product ions [13] such as peptide backbone products, side-chain losses, and charge reduced species which are single peaks. Each of the peptides cleaved at the S-S bond is structurally interrogated via MS³-CID where the product ion spectra of each peptide half form the basis for their identification and hence the identification of an interacting protein. We have named this new cross-linking strategy that identifies interacting proteins as Isotope Tagging of Interacting Proteins (iTIP). In this study we present iTIP strategy applied to map the distance between several lysine residues of Holomyoglobin.

Experimental Section

Materials

All chemicals and solvents were of the highest grade and used without further purification. 3-Mercaptopropionic acid, 2,2'-dithiodipyridine and dimethylformamide (DMF) were purchased from Acros. Holomyoglobin (horse heart) and Ubiquitin (human erythrocytes), Dicyclohexylcarbodiimide (DCC) and ethanol were purchased from Sigma-Aldrich. *N*-Hydroxysulfosuccinimide sodium salt (NHSS) was purchased from Fluka. Acetic acid and ethyl acetate were purchased from Fisher Scientific. 2,2',3,3'-Tetradeuterium-3-mercaptopropionic acid was purchased from Creative Molecules Inc.

Mass Spectrometry Analysis

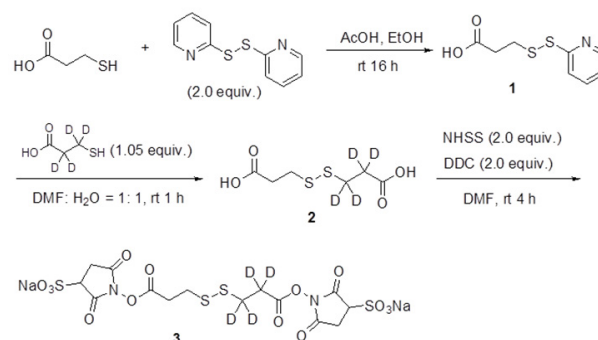


Figure 1: The synthetic procedure for generating d4-DTSSP

Synthesis of Asymmetric d4-DTSSP

Figure 1 shows the overall synthetic scheme for generating asymmetric d4-DTSSP. The disulfide **1** was synthesized as described by Xie et al. [14] Disulfide **1** (16.9 mg, 79 μ mol), THF (0.4 mL), H₂O (0.3 mL) and 2,2',3,3'-Tetradeuterium-3-mercaptopropionic acid (83 μ L, 1 M in D₂O, 83 μ mol) were added successively to a vial at room temperature. The reaction mixture was stirred for 1 hr and then concentrated under reduced pressure. Flash chromatography (CH₂Cl₂: MeOH = 95:5 with 0.4 % formic acid) over silica gel gave compound **2** as a white solid (9.3 mg, 55 %). NHSS (8.7 mg, 40 μ mol) and DCC (8.3 mg, 40 μ mol) were added successively to a solution of compound **2** (4.3 mg, 20 μ mol) in DMF (0.3 mL) at room temperature. The resulting solution was stirred for 4 hrs and then subjected to centrifugation. The supernatant was collected, and 1.2 mL of ethyl acetate was added. The resulting mixture was centrifuged. The precipitate was collected and washed by ethyl acetate three times. After drying by SpeedVac, the cross-linker **3** was obtained as a pale white solid (7.0 mg, 57 %). Figure 1a describes the synthetic scheme used to generate d4-DTSSP. The synthetic product was fully characterized by NMR ¹H NMR (CDCl₃, 400 MHz) δ 3.11-3.15 (m, 2H), 3.19-3.26 (m, 4H), 3.40 (d, *J* = 9.2 Hz, 1H), 3.44 (d, *J* = 9.6 Hz, 1H), 4.33 (d, *J* = 8.8 Hz, 1H), 4.52 (d, *J* = 8.8 Hz, 1H), and high resolution mass spectrometry data (Figure 1b) that compares commercially available DTSSP, d8-DTSSP and newly synthesized asymmetric d4-DTSSP.

