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Developing New Isotope-Coded Mass Spectrometry-Cleavable Cross-Linkers for Elucidating Protein Structures

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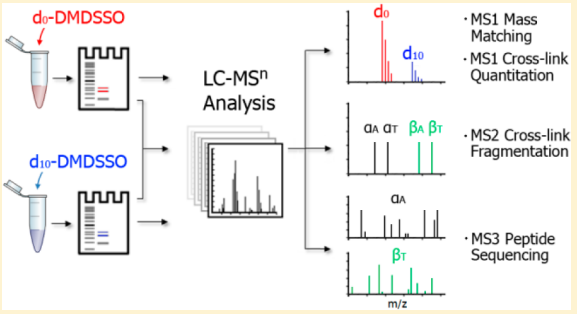
Supporting Information

ABSTRACT: Structural characterization of protein complexes is essential for the understanding of their function and regulation. However, it remains challenging due to limitations in existing tools. With recent technological improvements, cross-linking mass spectrometry (XL-MS) has become a powerful strategy to define protein−protein interactions and elucidate structural topologies of protein complexes. To further advance XL-MS studies, we present here the development of new isotope-coded MS-cleavable homobifunctional cross-linkers: d_0- and d_{10}-labeled dimethyl disuccinimidyl sulfoxide (DMDSSO). Detailed characterization of DMDSSO cross-linked peptides further demonstrates that sulfoxide-containing MS-cleavable cross-linkers offer robust and predictable MS2 fragmentation of cross-linked peptides, permitting subsequent MS3 analysis for simplified, unambiguous identification. Concurrent usage of these reagents provides a characteristic doublet pattern of DMDSSO cross-linked peptides, thus aiding in the confidence of cross-link identification by MS^n analysis. More importantly, the unique isotopic profile permits quantitative analysis of cross-linked peptides and therefore expands the capability of XL-MS strategies to analyze both static and dynamic protein interactions. Together, our work has established a new XL-MS workflow for future studies toward the understanding of structural dynamics of protein complexes.



Protein complexes represent essential functional entities in cells for carrying out multiple biological processes including translation, replication, cell division, and cell cycle control. Protein−protein interactions are integral in modulating the assembly, structure, and function of protein complexes. Perturbations of endogenous protein−protein interactions can result in deleterious effects on cellular activities and lead to human disease. In recent years, protein−protein interaction interfaces have become a new and attractive platform for therapeutics.[1] Therefore, characterization of structures and interaction dynamics of protein complexes is critical to understanding their function and regulation, thus unraveling molecular mechanisms underlying human pathologies and providing insight on potential targets for drug development. Traditional structural tools such as nuclear magnetic resonance (NMR) and X-ray crystallography are able to yield detailed, high-resolution information on protein structures. However, these technologies have difficulty in analyzing heterogeneous and dynamic protein complexes. Following decades of method development alongside technological advances in mass spectrometry, cross-linking mass spectrometry (XL-MS) has emerged as a powerful strategy not only for mapping protein interaction networks[2−4] but also for structural elucidation of protein complexes.[5−8] The cross-links between proteins can be used to derive topological ordering of protein complexes by computational modeling.[8,9] In addition, spatial distances between cross-linked residues can be converted to distance restraints for protein homology modeling.[6]

The major challenges in XL-MS studies are the detection of low-abundance cross-linked peptides and their unambiguous identification. The complexity in peptide mixtures often impedes MS detection of cross-linked peptides due to the presence of significantly more abundant noncross-linked peptides. In addition, heterogeneous populations of cross-linked products, i.e., interlinked, intralinked, and dead-end modified peptides further complicates the analysis. To facilitate the detection of cross-linked peptides, one strategy is to selectively enrich cross-linked products for MS analysis using enrichable cross-linkers containing either an affinity tag (e.g., biotin tag)[10,11] or a chemical handle that allows subsequent addition of an affinity tag through chemical conjugation.[12] Another strategy is to incorporate stable isotopes in cross-linked peptides to generate characteristic isotopic profiles, thus separating them from noncross-linked peptides.[6,7,10,13−16] This differentiation can be achieved by first carrying out enzymatic digestion of cross-linked proteins in ^{16}O and ^{18}O water, respectively, and then mixing prior to MS analysis.[13] Although effective, enzymatic incorporation of ^{18}O is troublesome as its

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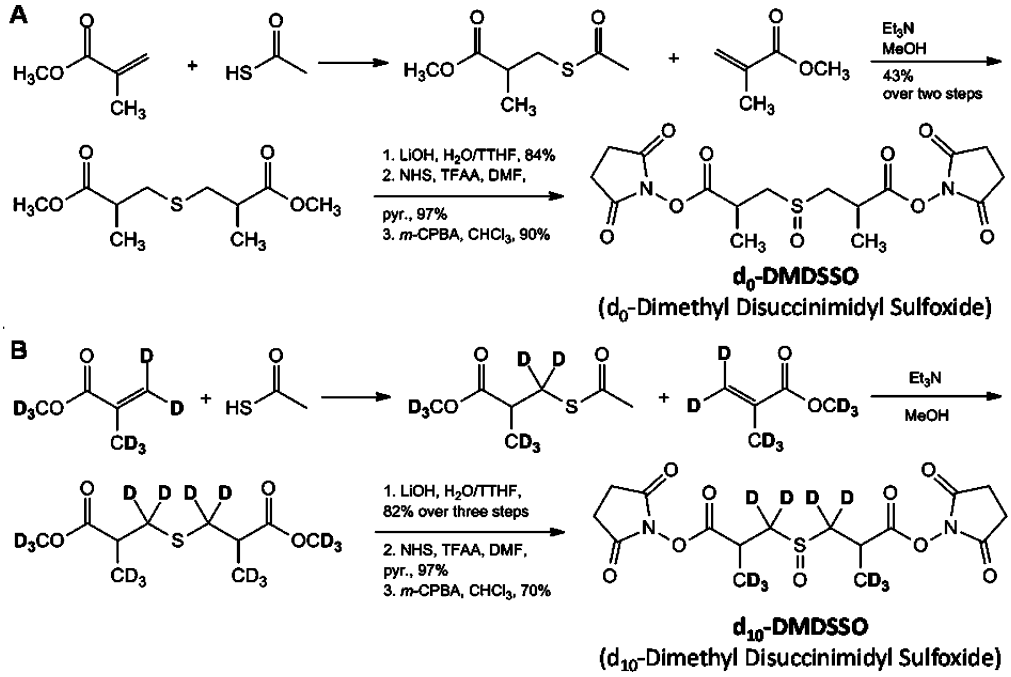

