Developing an Acidic Residue Reactive and Sulfoxide-Containing MS-Cleavable Homobifunctional Cross-Linker for Probing Protein–Protein Interactions

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Supporting Information

ABSTRACT: Cross-linking mass spectrometry (XL-MS) has become a powerful strategy for defining protein–protein interactions and elucidating architectures of large protein complexes. However, one of the inherent challenges in MS analysis of cross-linked peptides is their unambiguous identification. To facilitate this process, we have previously developed a series of amine-reactive sulfoxide-containing MS-cleavable cross-linkers. These MS-cleavable reagents have allowed us to establish a common robust XL-MS workflow that enables fast and accurate identification of cross-linked peptides using multistage tandem mass spectrometry (MS²). Although amine-reactive reagents targeting lysine residues have been successful, it remains difficult to characterize protein interaction interfaces with little or no lysine residues. To expand the coverage of protein interaction regions, we present here the development of a new acidic residue-targeting sulfoxide-containing MS-cleavable homobifunctional cross-linker, dihydrazide sulfoxide (DHSO). We demonstrate that DHSO cross-linked peptides display the same predictable and characteristic fragmentation pattern during collision induced dissociation as amine-reactive sulfoxide-containing MS-cleavable cross-linked peptides, thus permitting their simplified analysis and unambiguous identification by MS². Additionally, we show that DHSO can provide complementary data to amine-reactive reagents. Collectively, this work not only enlarges the range of the application of XL-MS approaches but also further demonstrates the robustness and applicability of sulfoxide-based MS-cleavability in conjunction with various cross-linking chemistries.
dimethyl disuccinimidyl sulfoxide (DMDSSO), azide-tagged acid-cleavable disuccinimidyl bissulfoxide (Azide-A-DSBSO) (Figure 1A–C). These MS-cleavable reagents contain symmetric MS-labile C–S bonds (adjacent to the sulfoxide group) that can be selectively and preferentially fragmented prior to peptide backbone cleavage during collision induced dissociation (CID). Such fragmentation is robust and predictable, occurring independently of cross-linking types, peptide charges, and sequences. Ultimately, this unique feature enables simplified and unambiguous identification of cross-linked peptides by MS analysis and conventional database searching tools. Our newly developed sulfoxide-containing, MS-cleavable cross-linkers have been successfully applied not only to define protein–protein interactions and elucidate structures of protein complexes in vitro and in vivo but also to quantify structural dynamics of protein complexes.

In current XL-MS studies, amine-reactive reagents targeting lysine residues are the most widely used compounds for successful elucidation of protein structures. This is due to their effective and specific cross-linking chemistry as well as frequent occurrence of lysine residues in protein sequences, especially at surface-exposed regions of protein structures. However, it remains challenging to characterize protein interaction interfaces with little to no lysine residues. Therefore, there is a necessity for the development of additional cross-linking chemistries in order to increase the coverage of structural information obtainable from XL-MS experiments, particularly in systems where protein interacting regions are refractive to amine-specific cross-linking. Although several types of cross-linkers targeting other amino acids (e.g., sulhydryl-reactive and nonselective photoreactive) reagents are commercially available, their applications in studying protein–protein interactions thus far are very limited. For instance, sulphydryl-reactive cross-linking reagents with specific chemistries targeting cysteine residues have not been widely adopted, most likely owing to the relatively low occurrence of cysteine residues and their participation in forming disulfide bonds in protein structures. In comparison, although photochemical cross-linking reagents can improve the coverage of protein interaction contacts by reacting with any amino acids nonspecifically, the resulting cross-linked products are often unpredictable, making their unambiguous MS identification even more difficult. In addition, nonspecific cross-linking has a higher chance of introducing more nonspecific interactions. Therefore, a specific cross-linking chemistry targeting other amino acid residues abundant at protein interaction sites would be ideal for complementing lysine targeting cross-linkers. While hydrophobic amino acid residues often constitute the cores, charged hydrophilic residues such as lysine, arginine, aspartic acid (Asp), and glutamic acid (Glu) often occupy surface-exposed regions of protein complexes, making them ideal targets for mapping protein interactions. According to a recent SwissProt database release, aspartic and glutamic acids comprise roughly 12.2% of all amino acid residues, compared to the 5.8% of lysines. Therefore, acidic residues (i.e., aspartic and glutamic acids) represent high potential targets for cross-linking studies due to their abundance and prevalence at interaction interfaces.