Protein Cross-Linking and Proteolysis

Protein cross-linking was conducted on Bovine Ubiquitin and Equine Holomyoglobin using the manufacturer's protocol described for DTSSP cross-linkers (Pierce Inc.) The protein solution was buffer exchanged to 25 mM ammonium bicarbonate (ABC) and trypsinized at an enzyme to protein mole ratio of 1:50 at 37 oC in a 3M spin-filter device (Millipore Inc.). Peptides were then desalted using PepClean spin filters (Pierce Inc.) and reconstituted in an aqueous solution of 0.1% formic acid.

Data dependent LC-MS/MS was carried out on a LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific) coupled to an Eksigent 2D nano ultra LC system as described previously [15]. The targeted experiments consisted of ETD-MS/MS, followed by MS3 experiment with minimum of two CID-MS/MS scans or two HCD-MS/MS scans for each polypeptide light/heavy chain. A 2 Da isolation window was used to mass select precursor ions while 1 Da isolation window was used to mass select product ions. All ETD reactions were performed for mutual storage of peptide cations with fluoranthene anions for 100 ms. All CID experiments were performed at normalized collision energy of 32 eV. Mass spectra were processed by filtering MS³-CID or -HCD spectra, and peptide identification was performed on the filtered MS³ spectra by Mascot (Matrix Science Inc.) against a human Uniprot database (V3.63). Peptides were confidently identified using a target-decoy approach [16,17] at a 1% false discovery rate (FDR). A precursor ion mass tolerance of 10 p.p.m. and a product ion mass tolerance 0.5 Da were used during the initial search with a maximum of two missed trypsin cleavages. Variable modifications included methionine oxidation and customized cross-linker associated modifications with following empirical formulas: C₃OD₄S, C₃OD₄SH, C₃OH₅S, C₃OH₅S at lysine and N-terminus. All search results were filtered for precursor masses to be within a 6 p.p.m mass accuracy. MS2-ETD and MS2-CID spectra were manually annotated based identifications obtained of the MS³ spectra.

Results and Discussion

Rationale for Cross-linker Design and Isotope Tagging of Interacting Proteins (iTIP) Strategy

It has been demonstrated that electron transfer dissociation (ETD) provides facile cleavage of peptide backbone bonds [13,18], disulfide bonds of oxidized cysteine residues, [12] and several cross-linked polypeptides [10]. Based on these observations, we rationalized that an ETD cleavable cross-linker consisting of a disulfide bond could be quite useful in studying inter-linked polypeptides in cross-linking-based tandem mass spectrometry experiments. Commercially available disulfide carrying cross-linkers such as DTSSP and DSP, and their deuterated analogs are structurally symmetrical across the disulfide bond: with a two-fold symmetry, each constituent peptide chain results in identical mass-tags in ETD-MS² spectra. Our new design of asymmetric d4-DTSSP is structurally asymmetric and cleavage at the disulfide bond results in peptide chains that are dissimilar in mass-tags in ETD-MS² spectra.

Figure 2a shows the structure of our asymmetric d4-DTSSP cross-linker with a design strategy based on a mass off-set or ($\Delta m = 4$ Da) around the S-S bond. The bidirectional orientation of the cross-linker facilitates two fragments in