Figure 1. Chemical synthesis schemes for (A) d_0-DMDSSO and (B) d_{10}-DMDSSO.

labeling efficiency relies heavily on peptide sequences. Interestingly, performing protein cross-linking in ^{18}O water can result in the incorporation of one ^{18}O to dead-end modified peptides but not to other types of peptides, thus effectively distinguishing them from intralinked and interlinked peptides.[10] However, a common practice to produce cross-linked peptides as isotopic pairs for easy identification is to cross-link proteins with a 1:1 mixture of nonlabeled and labeled cross-linkers.[6,7,14−17]

Apart from the detection of cross-linked peptides, unambiguous identification of interlinked peptides by peptide sequencing is challenging when noncleavable cross-linkers are used. This is due to the difficulty in interpreting convoluted tandem mass spectra resulted from the fragmentation of two interlinked peptides. Despite recent innovation in bioinformatics tools that have been developed to better dissect fragmentation data of interlinked peptides,[18−22] further improvements are required to make it as generally applicable as that for identifying single peptide sequences. To circumvent these problems, various types of cleavable cross-linkers, e.g., MS-, photo-, and chemical-cleavable reagents, have been developed to facilitate MS identification of cross-linked peptides. Among them, MS-cleavable reagents appear to be most attractive for XL-MS studies,[11,12,17,23,24] owing to their unique capability of fragmenting cross-links during collision-induced dissociation (CID) and thus facilitating subsequent peptide sequencing for unambiguous identification. Recently, we have developed a novel MS-cleavable homobifunctional NHS ester, disuccinimidyl sulfoxide (DSSO), in which the MS-cleavable C−S bond cleaves preferentially during MS2 analysis prior to the breakage of the peptide backbone.[12] This cleavage permits robust, reliable, and characteristic CID-induced fragmentation of cross-linked peptides unique to their cross-linking types, generating distinct MS2 fragment ions for subsequent MS3 sequencing. This novel integrated workflow has proven to be effective for fast and accurate identification of cross-linked peptides using conventional bioinformatics tools and has been successfully applied to elucidate structures of proteasome complexes.[8,12]

To further advance XL-MS studies of protein complexes, we have developed a pair of new isotope-coded DSSO derivatives, i.e., d_0- and d_{10}-labeled dimethyl-disuccinimidyl sulfoxide (DMDSSO). Incorporation of deuterium labeling into our robust sulfoxide-containing MS-cleavable cross-linker adds new features that not only enhance the detection and identification of cross-linked peptides but also provide the capability of quantifying cross-linked peptides. Here we present the detailed characterization of DMDSSO-based cross-linking strategy using synthetic peptides and model protein cytochrome C. We have compared MS^n analyses of d_0- and d_{10}-DMDSSO cross-linked peptides and performed quantitative assessments of cross-linked peptides with different sample preparation strategies.

EXPERIMENTAL PROCEDURES

Materials and Reagents. General chemicals were purchased from Fisher Scientific or VWR International, bovine heart cytochrome C (98% purity) from Sigma-Aldrich, and Ac-Myelin peptide (Ac-ASQKRPSQRHG, 92.7% purity) from American Peptide (Sunnyvale, CA).

Synthesis and Characterization of d_0-DMDSSO and d_{10}-DMDSSO. The synthesis of DMDSSO was depicted in Figure 1. Briefly, the preparation of d_0-DMDSSO began with addition of thioacetic acid to methyl methacrylate. Methanol and triethylamine were added to the mixture along with another equivalent of methyl methacrylate to afford the symmetrical diester in one pot. The diester was hydrolyzed with lithium hydroxide in THF/H_2O before coupling with NHS, in the presence of trifluoroacetic anhydride, pyridine, and DMF.[25] Lastly, oxidation of the sulfide to the sulfoxide yielded the desired linker as described.[12] The preparation of d_{10}-DMDSSO was carried out similarly, beginning with commercially available d_8-methyl methacrylate. The details of the chemical characterization are described in the Supporting Information.

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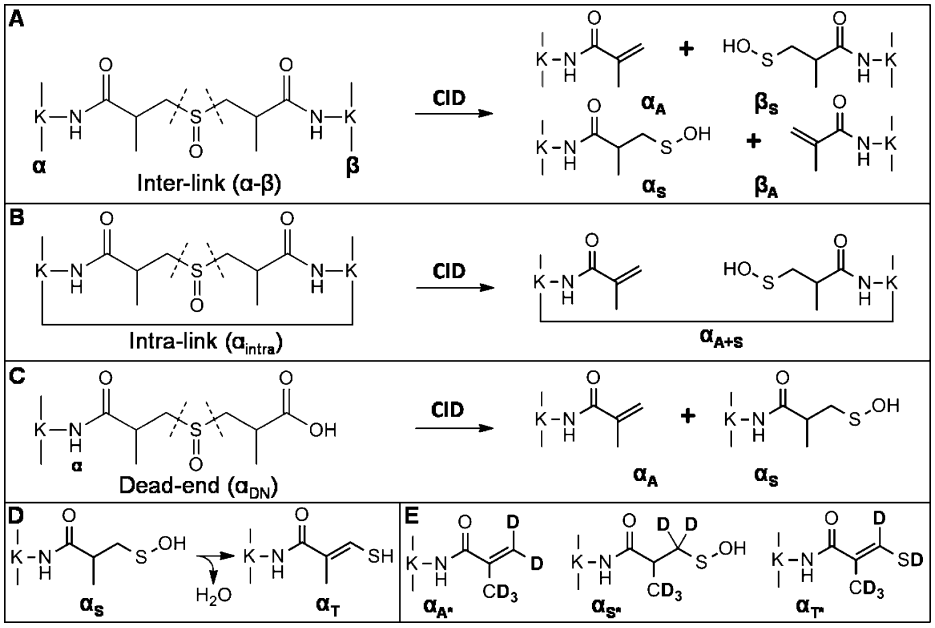