A recent study by Leitner et al. has demonstrated the feasibility of acidic residue-specific cross-linking chemistry to study protein interactions using noncleavable homobifunctional dihydrazide cross-linkers in conjunction with the coupling reagent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). This methodology is an improvement on the acidic residue cross-linking chemistry involving the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) that requires the cross-linking reaction to occur at a pH of 5.5. In comparison, DMTMM coupling with dihydrazide cross-linkers is compatible with proteins at neutral pH (7.0–7.5) and therefore better suited for studying the structures of proteins and protein complexes under physiological conditions. However, this cross-linking strategy remains susceptible to the challenges associated with traditional cross-linking reagents in unambiguously identifying cross-linked peptides and their linkage sites. Because of the increased prevalence of Asp and Glu in protein sequences, the accurate and unambiguous identification of peptides containing noncleavable dihydrazide cross-linked acidic residues would be intrinsically more complicated than the identification of lysine cross-linked peptides. To simplify MS analysis and facilitate the identification of acidic residue cross-linked peptides, we have developed a sulfoxide-containing MS-cleavable acidic residue-specific homobifunctional cross-linking reagent, dihydrazide sulfoxide (DHSO, a.k.a. 3,3′-sulfynyl[(propenylhydrazide)]. This reagent adopts the same MS-labile sulfoxide chemistry as our previously developed amine-reactive MS-cleavable cross-linkers (i.e., DSSO, DMDSSO, and Azide-A-DSBSO), thus

Figure 1. Sulfoxide-containing MS-cleavable cross-linkers: (A) DSSO, (B) DMDSSO, and (C) Azide-A-DSBSO. (D) Synthesis scheme of MS-cleavable cross-linker DHSO. (E) Characteristic MS fragmentation of DHSO interlinked heterodimer αβ.
enabling robust and unambiguous identification of cross-linked peptides via the same XL-MS\(^n\) workflow.\(^{14-16}\) DHSO represents a novel class and the first generation of acidic residue-targeting cross-linking reagents with MS-cleavability. We expect that DHSO-based XL-MS strategies will become an invaluable tool in providing a complementary subset of cross-linking data toward a comprehensive structural elucidation of protein complexes by XL-MS.

### EXPERIMENTAL PROCEDURES

#### Materials and Reagents

General chemicals were purchased from Fisher Scientific or VWR International. Bovine serum albumin (\(\geq 96\%\) purity), myoglobin from equine heart (\(\geq 90\%\) purity), and DMTMM (\(\geq 96\%\) purity) were purchased from Sigma-Aldrich. Ac-SR8 peptide (Ac-SAKAYEHR, 98.22\% purity) was custom ordered from Biomatik (Wilmington, DE).

DHSO Cross-Linking of Synthetic Peptides. DHSO was synthesized as described (Figure 1D and Supplemental Methods). Synthetic peptide Ac-SR8 was dissolved in DMSO to 1 mM and cross-linked with DHSO in a 1:1 molar ratio of peptide to cross-linker in the presence of 1 equiv of diisopropylethylamine and DMTMM. The resulting samples were diluted to 10 pmol/\(\mu\)L in 3% ACN/2% formic acid prior to MS\(^n\) analysis.

DHSO Cross-Linking of Equine Myoglobin and Bovine Serum Albumin. A volume of 50 \(\mu\)L of 50 \(\mu\)M BSA or 200 \(\mu\)M myoglobin in PBS buffer (pH 7.4) was reacted with DHSO in molar ratios of 1:5, 1:10, 1:20, and 1:30. The cross-linking reaction was initiated by adding equivalent concentrations of DHSO and DMTMM to protein solutions, reacted for 1 h at room temperature.