the ETD spectrum for each constituent chain by tagging of the heavy c-b segment or the light a-b segment of the cross-linker to an amine group of an N-terminus or lysine residue of a peptide via acylation chemistry. The cleavage site or the disulfide bond of the cross-linked peptide is shown as **b**. **Figure 2b** is a schematic of the ETD-MS/MS spectrum of d4-DTSSP cross linking α and β peptide halves. The cross-linker can orient randomly to give a 1:1 mixture of the deuterated arm or a protonated arm of the linker tagged at each polypeptide half upon ETD. **Figure 2c** shows the ETD product ion spectrum of polypeptide of Ubiquitin tryptic peptides inter-linked with asymmetric d4-DTSSP. However, due to the asymmetry on either side of the S-S bond, and due to the bidirectional orientation of the cross-linkers during the cross-linker reaction step, we observe a doublet of peaks for both α and β chains, labeled as α -L/ α -H and β -L/ β -H. We rationalize that cross-linkers with asymmetric labeling are analytically useful as they encode isotopic tags of cross-linked product ions to be readily distinguished from other cleavages due to dissociation of the cross-linker. The mass spectra of isotope coded cross-linked peptides consist of two distinct fragment ions for each constituent peptide chain with a specific mass signature that is observed as a doublet of peaks in the ETD-MS² spectrum. **Figure 2c** shows the structure of the commercially available DTSSP cross-linker that has no mass off-set around the S-S bond as in Figure 2a. The cleavage site is shown as **b** or the disulfide bond of the cross-linked peptide. The schematic shows the ETD-MS/MS spectrum of DTSSP cross linking α and β peptide halves resulting in the tagging of the protonated arm of the linker upon ETD. **Figure 2d** shows the ETD product ion spectrum of an inter-linked polypeptide resulting from a cross-linking reaction of ubiquitin and commercially available DTSSP. The product ions consist mainly of α and β chains that are single isotope clusters resulting from the direct cleavage of the DTSSP disulfide bond. The product ion spectrum looks quite like Figure 2c except there is no signature of a doublet.

The ETD-MS² spectra most often are difficult interpret as the most search algorithm considers a base-peptide that is modified though the cross-linker with partner-peptide. This analysis gives ambiguities in the interpretation due to concomitant dissociation of both base- and partner-peptides. To circumvent this problem, we next, applied each peptide half to CID-MS³. The CID spectra eliminates the ambiguities associated with multiple product ions in a single spectrum and provides comprehensive sequence coverage of each polypeptide chain. We call this entire workflow as isotope tagging of interaction proteins or iTIP that first crosslinks a protein with the d4-DTSSP crosslinker followed by proteolytic digestion and LC-MS analysis of tryptic peptides with data dependent ETD-MS² scan followed by diagnostic ion detection followed by CID-MS³ scan.