Figure 2. Characteristic MS2 fragmentation patterns for DMDSSO cross-linked peptides. MS2 fragmentation of (A) d_0-DMDSSO interlinked heterodimer $\alpha-\beta$. (B) d_0-DMDSSO intralinked peptide α_{intra}. (C) Dead-end modified peptide α_{DN}. (D) The conversion scheme of α_S to α_T. (E) Illustrations of α_{A*}, α_{S*}, and α_{T*} fragments with lysines modified with d_{10}-DMDSSO remnants.

Cross-Linking of Synthetic Peptides with d_0- and d_{10}-DMDSSO. Synthetic peptide Ac-Myelin was dissolved in DMSO to 1 mM and cross-linked with either d_0- or d_{10}-DMDSSO in a 1:1 molar ratio of peptide to cross-linker in the presence of 1 equiv of diisopropylethylamine. The resulting samples were diluted to 5 pmol/μL in 3% ACN/2% formic acid for MS analysis.

Cross-Linking of Cytochrome C with d_0- and d_{10}-DMDSSO. A volume of 40 μL of 200 μM cytochrome C in PBS buffer (pH 7.4) was reacted with d_0- or d_{10}-DMDSSO in a molar ratio of 1:10 (protein−cross-linker) for 2 h at room temperature and quenched with excess ammonium bicarbonate. Samples were then subjected to SDS-PAGE and visualized by Coomassie blue. The dimerized bands were excised, reduced with TCEP for 30 min, and alkylated with chloroacetamide for 45 min in the dark, and then digested with trypsin at 37 °C overnight. Peptide digests were extracted, concentrated, and reconstituted in 3% ACN/2% formic acid for MS analysis.

Liquid Chromatography−Multistage Tandem Mass Spectromtery (LC MS^n). DMDSSO cross-linked peptides were analyzed by LC−MS^n utilizing an LTQ-Orbitrap XL MS (ThermoFisher, San Jose, CA) coupled online with an Eksigent NanoLC system (Dublin, CA) as previously described.[12] Each MS^n experiment has a duty cycle of 1.3 s, consisting of one MS scan in FT mode (350−1400 m/z, resolution of 60 000 at m/z 400) followed by two data-dependent MS2 scans in FT mode (resolution of 7500) with normalized collision energy at 15% on the top two MS peaks with charges at 3+ or up, and three MS3 scans in the LTQ with normalized collision energy at 35% on the top three peaks from each MS2.

Data Analysis of Cross-Linked Peptides. Data processing of LC−MS^n spectra was carried out as described.[12] MS3 data was subjected to a developmental version of Protein Prospector (v. 5.10.10) for database searching, using Batch-Tag against cytochrome C sequence (SwissProt accession no. P62894) with mass tolerances for parent ions and fragment ions set as ±20 ppm and 0.6 Da, respectively. Trypsin was set as the enzyme with four maximum missed cleavages allowed. Protein N-terminal acetylation, methionine oxidation, N-terminal conversion of glutamine to pyroglutamic acid, asparagine deamidation, and cysteine carbamidomethylation were selected as variable modifications. In addition, three defined modifications on uncleaved lysines and free protein N-termini were selected: alkene (A, C_4H_4O, + 68 Da; or A*, $C_4H_{-1}D_5O$, + 73 Da), sulfenic acid (S, $C_4H_6O_2S$, + 118 Da; or S*, $C_4H_1D_5O_2S$, + 123 Da), and unsaturated thiol (T, C_4H_4OS, + 100 Da; or T*, $C_4H_{-1}D_5OS$, + 105 Da) modification, due to remnant moieties for d_0- (i.e., A, S, T) or d_{10}-DMSSO (i.e., A*, S*, T*) cross-linker, respectively. Initial acceptance criteria for peptide identification required a reported expectation value ≤0.1.

MS-Bridge was used to confirm the identification of cross-linked peptides by mass mapping against bovine cytochrome C with the parent mass error set as ±10 ppm.[12] The in-house program Link-Hunter is a revised version of the previously written Link-Finder program, designed to automatically validate and summarize cross-linked peptides based on MS^n data and database searching results as previously described.[8,12]

RESULTS AND DISCUSSION

Design and Synthesis of New Isotope-Coded DSSO Derivatives. In order to further facilitate MS identification of cross-linked peptides and allow quantitative determination of structural dynamics of protein complexes, we aimed to generate deuterium labeled MS-cleavable cross-linkers. Given our previous success of DSSO-based XL-MS strategies in protein structural characterization,[8,12] we first attempted to produce d_4-DSSO by introducing deuterium at the positions alpha to the carbonyls through deuterium exchange (Supplementary Figure 1 in the Supporting Information). Although feasible, complete labeling was problematic due to slow exchange. Additionally, labeling with four deuteriums proved to be insufficient for effective separation of highly charged d_0/d_4-DSSO cross-linked peptide pairs (4+ and above) during MS^n analysis. Therefore,