Digestion of DHSO Cross-Linked Proteins. Cross-linked protein samples were subjected to either SDS-PAGE followed by in-gel digestion or directly digested in solution prior to MS analysis (Supplemental Methods).

**Liquid Chromatography–Multistage Tandem Mass Spectrometry (LC–MS\(^n\)) Analysis.** DHSO cross-linked peptides were analyzed by LC–MS\(^n\) utilizing an Easy-nLC 1000 (Thermo Fisher, San Jose, CA) coupled online to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher, San Jose, CA). Multi-stage tandem mass spectrometry (Supplemental Methods). A+T fragmentation of DHSO cross-linked peptides will behave similarly during MS\(^n\) analysis even though their residue-targeting functional groups are different.

To elaborate this process, Figure 1E and Figure S-1B,C illustrate the predicted MS\(^2\) fragmentation patterns of DHSO interlinked, intralinked, and dead-end modified peptides, respectively. For a DHSO interlinked peptide \(\alpha/\beta\), the cleavage of one of the two symmetric C–S bonds would result in one of the two predicted peptide fragment pairs (i.e., \(\alpha_{\text{A}}/\beta_{\text{T}}\) or \(\alpha_{\text{T}}/\beta_{\text{A}}\)). The resulting \(\alpha\) and \(\beta\) peptide fragments are modified by complementary cross-linker remnant moieties, i.e., alkene (A) or sulfenic acid (S). However, the sulfenic acid moiety can undergo dehydration to become a more stable unsaturated thiol moiety (i.e., \(T\)) (Figure S-1D). This conversion has been commonly observed in amine-reactive, sulfoxide-containing MS-cleavable cross-linked peptides, thus leading to the detection of \(\alpha_{\text{T}}/\beta_{\text{T}}\) and \(\alpha_{\text{A}}/\beta_{\text{A}}\) pairs instead as the four dominant MS\(^2\) fragment ions.\(^{14-16}\) Therefore, these two MS\(^2\) fragment pairs (i.e., \(\alpha_{\text{T}}/\beta_{\text{T}}\) and \(\alpha_{\text{A}}/\beta_{\text{A}}\)) are expected for a DHSO cross-linked heterodimer as well (Figure 1E), which can then be subjected to MS\(^3\) analysis for unambiguous identification of cross-linked peptide sequences and cross-linking sites. For a DHSO intralinked peptide \(\alpha_{\text{inter}}\), in which proximal D or E amino acid residues are cross-linked within the same peptide, one peptide fragment (i.e., \(\alpha_{\text{A1}}\)) is expected in MS\(^3\) analysis (Figure S-1B). In reality, this particular ion would
represent two populations of ion species that have identical peptide sequences and $m/z$ values but transposed DHSO remnant-modified acidic residues. Lastly, a DHSO dead-end modified peptide $\alpha_{DN}$ would potentially fragment into two ion species during MS$^2$ analysis. Depending on the position of the cleaved C=S bond, $\alpha_A$ or $\alpha_T$ fragments would be observed, resulting in a pair of daughter ions detected during MS$^2$ (Figure S-1C). The distinct MS$^2$ fragmentation patterns of sulfide-containing MS-cleavable cross-linked peptides result in predictable mass relationships between parent ions and their respective fragments. These mass relationships are utilized as an additional verification of cross-linked peptide identification at Figure 2. MS$^n$ analysis of DHSO interlinked Ac-SR8 homodimer. MS$^3$ spectra of DHSO interlinked Ac-SR8 at two different charge states: (A) $[\alpha-\alpha]^{4+}$ ($m/z$ 548.76234$^+$) and (B) $[\alpha\alpha]^{5+}$ ($m/z$ 459.21175$^+$). MS$^3$ spectra of MS$^2$ fragment ions detected in part A: (C) $\alpha_A$ ($m/z$ 536.27$^+$) and (D) $\beta_T$ ($m/z$ 552.26$^+$).