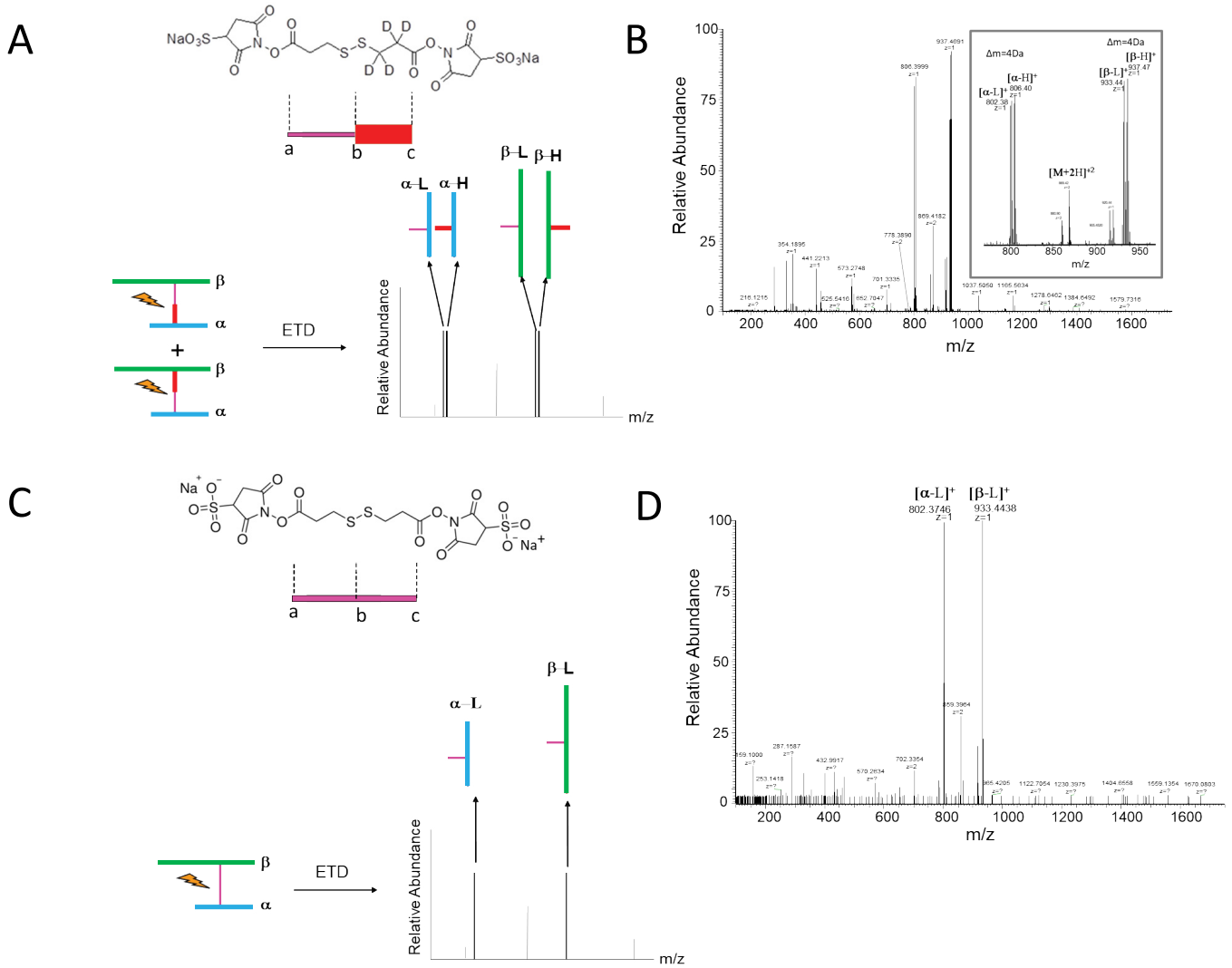


Figure 2: Cross-linking reagents and MS²-ETD of an interlinked peptide (A) Asymmetric d4-DTSSP structure showing amine reactive carbonyl carbons at a, c and ETD cleavable disulfide bond at b (top). A scheme of ETD-MS/MS of d4-DTSSP inter-linked peptide (bottom). Note: constituent α -L and α -H polypeptide doublet and β -L and β -H polypeptide doublet. (B) ETD-MS/MS of d4-DTSSP inter-linked Ubiquitin peptide. (Inset shows α -L, α -H doublet and β -L, β -H doublet. (C) DTSSP structure showing amine reactive carbonyl carbons at a, c and ETD cleavable disulfide bond at b (top). A scheme of ETD-MS/MS of DTSSP inter-linked peptide (bottom). Note: constituent α -L polypeptide β -L polypeptide. (B) ETD-MS/MS of DTSSP inter-linked Ubiquitin peptide from Ubiquitin. (Inset shows α -L, β -L chains). (C) ETD-MS/MS spectrum of the same polypeptide pairs interlinked via asymmetric d4-DTSSP. Inset shows dissociation of the cross-linker to yield the constituent α -L and α -H polypeptide and β -L and β -H polypeptide.

We next applied iTIP workflow to map the distances between lysine residues of Holomyoglobin using the d4-DTSSP crosslinking reagent.

Figure 3a shows an ETD-MS/MS of $[M+3H]^{3+}$ precursor ion $m/z = 814.4$ Da of an interlinked cross-linked polypeptides that was derived from d4-DTSSP cross-linking reactions with Holomyoglobin. Unlike Figure 1b, where disulfide cleavage products were the dominant peaks, the ETD products associated with the disulfide cleavage products are less conspicuous. The product ions make up of a distribution of charge reduced species $[M+4]^{2+}$ and a series of backbone cleavage product ions that gives rise to c- and •z-type ions, and two doublets of

fragment ions corresponding to the cleavage of isotope coded disulfide-linked cross-linker. The doublet peaks are the only diagnostic features in the mass spectrum that is informative of an interlinked polypeptide with two chains. The doublet of peaks of $m/z = 685.42$, and $m/z = 689.44$ have a nominal mass difference of 4 Da, corresponding to the ΔM generated by the dissociation across the disulfide of an asymmetric d4-DTSSP cross-linker. Close examination of the isotope clusters of both these peaks shows an isotope spacing of ~ 1 Da that is indicative of a singly charged fragment ions. Similarly, the doublet of $m/z = 1749.84$ and $m/z = 1753.97$ are separated by a nominal mass difference of 4 Da with each peak having