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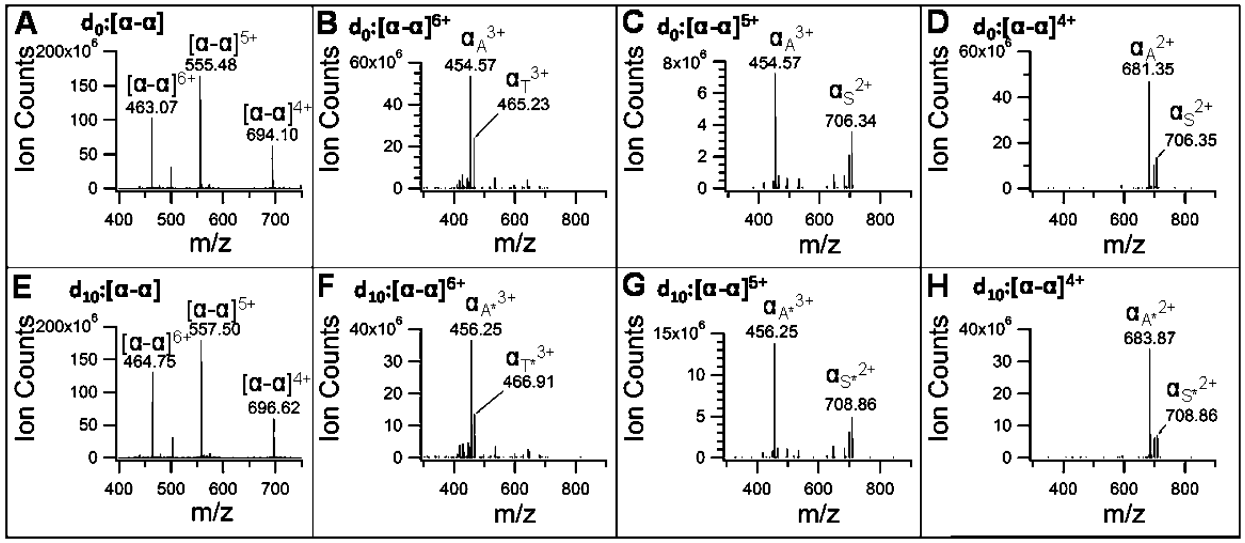

Figure 3. MS^n analyses of d_0- and d_{10}-DMDSSO interlinked Ac-Myelin peptides. (A) MS spectrum of d_0-interlinked Ac-Myelin. (B–D) MS2 spectra of d_0-interlinked Ac-Myelin at three different charge states: (B) $[\alpha-\alpha]^{6+}$, (C) $[\alpha-\alpha]^{5+}$, and (D) $[\alpha-\alpha]^{4+}$. (E) MS spectrum of d_{10}-interlinked Ac-Myelin. (F–H) MS2 spectra of d_{10}-interlinked Ac-Myelin at three different charge states: (F) $[\alpha-\alpha]^{6+}$, (G) $[\alpha-\alpha]^{5+}$, and (H) $[\alpha-\alpha]^{4+}$.

d_8-labeled DSSO would be ideal; however, incorporation of eight deuteriums in DSSO appeared to be less practical due to cost and experimental difficulties. To circumvent this problem, we have designed a new derivative of DSSO, dimethyl disuccinimidyl sulfoxide (DMDSSO). With the commercial availability of methyl methacrylate and d_8-methyl methacrylate, the synthesis of d_0- or d_{10}-DMDSSO is economical and straightforward (Figure 1). Similar to DSSO, DMDSSO also has an ideal length (an average extended length of 9.3 Å) for structural proteomics studies.

Expected CID Fragmentation Patterns of d_0- and d_{10}-DMDSSO Cross-linked Peptides. Three types of cross-linked products can result from the digestion of cross-linked proteins: interlinked, intralinked, and dead-end modified peptides. Previously we have shown that DSSO cross-linked peptides display characteristic fragmentation patterns during MS2 analysis due to preferential cleavage of CID-cleavable C–S bonds adjacent to the sulfoxide.[12] Aside from two additional methyl groups, DMDSSO has a structure very similar to DSSO, with two symmetric MS-cleavable C–S bonds. Therefore, we expect that DMDSSO cross-linked peptides will display the same characteristic MS2 fragmentation patterns as DSSO cross-linked peptides. Since deuterium labeling should not interfere with peptide fragmentation, d_0- and d_{10}-DMDSSO cross-linked peptides would behave similarly during MS^n analysis. For simplicity, we use d_0-DMDSSO cross-linked peptides to illustrate their predicted fragmentation patterns (Figure 2). Prior to peptide backbone fragmentation, MS2 analysis selectively cleaves either of the two symmetric C–S bonds in the linker region of DMDSSO cross-linked peptides, yielding peptide fragments with predictable modifications (due to the remnants of DMDSSO) on cross-linked lysine residues. For a d_0-DMDSSO interlinked peptide $\alpha-\beta$, cleavage of a C–S bond leads to physical separation of the two interlinked peptides into a pair of peptide fragments (i.e., α_A/β_S or α_S/β_A), in which α and β peptide fragments are modified by two complementary cross-linker remnant moieties, i.e., alkene (A) and sulfenic acid (S) (Figure 2A). Thus, the resulting MS2 peptide fragments can be subjected to MS3 sequencing for unambiguous identification of interlinked peptides.[12] For a d_0-DMDSSO intralinked peptide α_{intra}, one peptide fragment (i.e., α_{A+S}) is anticipated, carrying an alkene- and a sulfenic acid-modified lysine, respectively (Figure 2B). This MS2 fragment ion α_{A+S} actually represents two different ion species that have identical peptide sequences and m/z values but transposed DMDSSO remnant-modified lysine residues. For a d_0-DMDSSO dead-end modified peptide (α_{DN}), two peptide fragments (i.e., α_A and α_S) are expected (Figure 2C). It is noted that the sulfenic acid moiety often undergoes dehydration to become a more stable and dominant unsaturated thiol moiety (i.e., T, + 100 Da) as previously described (Figure 2D).[12] This conversion does not appear to complicate data analysis as observed for DSSO cross-linked peptides.[12] In comparison to d_0-DMDSSO cross-linked peptides, fragmentation patterns of d_{10}-DMDSSO cross-linked peptides should be the same except all of the d_{10}-DMDSSO remnants (i.e., A*, alkene; S*, sulfenic acid; or T*, unsaturated thiol) are 5 Da higher in mass due to the presence of 5 deuteriums after cleaving the C–S bond (Figure 2E). In addition to distinct MS2 fragmentation patterns, DMDSSO cross-linked peptides have fixed mass relationships between parent ions and their respective fragment ions, similar to those of DSSO cross-linked peptides,[12] thus providing an additional confirmation of the identified cross-linked peptides at the MS2 level. Together with MS3 sequencing and MS1 mass matching, three different types of evidence can be obtained for the identification of DMDSSO cross-linked peptides with significantly improved confidence and accuracy.