Figure 3. MS$^n$ analysis of a representative DHSO interlinked myoglobin peptide. (A) MS spectrum of the interlinked peptide $\alpha-\beta$ ($m/z$ 517.27035$^+$). (B) MS$^2$ spectrum of the interlinked peptide detected in part A. MS$^3$ spectra of MS$^2$ fragment ions: (C) $\alpha_A$ ($m/z$ 429.74$^+$) and (D) $\beta_T$ ($m/z$ 569.63$^+$).
the MS² level. Along with mass fingerprinting by MS¹ and peptide sequencing by MS³, three lines of evidence can be obtained and integrated to accurately identify DHSO cross-linked peptides using the identical MS⁴ workflow that has been developed for the analysis of DSSO, DMDSSO, and DSBSO cross-linked peptides.¹⁴⁻¹⁶

Characterization of DHSO Cross-Linked Model Peptides by MS⁵ Analysis. Despite the similarities in spacer arm structure to DSSO, it is necessary to verify whether DHSO cross-linked peptides indeed fragment as described above during MS⁵ analysis (Figure 1E). Initial characterization of DHSO was performed on a synthetic peptide containing a single acidic residue, Ac-SR8 (Ac-SAKAYEHR). Interlinked Ac-SR8 homodimer was detected as quadruply charged (m/z 548.76234+) and quintuply charged (m/z 439.21175+) ion species, respectively. Since the two peptide sequences of interlinked homodimer are the same, only one pair of MS⁵ fragment ions (i.e., α₁/α₂) would be expected. Indeed, MS⁵ analysis of the quadruply charged parent ion produced a pair of dominant fragment ions α₁/α₂ (m/z 536.272+/552.262+), demonstrating effective physical separation of the two cross-linked peptides as expected (Figure 2A). Similarly, MS⁵ analysis of the quintuply charged parent ion (m/z 439.21175+) yielded a single pair of dominant fragment ions α₁/α₂ (m/z 357.853+/357.853+) as well (Figure 2B), demonstrating the characteristic fragmentation independent of peptide charges as expected. Subsequent MS⁶ analysis of the α₅ (m/z 536.272+) fragment ion (Figure 2C) resulted in a series of y and b ions that unambiguously confirmed the peptide sequence as Ac-SAKAYE₆HR, in which the glutamic acid was modified with a DHSO alkene (A) moiety. Similarly, MS⁶ analysis of the α₇ fragment (m/z 552.262+) determined its identity as Ac-SAKAYE₆HR, in which the glutamic acid was modified with a DHSO unsaturated thiol (T) moiety (Figure 2D). Therefore, the cross-linked peptide was identified as [Ac-SAKAYE₆HR] interlinked to [Ac-SAKAYE₆HR] through E₆ in both peptides. This result indicates that DHSO interlinked peptides indeed display the same characteristic MS⁶ fragmentation as sulfoxide-containing lysine interlinked peptides and can be analyzed using the same data analysis workflow as previously described.¹⁴⁻¹⁶

Characterization of DHSO Cross-Linked Model Proteins by MS⁷ Analysis. To evaluate the capability of DHSO for protein cross-linking in vitro, we used equine myoglobin and bovine serum albumin (BSA) as our model proteins. These two proteins contain above-average acidic residue content (16.3% and 13.6%, respectively), making them well suited for evaluating DHSO cross-linking. In addition, BSA was employed previously for acidic residue cross-linking by noncleavable dihydrazides.²¹ To identify DHSO cross-linked peptides in myoglobin and BSA, we have performed in-gel digestion of gel-separated DHSO cross-linked proteins or in solution digestion of DHSO cross-linked proteins followed by peptide SEC as illustrated (Figure S-2). The resulting peptides were subjected to LC–MS⁸ analysis. Figure 3A displays the MS⁷ spectrum of an exemplary interlinked peptide (αβ) (m/z 517.27035+) identified from myoglobin. Its MS⁸ analysis resulted in the detection of two peptide fragment pairs, i.e., α₅/β₁ (m/z 429.743+/569.635+) and α₅/β₆ (m/z 445.723+/559.645+) (Figure 3B), characteristic for DHSO interlinked heterodimers. MS⁸ analysis of α₅ (m/z 429.743+) (Figure 3C) determined its sequence as ASE₅DLKK, in which the glutamic acid residue at
the third position from the N-terminus was modified with an alkene moiety. MS² analysis of $\beta_2$ ($m/z$ 692.8475) identified its sequence as VEAD₁-IAGHGQEVLIR, with the aspartic acid residue at the fourth position from the N-terminus carrying an unsaturated thiol moiety (Figure 3D). Collectively, the interlinked peptide was unambiguously identified as $[^{18}V_{EAD₁-IAGHGQEVLIR}]^{58}S_{EAD₃DLKK}$ cross-linked to $^{58}S_{EAD₃DLKK}$, describing an interlink formed between D21 and E60 of equine myoglobin.