an isotope spacing of 1 Da. The isotope spacing shows that a singly charged fragment has a ΔM of 4 Da generated via a cleavage across a d4-DTSSP cross-linker. These diagnostic doublets of peaks are therefore related to a peptide cross-linked by an asymmetric d4-DTSSP. Further, both these doublets we believe are complementary fragments generated via the single dissociation of charged reduced radical cations: $[M+2]^{2+}$ at the disulfide bond since cleavage resulted in the conservation of the overall charge state of the charged reduced radical cation. The constituent peptide chains with their corresponding charge states for doublet pairs are labeled as $[\alpha-L]^+ / [\alpha-H]^+$ and $[\beta-L]^{2+} / [\beta-H]^{2+}$. **Figure 3b** show CID-MS/MS of $[M+3H]^{3+}$ precursor ion $m/z = 814.4$ Da of an interlinked cross-linked polypeptides that was derived from d4-DTSSP cross-linking reactions with Holomyoglobin. Unlike Figure 3a, where ETD results in facile cleavage of the crosslinker at the disulfide bond, CID produced more dominant peptide backbone cleavage products (i.e., $\alpha+\beta y_9^+$, $\alpha+\beta z_{10}^{2+}$). The CID cleavage of disulfide bond produced low abundant α and β chains of the polypeptide that were difficult to resolve as a pair of doublets $[\alpha-L]^+ / [\alpha-H]^+$ and $[\beta-L]^{2+} / [\beta-H]^{2+}$ and hence not as diagnostic as the ETD cleavage. Next, we confirmed the identity of the α and β chains via MS3 using CID. CID of singly and doubly charged product ions can efficiently generate fragments compared to ETD. Therefore, we first mass selected the ETD generated $m/z = 685.42$ $[\alpha-L]^+$ and

increase the collision energy to obtain a product ion spectrum (**Figure 3c**). In a similar fashion, we mass selected the ETD generated $m/z = 1749.84$ $[\beta-L]^+$ and increased the collision energy to obtain the product ion spectrum (**Figure 3d**). The MS3-CID spectra are filtered and subjected to database search making up of the Myoglobin sequence along with the lysine modification masses of the cleaved crosslinker that leads to the identification α and β chains. Furthermore, the modification associated with the crosslinker is localized at Lysine-99 of the α -chain and Lysine-43 of the β chain facilitates the mapping of distances between Lysine residues.

Identification of Interacting Peptide Pairs

The iTIP approach readily generated diagnostic fragment ions upon ETD of d4-DTSSP cross-linked peptides that can be structurally interrogated via a CID step followed by mapping of lysine residues that are within 12 Å apart from each other in the protein's native conformation. Mapping residue distances using this approach benefits from obtaining Two MS3 fragmentation spectra that confirm the identity of each interpeptide chain. In some instances, cross-linking of a given lysine residue can take place with multiple lysine residues that further improves the confidence. We used an inter-linked cross-linked peptide that had a common base-peptide but had two different partner-peptides to demonstrate the confidence boost you get in the connectivity map.