Characterization of DMDSSO Cross-Linked Model Peptides by MS^n Analysis. We first performed DMDSSO cross-linking on synthetic peptide Ac-Myelin. Under our experimental conditions, the resulting cross-linked products were primarily interlinked Ac-Myelin homodimer ($\alpha-\alpha$), which were detected as a series of multiply charged ions for d_0-DMDSSO (m/z 462.9033^{6+}, 555.2822^{5+}, 693.8497^{4+}) and d_{10}-DMDSSO (m/z 464.5796^{6+}, 557.2951^{5+}, 696.3656^{4+}), respectively (Figure 3A,E). There is a 10 Da mass difference between d_0- and d_{10}-labeled cross-linked peptides due to incorporation of 10 deuteriums in d_{10}-DMDSSO. As shown in Figure 3B, MS2 analysis of the sextuply charged d_0-interlinked Ac-Myelin (d_0, $\alpha-\alpha^{6+}$) yielded a pair of dominant fragment ions (α_A/α_T),

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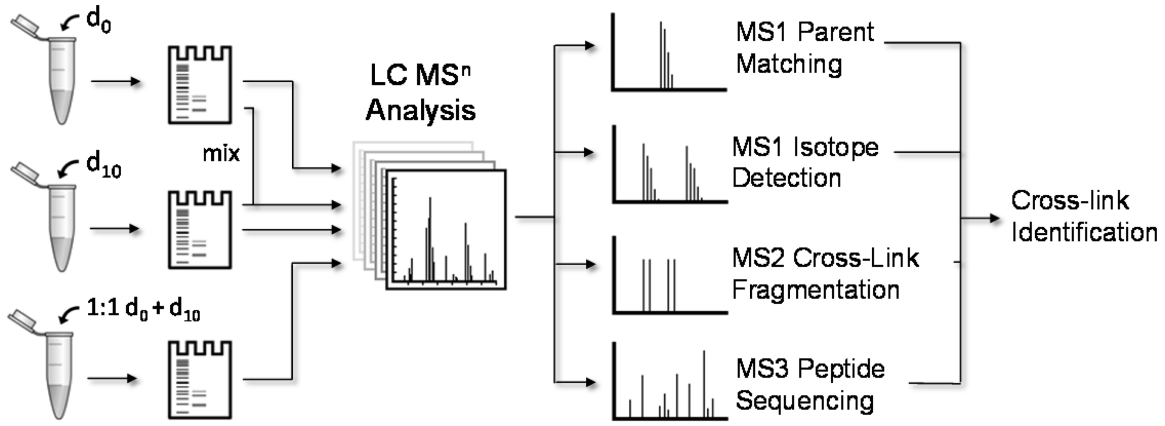

Figure 4. General workflow for the analysis and identification of d_0/d_{10} DMDSSO cross-linked cytochrome C peptides.

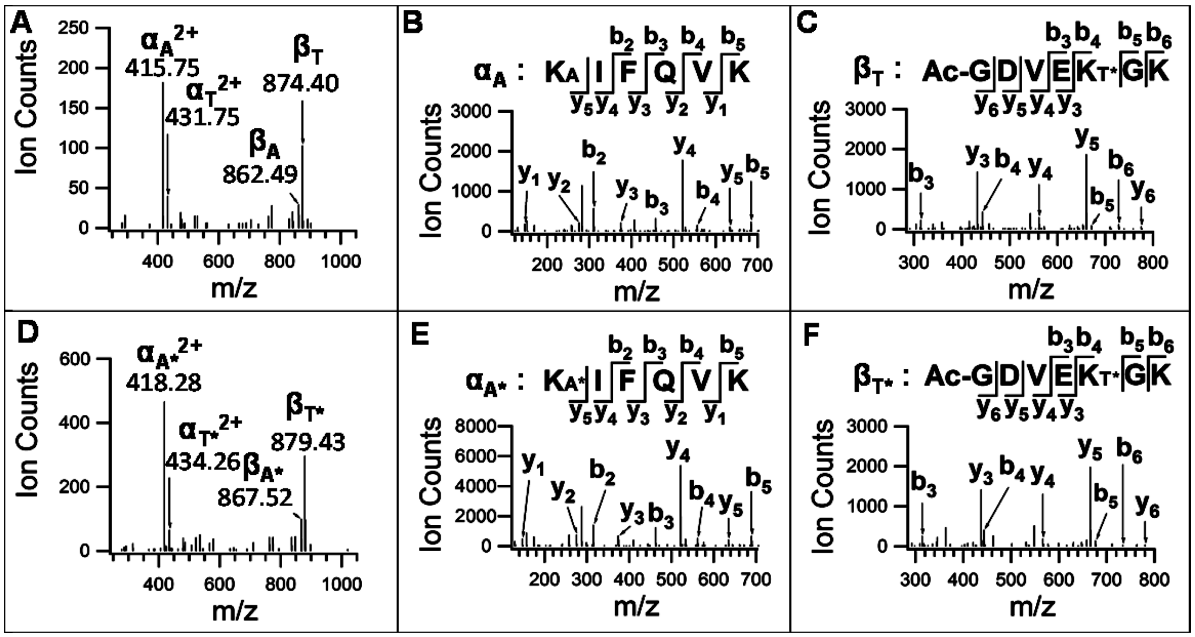

Figure 5. MS^n analysis of d_0/d_{10}-DMDSSO interlinked cytochrome C peptides. (A) MS2 spectrum of a d_0-interlinked cytochrome C peptide $\alpha-\beta$ (m/z 574.6436^{3+}). MS3 spectra of its MS2 fragment ions (B) α_A (m/z 415.76^{2+}) and (C) β_T (m/z 874.40). (D) MS2 spectrum of a corresponding d_{10}-interlinked cytochrome C peptide $\alpha-\beta$ (m/z 577.9993^{3+}). MS3 spectra of its fragment ions (E) α_{A*} (m/z 418.28^{2+}) and (F) β_{T*} (m/z 879.43).