Figure S-3 displays MS³ analysis of a representative DHSO interlinked BSA peptide, which was measured as a quadruply charged ion ($m/z$ 692.8475) in MS¹ (Figure S-3A). Its MS² spectrum revealed two pairs of complementary MS² fragment ions, i.e., $\alpha_\beta/\beta_\alpha$ and $\alpha_\gamma/\beta_\delta$ (Figure S-3B), further demonstrating the robust fragmentation expected of DHSO interlinked peptides. Together with MS³ sequencing of MS² fragments $\alpha_\delta$ ($m/z$ 616.3211) and $\beta_\gamma$ ($m/z$ 760.3623) (Figure S-3C,D), this DHSO interlinked peptide was unambiguously identified as $[^{16}L_{YNE₆₁LTEL_FK₇₅}]^{89}L{HTLFGDE₄₁LC{K}^{100}}$, in which residue E69 cross-linked to residue E97 in BSA.

In addition to interlinked peptides, intralinked peptides were also observed as a result of DHSO cross-linking of our model proteins. For example, MS² fragmentation of an intralinked myoglobin peptide (Figure S-4) produced a single fragment ion peak $\alpha_{A_{XT}}$ ($m/z$ 514.0244) 18 Da less than its parent ion, consistent with the expected fragmentation pattern described in Figure S-1B following dehydration of the sulfinic acid moiety to an unsaturated thiol moiety. Analysis of the $\alpha_{A_{XT}}$ ion in subsequent MS³ analysis (Figure S-4C) yielded a series of b and y ions permitting the unambiguous identification of two peptides sharing identical sequences but transposed alkene and unsaturated thiol moieties: $^{105}Y_{LE₇₁F_{ISDF₁₃₁AIHVLHSK}^{11₉}}$ and $^{0₉}Y_{LE₇₁F_{ISDF₁₃₁AIHVLHSK}^{11₉}}$, indicating an intralink between residues E106 and D110.

MS² fragmentation of a myoglobin dead-end modified peptide ($m/z$ 604.3095) resulted in the detection of a single pair of fragment ions $\alpha_\delta/\alpha_\epsilon$ ($m/z$ 559.3006/569.9604) (Figure S-5), consistent with the expected fragmentation pattern described in Figure S-1C. These fragment ions were then identified by MS³ analysis as $[^{18}V_{E₄₁ADIAHGQEV_LIR}]^{58}S_{EAD₃DLKK}$ and $^{18}V_{E₄₁ADIAHGQEV_LIR}$, respectively, representing a dead-end cross-link located on E19 of myoglobin (Figure S-5C,D).

In total, LC–MS³ analysis of DHSO cross-linked myoglobin identified 33 unique interlinked peptides, representing 32 unique DIE-DIE linkages (Table S-1). Similarly, 62 unique DHSO interlinked BSA peptides were identified, describing 69 unique DIE-DIE linkages (Table S-2). Collectively, the results presented thus far indicate that DHSO can effectively cross-link acidic residue containing peptides and proteins in the presence of DMTMM at neutral pH. More importantly, our results have demonstrated that DHSO cross-linked peptides indeed exhibit the same characteristic MS³ fragmentation patterns as expected to allow their facile and accurate identification.