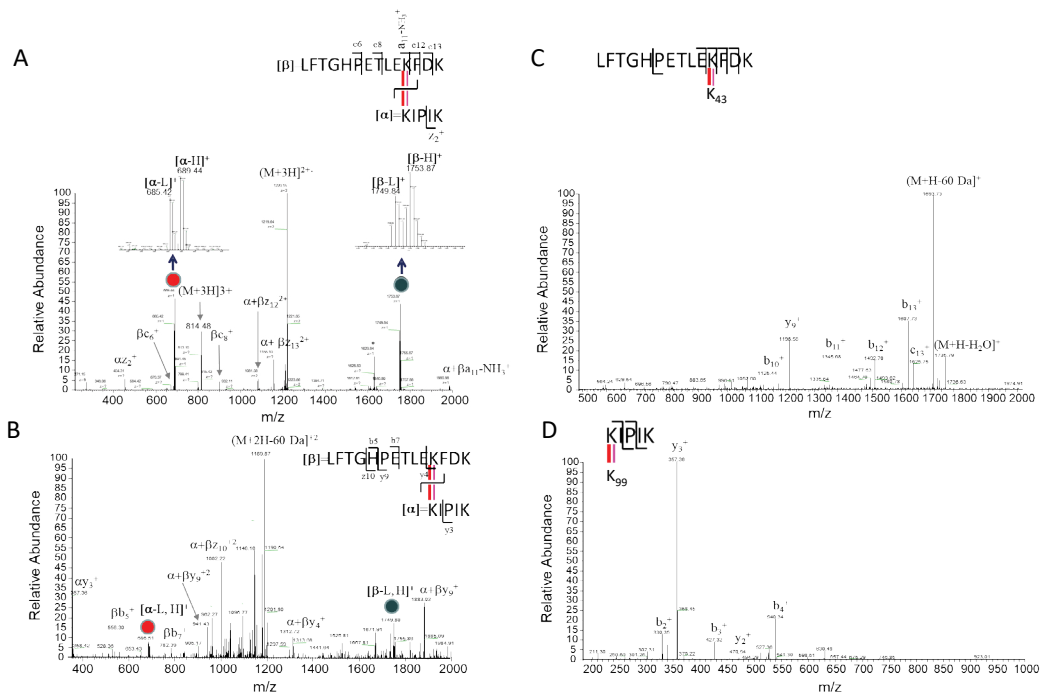


Figure 3: MS2 and MS3 product ion spectra of d4-DTSSP crosslinked to Holomyoglobin (A) ETD-MS/MS of product ion spectrum of a triply charged inter-linked peptide. Insets show the dissociation products associated with the cross-linker that is seen as an isotope coded mass tag with each constituent chain labeled as α -L and α -H doublet and β -L and β -H doublet. (B) CID-MS/MS of product ion spectrum of a triply charged inter-linked peptide. (C) CID-MS3 of β -H chain and the corresponding sequence map of each polypeptide depicting CID fragmentation and location of lysine residues that are cross-linked. (D) CID-MS3 of α -H chain and the corresponding sequence map of each polypeptide depicting CID fragmentation and location of lysine residues that are cross-linked.

Figure 4a shows an ETD-MS/MS of $[M+4H]^{4+}$ precursor ion $m/z = 628.3$ Da of an interlinked cross-linked polypeptides derived from d4-DTSSP cross-linking reactions with Holomyoglobin. The product ions comprise of a distribution of charge reduced species $[M+4]^{3+}$, $[M+4]^{2+}$, a series of backbone cleavage product ions that gives rise to c- and •z-type ions, and three doublets of fragment ions corresponding to the cleavage of isotope coded disulfide-linked cross-linker. The doublet peaks are the only diagnostic features in the mass spectrum that is informative of an interlinked polypeptide with two chains. The doublet of peaks at $m/z = 759.4$, and $m/z = 763.4$ have a nominal mass difference of 4 Da, corresponding to the ΔM generated by the dissociation across the disulfide of an asymmetric d4-DTSSP cross-linker. Close examination of the isotope clusters of both these peaks shows an isotope spacing of ~ 1 Da showing that they are singly charged fragment ions. Similarly, the doublet of $m/z = 875.2$ and $m/z = 877.2$ are separated by a nominal mass difference of 2 Da with each peak having an isotope spacing of 0.5 Da. The isotope spacing shows that a doubly charged fragment has a ΔM of 4 Da generated via