demonstrating effective separation of the interlinked homodimer as expected. Similarly, the α_{A*}/α_{T*} ion pair was also detected as the most abundant ions in MS2 spectrum for d_{10}-interlinked Ac-Myelin peptide (d_{10}, $\alpha-\alpha^{6+}$) (Figure 3F), indicating no interference from deuterium labeling. MS2 analyses of quadruply- and quintuply-charged Ac-Myelin peptides also resulted in one pair of fragment ions (d_0, α_A/α_S; d_{10}, α_{A*}/α_{S*}) (Figure 3C,D,G,H), in which α_S or α_{S*} appears to be more dominant than α_T or α_{T*}, respectively, in contrast to the fragmentation of sextuply charged interlinked peptides (Figure 3B,F). This observation may be due to the susceptibility of highly charged species to fragmentation when the same energy is applied to all precursor ions during CID analysis regardless of their charge. Such fragmentation behavior was previously observed for DSSO interlinked Ac-Myelin peptides as well.[12] MS3 sequencing of α_A, α_T, α_{A*}, and α_{T*} fragment ions confirmed the peptide sequences of d_0- and d_{10}-interlinked Ac-Myelin peptides unambiguously (Supplementary Figure 2 in the Supporting Information), and none of the DMDSSO remnants appear to complicate peptide sequencing by MS3. Taken together, the results have proven that addition of methyl substituents in the linker region does not change the unique fragmentation of sulfoxide-containing MS-cleavable cross-linked peptides, and the preferential cleavage of C−S bonds is independent of peptide charges. Thus, MS^n analysis of DMDSSO cross-linked peptides can be performed the same way as that of DSSO cross-linked peptides.[12]

Characterization of DMDSSO Cross-Linked Cytochrome C by MS^n Analysis. We next evaluated the applicability of d_0- and d_{10}-DMDSSO for protein cross-linking. Model protein cytochrome C has been extensively used to test various new cross-linking strategies due to the large number of lysine residues relative to its size.[12,26] In this work, DMDSSO cross-linked cytochrome C was separated by 1-D SDS-PAGE and visualized by Coomassie blue staining. In comparison to DSSO, d_0- and d_{10}-DMDSSO showed comparable efficiency in protein cross-linking (Supplementary Figure 3 in the Supporting Information). The general workflow for analyzing cross-linked cytochrome C is illustrated in Figure 4. As shown, we first analyzed in-gel digests of d_0- and d_{10}-DMDSSO dimerized

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cytochrome C separately. Figure 5A,D displays the respective MS2 spectra of a selected pair of d_0- and d_{10}-DMDSSO interlinked cytochrome C peptides (m/z 574.6436^{3+}, 577.9993^{3+}), in which two pairs of peptide fragment ions (d_0, α_A/β_T and α_T/β_A; d_{10}, α_{A*}/β_{T*} and α_{T*}/β_{A*}) were detected, demonstrating characteristic fragmentation pattern of interlinked heterodimeric peptides. The most dominant fragment pair ions, α_A/β_T for d_0- and α_{A*}/β_{T*} for d_{10}-labeled interlinked peptides, were subsequently subjected to MS3 analysis (Figure 5BC,E,F). On the basis of the series of y and b ions detected, the sequences of α_A (m/z 415.76^{2+}) and α_{A*} (m/z, 418.28^{2+}) were determined as K_AIFQVK and K_{A*}IFQVK, respectively, in which the N-terminal K is modified with the alkene moiety. Similarly, MS3 analysis of the corresponding β_T (m/z 874.40) and β_{T*} (m/z 879.43) identified their sequences as Ac-GDVEK$_T$GK and Ac-GDVEK$_{T*}$GK, respectively, where the K at the fifth position from N-terminus is modified with the thiol moiety. Together with mass mapping of the parent ions using MS-Bridge, the interlinked peptides were unambiguously determined as [Ac-^{1}GDVEKGK^7 interlinked to ^{8}KIFQVK^{13}], in which a cross-link was formed between K5 and K8 in cytochrome C.

In addition to interlinked peptides, we have also identified DMDSSO intralinked and dead-end modified cytochrome C peptides, and their MS2 fragmentation patterns are the same as depicted in Figure 2. For example, MS2 analysis of a selected d_0-intralinked cytochrome C peptide (m/z 621.3203^{3+}) yielded a single dominant fragment ion (α_{A+T}, m/z 615.32^{3+}) (Supplementary Figure 4A in the Supporting Information). Similarly, its corresponding d_{10}-labeled cross-linked peptide (m/z 624.6746^{3+}) also generated the same type of MS2 fragment ion (α_{A*+T*}, m/z 618.67^{3+}) (Supplementary Figure 4B in the Supporting Information), corroborating well with the predicted fragmentation unique to intralinked peptides. As for dead-end modified peptides, they are expected to generate two distinct MS2 fragment ions (Figure 2C). Such characteristic fragmentation was observed for DMDSSO dead-end peptides as demonstrated by MS2 spectra of a selected pair of d_0- (m/z 546.6116^{3+}) and d_{10}-dead-end (m/z 549.9661^{3+}) modified cytochrome C peptides, in which a pair of fragment ions α_A/α_T and α_{A*}/α_{T*} were detected, respectively (Supplementary Figure 4C,D in the Supporting Information). Taken together, the results further demonstrate that DMDSSO cross-linked peptides indeed produce specific MS2 fragmentation patterns that are predictable and reliable for the determination of their cross-link types, which allows subsequent MS3 analysis of unique MS2 fragments for unambiguous identification of cross-linked peptides. These features are consistent with those of DSSO cross-linked peptides,[12] further attesting the power and general applicability of sulfoxide-containing MS-cleavable cross-linkers in XL-MS studies.