**DHSO Cross-Linking Maps of Myoglobin and BSA.** In order to assess the efficacy and sequence coverage of DHSO cross-linking on our model proteins, we generated cross-linking maps of myoglobin and BSA based on their identified DHSO interlinked peptides. The secondary structures of equine myoglobin comprise of eight $\alpha$-helices and one short $\beta_{10}$ helix (PDB 1DWR) (Figure 4A). The globular nature of myoglobin suggests that many of the helices are in close proximity to one another in a three-dimensional space. The DHSO cross-link map of myoglobin based on the 33 unique DI-E-DIE linkages is illustrated in Figure 4B, describing numerous intra- and inter-secondary structure interactions (i.e., $\alpha_1$-$\alpha_5$, $\alpha_1$-$\alpha_8$, $\alpha_2$-$\alpha_4$, $\alpha_3$-$\alpha_4$, $\alpha_4$-$\alpha_5$, $\alpha_4$-$\alpha_8$, $\alpha_6$-$\alpha_8$, $\alpha_7$-$\alpha_8$, and $\alpha_8$-$\alpha_8$). To further evaluate the identified cross-links, we mapped the cross-linked residues onto the crystal structure of myoglobin and calculated the distances between their alpha carbons (Ca-Ca distances) (Figure 4D,F). Considering the spacer length of DHSO (12.4 Å) and the distances contributed by DIE side chains (3.8 Å/4.9 Å, respectively), as well as backbone flexibility and structural dynamics, the upper limit for the Ca-Ca distances between DHSO cross-linked acidic residues is estimated to be ~30 Å. Therefore, we have set the distance threshold for cross-linkable DIE residues as 30 Å. A total of 27 of the 32 myoglobin DHSO cross-links were mapped in the structure, with 26 having Ca-Ca distances <30 Å and one link slightly over the maximum distance at 31.1 Å. The remaining 5 linkages were not mapped on to the structure because they were identified as sites of oligomerization, in which identical residues or peptide sequences were cross-linked together.

Similarly, a DHSO cross-link map of BSA was generated based on the 69 unique DIE-DIE linkages (Figure 5A). When mapped to a previously published BSA crystal structure (PDB 4FSS), 64 out of 69 BSA linkages (93%) were calculated to have Ca-Ca distances below 30 Å (Figure 5A,C). Structural flexibility and/or oligomerization of BSA likely contribute to the other five identified linkages found to be >30 Å. As shown in Figure 5A, DHSO interlinks were distributed throughout the primary sequence of BSA, with regions of dense cross-link clusters identified in regions with higher $\alpha$-helix density. This even distribution is likely due to the dispersion of aspartic acid and glutamic acid residues throughout the protein. Collectively, our results suggest that DHSO cross-linking yields cross-links within expected distance constraints useful for structural elucidation for computational modeling in the same way as lysine cross-linked data.

**Comparison of MS-Cleavable and Noncleavable Acidic Residue Cross-Linking.** Previously, two noncleavable acidic residue cross-linkers, i.e., adipic acid dihydrazide (ADH) and pimetic acid dihydrazide (PDH), were used for probing the structure of bovine serum albumin, which resulted in the identification of 27 and 35 unique acidic residue linkages,

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A comparison of the linkage maps generated for DHSO, ADH, and PDH cross-linking of BSA (Figure S A–C) revealed a high degree of similarity in proximally cross-linked regions. Apart from covering interaction regions cross-linked by ADH and PDH, DHSO cross-linking resulted in 34 additional unique DIE-DIE linkages. These unique DHSO cross-links are generally clustered in regions of particularly high acidic residue density, such as the regions between D25 and D97, E250 and E344, and D405 to E494 (Figure S A–C). Limitations in bioinformatics software for analyzing noncleavable cross-linked peptides have been previously noted, which made the accurate identification of acidic residue cross-linked peptides considerably more challenging due to their higher frequency and corresponding increase in search space. In contrast, CID induced cleavage of DHSO cross-linked peptides during MS2 and corresponding increase in search space. In contrast, CID induced cleavage of DHSO cross-linked peptides during MS2 cleavage resulted in much more similar cross-linking profiles, meaning that similar interactions within BSA were identified (Figure S A,D). This is most likely owing to the fact that BSA has more evenly dispersed distribution of lysine, aspartic acid, and glutamic acid residues throughout the protein sequence. Thus, combined usage of DHSO and DSSO can strengthen the validity of the cross-links identified by any of the two reagents individually. More importantly, this will generate complementary structural information to facilitate a more comprehensive understanding of protein structures.