a cleavage across a d4-DTSSP cross-linker. Related, these diagnostic doublets of peaks are related to a peptide cross-linked by an asymmetric d4-DTSSP. Further, both these doublets we believe are complementary fragments generated via the single dissociation of charged reduced radical cations: $[M+4]^{3+}$ at the disulfide bond since cleavage resulted in the conservation of the overall charge state of the charged reduced radical cation. The constituent peptide chains with their corresponding charge states for doublet pairs are labeled as $[\alpha-L]^+ / [\alpha-H]^+$ and $[\beta-L]^{2+} / [\beta-H]^{2+}$. Further, the doublet of $m/z = 1750.7$ and $m/z = 1754.7$ has a nominal mass difference of 4 Da with each peak having an isotope spacing of 1 Da, again indicating a singly charged fragment ion corresponding to the isotope coded product ions of the cross-linker. We believe the generation of these doublet peaks $[\beta-L]^+ / [\beta-H]^+$ was due to the sequential charge reduction of the doubly charged doublets $[\beta-L]^{2+} / [\beta-H]^{2+}$. The iTIP approach readily generated diagnostic fragment ions from cross-linked peptides that can be structurally interrogated via a CID step that is more suitable for doubly and singly charged peptides halves making up the cross-linked peptide halves.

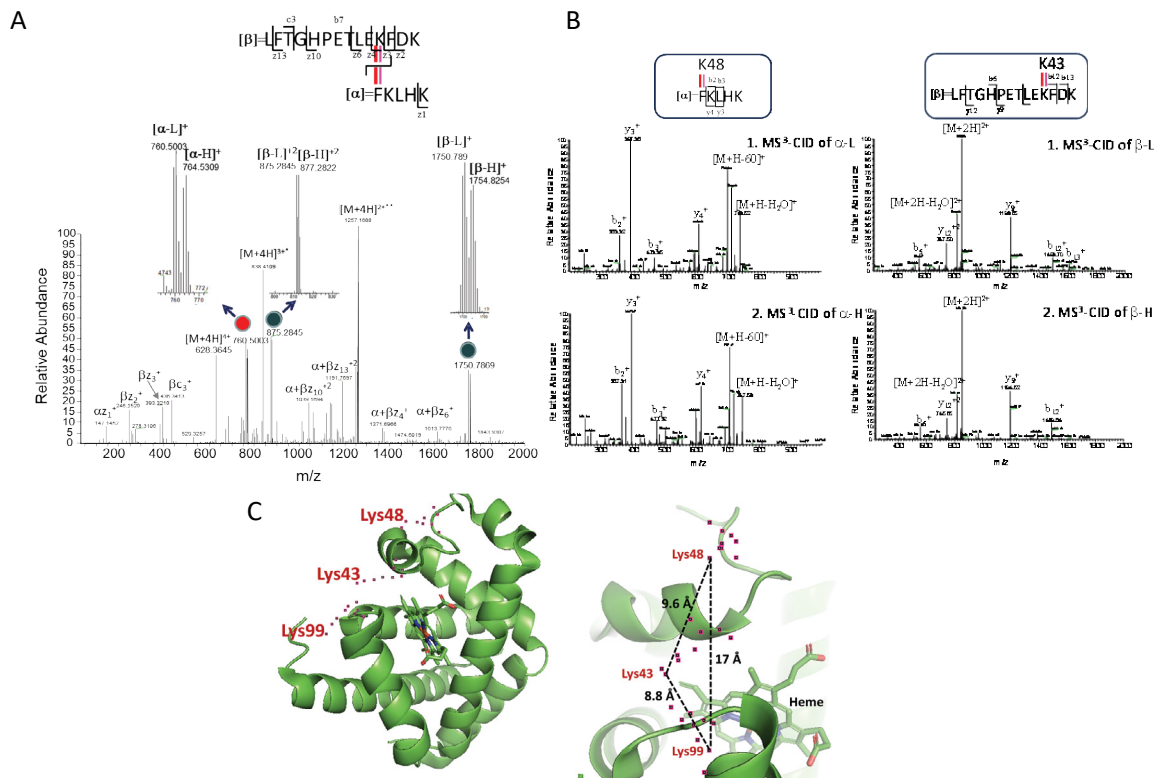


Figure 4: MS2 and MS3 product ion spectra of d4-DTSSP interpeptide crosslinks and mapping lysine residue distances to Holomyoglobin (A) ETD-MS/MS of product ion spectrum of a quadruply charged inter-linked peptide. Insets show the dissociation products associated with the cross-linker as an isotope coded mass tag with each constituent chain labeled as $\alpha-L$ and $\alpha-H$ doublet and $\beta-L$ and $\beta-H$ doublet. (B) CID-MS3 of $\alpha-L$, $\alpha-H$, $\beta-L$, $\beta-H$ chains with corresponding sequence maps of each polypeptide depicting CID fragmentation and location of cross-linked lysine residues. (c) Inter-residue distances of the crosslinked peptides mapped to the Holomyoglobin crystal structure (PDB code 1WLA). Note: Zoomed-in view shows the closest distances between Lysine-43, Lysine-99 pair, and Lysine-43-Lysine 48 pair are less than the 12 Å length of the d4-DTSSP spacer.

The selective identification of cross-linked peptides becomes straightforward and streamlined by the MS³ step. Here we show that the peptide doublets generated by ETD can be sequenced by conventional database assisted peptide search strategies. For example, the doublet peaks that is shown in Figure 3a were structurally interrogated via CID-MS³ and the spectra were subjected to a database assisted sequence determination of the interlinked polypeptides. The iTIP strategy also allows MS³-CID of both heavy/light chains of each peptide sequence which provides an added degree of confidence in their identification as interacting proteins or interaction sites within a protein. **Figure 4b** shows the resulting CID product ion spectra for the α -chain_L, α -chain_H, β -chain_L, and β -chain_H of the constituent cross-linked peptide chains of the