Detection of d_0/d_{10}-DMDSSO Cross-Linked Peptide Pairs. In order to further facilitate the detection and identification of cross-linked peptides, we next mixed the digests of d_0- and d_{10}-DMDSSO cross-linked cytochrome C at 1:1 for LC−MS^n analysis. When analyzed together, d_0- and d_{10}-DMDSSO cross-linked peptides should be detected as isotopic doublets in MS1 with defined mass differences ($\Delta(d_{10} - d_0) = n \times 10$ Da) depending on the number of cross-links (n) in a given cross-linked peptide. In contrast, noncross-linked peptides should be detected only as singlets. This provides additional confirmation to cross-linked peptides identified by MS^n. Not surprisingly, all of the cross-linked peptides identified display the expected isotopic doublets with a 10 Da mass difference, indicative of cross-linked peptides containing one cross-link. This can be exemplified by respective peptide pairs detected in MS1 for the three representative DMDSSO cross-linked cytochrome C peptides described above (Supplementary Figure 5A−C in the Supporting Information). Similar isotopic signatures can also be detected in MS2 if d_0- and d_{10}-DMDSSO cross-linked peptide pairs can be selected for CID analysis at the same time or their respective MS2 spectra can be merged together. The resulting MS2 isotopic doublets would have a mass difference of 5 Da because MS2 fragments of DMDSSO cross-linked peptides only carry five residual isotopic labels (Figure 2E). Similarly, the unique MS2 isotopic signature can be used to facilitate the identification and quantitation of cross-linked peptides; however, special software is needed for effective data analysis. Although quantitation at the MS1 level is often preferred due to sensitivity, the detection of multiple MS2 isotopic pairs can provide better statistics in quantitation.

In total, 33 unique interlinked cytochrome C peptides were identified, and 19 of them were identified based on MS^n analysis of both d_0- and d_{10}-DMDSSO-cross-linked peptides (Supplementary Table 1 and Supplementary Figure 7 in the Supporting Information). The remaining 14 interlinks were determined only by MS^n sequencing of either d_0- or d_{10}-DMDSSO-cross-linked peptides. Importantly, the detection of d_0/d_{10} peptide doublets confirms the existence of the same cross-linked peptides formed by both cross-linkers even if only one of the d_0 and d_{10} forms is analyzed by MS^n. These results demonstrate that isotope-coded cross-linkers further improve the identification of cross-linked peptides. The 33 identified interlinked peptides represent 26 unique K−K linkages in cytochrome C, C_α−C_α distances of which range from 5.3 to 26.2 Å based on the reported monomer crystal structure (PDB 2B4Z). These distances are well within the expected range of our cross-linkers (≤26 Å). However, it is noted that some of the identified cross-linked peptides more likely represent interprotein interlinks and may have larger spatial distances as the dimerized cytochrome C was analyzed here. For example, the peptide [^{39}KTGQAPGFSYTDANK^{53}] was determined to be interlinked with another peptide [^{39}KTGQAPGFSYTDANKNK^{55}] through K39 to K53 linkage (Supplementary Table 1 in the Supporting Information). Interestingly, these two interlinked peptides share a significant overlap in sequences, strongly suggesting an interprotein interlink between a cytochrome C dimer.

Previously, we have identified 14 interlinked cytochrome C peptides using DSSO cross-linking,[12] 8 of which have also been determined by d_0/d_{10}-DMDSSO cross-linking in this study. Although each study has resulted in several unique cross-linked peptides, it is noted that many of the identified interlinked lysines are located in very close proximity within the sequence of cytochrome C. For example, while K53 to K79 (11.6 Å) linkage was found with DSSO cross-linking, K55 to K73 (11.6 Å) was only identified by DMDSSO cross-linking. Because of the similar calculated distances within these cross-linked lysine residues and the closeness of K53 to K55 as well as the proximity between K73 and K79, we consider their interaction regions are similar. Therefore, we clustered 17 lysines of cytochrome C into 8 "groups", in which adjacent lysines are within a string of 6 amino acids (Supplementary Figure 6 in the Supporting Information). In comparison to the interlinks identified within these lysine groups, this work has mapped all of the interlinked regions determined by DSSO cross-linking.[12]

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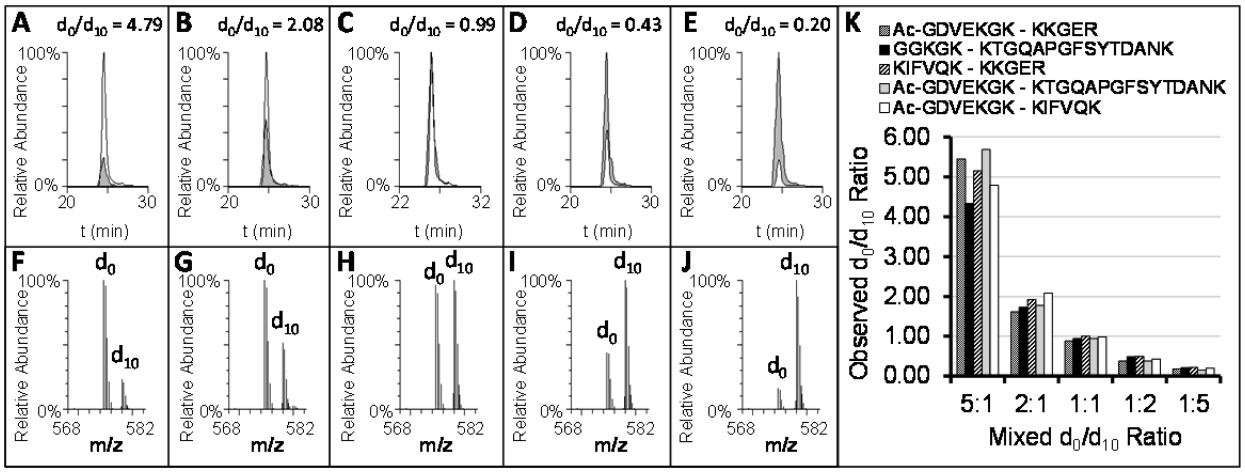