Comparison of DHSO and DSSO Cross-Linking. To assess the complementarity between acidic residue and primary amine cross-linking data, we examined the similarities and differences between DHSO and DSSO cross-linking of our selected model proteins. To this end, we also carried out LC– MS² analyses of DSSO cross-linked myoglobin and BSA, respectively. As summarized in Tables S 3 and S 4, 19 unique DSSO interlinked myoglobin peptides and 33 unique DSSO interlinked BSA peptides were identified. These linkages were then mapped onto their corresponding protein linear sequences (Figures 4C and 5D) and crystal structures (Figure 4E and Figure S 6B). As a result, all of myoglobin DSSO cross-links (Figure 4F) and 94% of BSA DSSO cross-links corresponded to Ca-Ca distances ≤ 30 Å (Figure S 6C). The two BSA cross-links that are outside the distance range may be a result of unexpected structural flexibility.

In the case of myoglobin, DSSO cross-linking identified several proximal helicase regions, such as α4-α5, α4-α7, α5-α8, α6-α8, and α5-3.10. In comparison, there is limited overlap between DHSO and DSSO cross-link maps except in regions containing α4-α5 and α6-α8 (Figure 4B,C), indicating that DHSO and DSSO cross-linking mapped different parts of interactions within myoglobin. The identified helicase interacting regions unique to DHSO or DSSO cross-linking correspond well with the number of cross-linkable residues and specific reactive chemistries. This is due to the fact that lysine and acidic residues are distributed unevenly across the myoglobin sequence. For example, the N-terminal region of myoglobin (residues 1–41) spanning helices α1 through α3 contains only one lysine but four glutamic acids and two aspartic acid residues. Therefore, profiling the interactions of the N-terminus within itself and with other parts of the protein will be difficult with amine-reactive cross-linking reagent such as DSSO. In contrast, acidic residue reactive cross-linker DHSO would be better suited for this purpose. Indeed, while DHSO was not able to cover this region as expected, DHSO cross-linking enabled the identification of 11 interlinked peptides describing multiple interactions between the N-terminus and other parts of the protein (i.e., α1-α5, α1-α8, α2-α4, α3-α4, and α3-α8). While DHSO provided exclusive data from the lysine scarce N-terminus, the lysine-rich 34 helix and many of the loop regions between the helical structures were better analyzed by DSSO due to the higher abundance of lysine residues in these regions. Together, these results demonstrate that acidic residue cross-linking can provide complementary structural information to that obtained using amine-reactive cross-linkers.

CONCLUSION

Here we report the development and characterization of a new acidic residue-targeting, sulfoxide-containing MS-cleavable cross-linker, dihydrazide sulfoxide (DHSO), which is a new derivative of our previously developed amine-reactive MS-cleavable reagent, DSSO. Our analyses here have proven that DHSO cross-linked peptides possess the same characteristics distinctive to peptides cross-linked by sulfoxide-containing amine-reactive cross-linkers, thus permitting their fast and accurate identification by MS² analysis. The unique features of DHSO will significantly facilitate cross-linking studies targeting acidic residues, which has been difficult in the past due to the large number of DIE present in protein sequences and complexity of their resulting cross-linked peptides for MS analysis. Comparison of DHSO and DSSO cross-linking confirms the need of expanding the coverage of protein interactions using cross-linkers targeting different residues, especially when the distribution of specific amino acids is uneven. In summary, this work further demonstrates the robustness and potential of our XL-MS technology based on sulfoxide-containing MS-cleavable cross-linkers and provides a viable analytical platform for the development of new MS-cleavable cross-linker derivatives to further define protein-protein interactions. The development of these new tools will aid in the goal of understanding the structural dynamics of protein complexes at the global scale in the future.

ASSOCIATED CONTENT

Supporting Information

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Supplemental methods, figures, and tables (PDF)

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The authors declare no competing financial interest.

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