two interacting lysine residues of Holomyoglobin. Each type of chain encoded in Light: C₃OH₄S, and Heavy: C₃OD₄S modification mass is discernable by its respective mass spacing of 4 Da. The sequence for each constituent polypeptide ion was identified using a standard database search with dynamic lysine modification as described in the experimental section. The polypeptide sequences were identified as myoglobin, and the modification associated with the crosslinker is localized at Lysine-48 of the α -chain and Lysine-43 of the β chain facilitates the mapping of distances between Lysine residues. **Figure 4c** shows how pair-wise distance between lysine residues are mapped to the native Holomyoglobin crystal structure (PDB code: 1WLA). Residue level connectivity is observed in our crosslinking experiments between Lysine-43 in LFTGHPETLEK₄₃FDK as base peptide and Lysine-99 and Lysine-48 in FK₄₈LHL and K₉₉IPIK partner peptide sequences, respectively. The pair-wise distance of Lysine-43/Lysine-48 residues is 9.6 Å and the distance between Lysine-43/Lysine-99 is 8.8 Å. These distances can be less in dynamic structure considering the flexibility of the lysine side chains, for a DTSSP cross-linker that has 12 Å spacer to react with these lysine residues. Lysine-48/Lysine-99 is 17 Å apart making DTSSP crosslinkers less effective to map these residue distances of Holomyoglobin.

Conclusions

We have developed a novel crosslinker (asymmetric d4 DTSSP) to accurately detect crosslinked peptides using diagnostic product ion signatures obtained by tandem mass spectrometry. We call the overall strategy of identifying and localizing putative lysine residues that belong to a single protein or complexes as Isotope Tagging of Interacting Proteins (iTIP). The iTIP procedure starts with cross-linking of whole protein molecules or complexes with d4-DTSSP, followed by proteolysis and tandem mass spectrometry involving ETD of the inter-peptide cross-links to generate isotope tagging patterns that serve as structural surrogates for identifying protein-protein interactions. The iTIP

technology is amenable to identifying lysine residues that constitute the interacting surfaces of proteins with a high degree of confidence as proved by CID-MS/MS of both light and heavy forms of the same peptide sequence. Structural tools become important for the discovery of novel drug targets and the development of multimodal biologics in the pharmaceutical industry. We foresee asymmetric d4-DTSSP analogs of variable linker lengths can be used to improve the resolution of biological structures. We envision that iTIP can be leveraged to solve many structural biology and structural proteomics questions.

Acknowledgement

The research was performed at The University of North Carolina at Chapel Hill. HPG conceived the idea, designed, and performed all MS experiments and wrote the paper. HPG is the inventor of the iTIP cross-linking method and is patented under US patent US 9,562,010 B2 and assigned to the University of North Carolina at Chapel Hill.

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