Figure 6. Quantitative analysis of d_0/d_{10}-DMDSSO cross-linked cytochrome C peptides. (A–E) Extracted ion chromatogram (XIC) overlays for a selected d_0- and d_{10}-interlinked peptide pairs (m/z 574.64^{3+}/578.00^{3+}) when the digests of d_0- and d_{10}-DMDSSO cross-linked peptides were mixed in the ratio of 5:1, 2:1, 1:1, 1:2, and 1:5, respectively. The shaded areas represent the XICs of d_{10}-interlinked peptides. (F–J) Representative MS spectra obtained for each corresponding overlaid XICs shown in parts A–E. (K) Observed ratios of d_0/d_{10} ion signals for the 5 selected interlinked cytochrome C peptides. Their sequences are shown in the inset.

In addition, 5 additional ones derived from 10 DMDSSO cross-linked peptides were identified, representing the most extensive cross-linking data on cytochrome C. These results are more likely attributed to combined improvements in sample preparation, data acquisition, and usage of two isotope-coded cross-linkers separately and simultaneously.

In contrast to previous analysis of the entire cross-linked cytochrome C mixture in which the monomeric form was the most abundant species,[12] here we have only focused on analyzing gel-separated cytochrome C dimer bands to decrease sample complexity. Because most noncross-linked tryptic peptides, dead-end modified, and intralinked cross-linked peptides have lower charges than interlinked peptides, we also modified data acquisition control to select only higher charged ions (i.e., 3+ and up) for MS^n analysis. This allows the instrument to carry out data-dependent MS^n acquisition toward potentially interlinked peptides. Importantly, the concurrent usage of the isotope-labeled cross-linkers permits easy detection of cross-linked peptides and increases the identification of interlinked peptides overall. Taken together, our current workflow has proven its effectiveness in identifying cross-linked peptides.

Quantitation of d_0/d_{10} Labeled Cross-Linked Peptides. In addition to assisting MS detection and identification of cross-linked peptides, we expect that isotope-coded cross-linkers can be used to study protein structural changes by quantifying relative abundances of nonlabeled and labeled cross-linked peptides. In order to do this, protein cross-linking has to be carried out using nonlabeled and labeled cross-linkers separately assuming their cross-linking efficiencies are similar. In our experiments, we have shown that cross-linking efficiency of cytochrome C by d_0- or d_{10}-DMDSSO is very similar (Supplementary Figure 3 in the Supporting Information) and equal mixing of the peptide digests of d_0- and d_{10} cross-linked cytochrome C led to DMDSSO cross-linked peptide doublets with relative ratios of 1 (Supplementary Figure 5A–C in the Supporting Information). Since previous XL-MS studies often cross-link proteins using a 1:1 mixture of nonlabeled and labeled cross-linkers to generate isotopic pairs, we wanted to compare whether equivalent results can be achieved using different sample preparation approaches. Therefore, we first cross-linked cytochrome C with a 1:1 mixture of d_0- and d_{10}-DMDSSO and then analyzed the resulting cross-linked peptide digests by MS (Supplementary Figure 5D–F in the Supporting Information). In comparison, corresponding d_0- and d_{10}-labeled cross-linked peptides display similar relative abundance ratios regardless of whether mixing was done before or after protein cross-linking. These results suggest that our isotopically labeled cross-linkers are indeed comparable in their ability to cross-link proteins and that the resulting d_0- and d_{10}-labeled cross-linked products behave similarly during sample preparation and MS^n analysis, thus providing flexibility of using these isotope-coded reagents in XL-MS studies.

To further explore the capability of d_0- and d_{10}-DMDSSO for quantitative analysis, we cross-linked cytochrome C with d_0- and d_{10}-DMDSSO separately, carried out their in-gel digestion, and then mixed the resulting peptide digests in five chosen d_0/d_{10} ratios (i.e., 5:1, 2:1, 1:1; 1:2, 1:5) prior to LC–MS^n analysis. In order to determine the relative abundance ratios, we manually obtained extracted ion chromatograms (XIC) for five selected d_0- and d_{10}-labeled cross-linked peptide pairs for each sample. As an example, Figure 6A–E illustrates the overlay of XICs for a representative d_0- and d_{10}-DMDSSO interlinked peptide pair in five samples mixed with different ratios, and the corresponding MS spectra are shown in Figure 6F–J. On the basis of the calculated area under XICs, its relative abundance (d_0/d_{10}) was determined as 4.79, 2.08, 0.99, 0.43, and 0.20, respectively, which correlates well with the initial sample mixing. In addition, the ratios obtained from peptide peak intensity are similar to those obtained using XIC, indicating that both approaches are sufficient for calculating relative abundance of cross-linked peptides. As shown in Figure 6K, the average ratios of the five selected cross-linked peptides for each sample corroborate very well with initial sample mixing. Collectively, these results have demonstrated the capability of quantifying cross-linked peptides using isotope-coded DMDSSO reagents.

■ CONCLUSIONS

We report here the development and characterization of new DSSO derivatives, a pair of isotope-coded MS-cleavable cross-

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linkers: d_0- and d_{10}-DMDSSO. DMDSSO cross-linked peptides preserve the same characteristic MS2 fragmentation patterns distinctive to cross-link types as DSSO cross-linked peptides, thus facilitating the detection and identification of cross-linked peptides. In combination with MS3 sequencing, MS1 mass mapping and isotopic profiling, the nature and identity of cross-linked peptides can be determined readily and unambiguously. This work further demonstrates the robustness of sulfoxide-containing MS-cleavable cross-linkers in XL-MS studies and provides a strong basis for us to further develop new DSSO derivatives and fully define protein−protein interactions. In addition, we have shown the flexibility in using d_0- and d_{10}-DMDSSO for quantitative analysis of cross-linked peptides, thus establishing a solid foundation for our future studies toward the understanding of structural dynamics of protein complexes.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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