Identification of Disulfide-Containing Chemical Cross-Links in Proteins Using MALDI-TOF/TOF-Mass Spectrometry

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Cross-linking can be used to identify spatial relationships between amino acids in proteins or protein complexes. A rapid and sensitive method for identifying the site of protein cross-linking using dithiobis(sulfosuccinimidyl propionate) (DTSSP) is presented and illustrated with experiments using murine cortactin, actin and acyl-CoA thioesterase. A characteristic 66 Da doublet, which arises from the asymmetric fragmentation of the disulfide of DTSSP-modified peptides, is observed in the mass spectra obtained under MALDI-TOF/TOF-MS conditions and allows rapid assignment of cross-links in modified proteins. This doublet is observed not only for linear cross-linked peptides but also in the mass spectra of cyclic cross-linked peptides when simultaneous fragmentation of the disulfide and the peptide backbone occurs. We suggest a likely mechanism for this fragmentation. We use guanidinylation of the cross-linked peptides with O-methyl isourea to extend the coverage of cross-linked peptides observed in this MALDI-MS technique. The methodology we report is robust and amenable to automation, and permits the analysis of native cystines along with those introduced by disulfide-containing cross-linkers.

Chemical modification in solution has been used to gain structural information about proteins for almost a century.[1] Evidence for the involvement of reactive amino acid side chains in diverse biological processes, including enzyme catalysis and protein–protein interactions, has been provided by this approach. Chemical modification using cross-linking reagents allows estimates of the proximity of the cross-linked amino acids, in turn providing information that can inform modeling of the three-dimensional structure of the protein or protein complex. Chemical cross-linking combined with mass spectrometric analysis has been sufficient for the production of moderate resolution models for proteins and protein complexes.[2–5] This complements the information provided by X-ray and NMR techniques and can also yield structural information for proteins or protein complexes that are refractory to these techniques.[5,6]

Our group has established a structural genomics pipeline in which we aim to obtain structural information about genes expressed in macrophages.[7] Only a small subfraction of proteins that are produced as soluble recombinant molecules can be crystallized, or are small enough to be amenable to NMR structure determination. In many cases, good approximations of secondary structural elements, known folds and domains, can be identified, but their position in space relative to each other is less clear. We therefore wished to use the large number of recombinant proteins obtained through our pipeline to gain some additional structural information, a goal which can potentially be achieved using chemical cross-linking.

Identification of cross-linked peptides commonly involves LC−MS analysis of enzymatic or chemical digests of the cross-linked protein (MS = mass spectrometry). The digests are prefractionated on reverse-phase or ion-exchange matrixes, and MS data is collected on the eluting peptidic species. Because the identification of cross-linked peptides cannot always rest on accurate mass determination alone, MS/MS (tandem mass spectrometry) data is frequently collected. Tandem mass spectra of cross-linked peptides are typically complex, and much effort has been put into the analysis of fragmentation patterns of cross-linked species, with automated analysis of MS/MS spectra of

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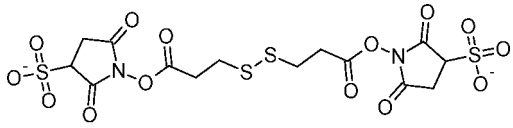
fractionated cross-linked protein digests being the ultimate goal.[8,9] Key variables influencing a successful outcome include the choice of cross-linker; the cross-linking reaction conditions; the choice of protein digestion methods; the method of peptide fractionation; and the mass spectrometric analysis of the protein digests.

The unequivocal identification of cross-linked peptides, which may only be present in low abundance in the complex digests of the proteins, is a technical challenge commonly addressed either through the use of affinity tags[10–12] to reduce the complexity of the digest or through the use of isotope tags.[13,14] For example, Hurst et al.[10] used the biotin-tagged trifunctional cross-linker sulfo-SBED to enrich digests in cross-linked peptides and identified the cross-linked peptides using MALDI-TOF-MS before and after reduction of this cleavable cross-linker (MALDI = matrix assisted laser desorptionionization; TOF = time of flight). On the other hand, Collins et al.[13] used the isotope tag approach, synthesizing an ^{18}O-containing cross-linker and reacting the protein with a mixture of both labeled and unlabeled cross-linker. Another example of the isotope tag approach is the use of ^{18}O-containing water during the digestion of cross-linked proteins; this approach has been used to facilitate the identification of native disulfide cross-linked peptides in complex protein digests.[14] In these isotope tag approaches, cross-linked peptides are identified by the isotope pattern produced by the presence of both labeled and unlabeled peptidic species.

We have adopted the cleavable cross-linker 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) (**I**) as the primary tool in our efforts to obtain spatial constraints for modeling proteins of unknown structure.



(I)

The disulfide bond is readily reduced, thus making identification of DTSSP-modified peptides more straightforward. In addition, following work by Bennett et al.[15] we reasoned that the reduced peptides would provide simpler fragmentation patterns making MS/MS data easier to interpret. During the course of these studies we have found that under MALDI-TOF/TOF-MS conditions the DTSSP-cross-linked species fragment in a distinctive way consistent with the facile asymmetric fragmentation of the disulfide. This distinctive fragmentation makes possible a rapid identification of DTSSP cross-linked peptides. Once identified, these can be validated by reduction and conventional tandem MS sequencing. We demonstrate that this method enables rapid and unambiguous identification of sites of cross-linking in proteins.

EXPERIMENTAL SECTION

Protein Production. The gene encoding murine cortactin (RefSeq NP_031829) was amplified by PCR from a mouse cDNA clone (NCBI AK084249) using a splice overlap strategy that fused a ribosome binding site, start codon and 6xHis-tag-coding region to the amino terminus. The gene was cloned into the Gateway expression vector pDEST15 (Invitrogen) following the manufacturer's instructions. The cortactin protein was expressed in *Escherichia coli* strain BL21 (DE3) pLysS at 30 °C for 24 h in rich autoinduction media (Novagen). The bacteria were lysed using the B-Per (Pierce) cell lysis reagent and purified on TALON metal affinity resin (Clontech) using 50 mM HEPES pH 7.4 containing 300 mM NaCl and 10 mM imidazole as the wash buffer and 50 mM HEPES pH 7.4 containing 300 mM NaCl and 250 mM imidazole as the elution buffer. The eluted protein was further purified by size exclusion chromatography on a Superdex 200 16/60 column (GE Healthcare) using 50 mM HEPES pH 7.4 containing 300 mM NaCl as the mobile phase. The protein was concentrated to ~7 μM using a 10 kDa cutoff spin concentrator (Millipore) and frozen in liquid nitrogen in 50 μL aliquots in thin walled PCR tubes before being stored at −80 °C.

Protein Modification. A solution of murine cortactin (~7 μM) in 20 mM HEPES buffer, pH 7.0 was reacted with DTSSP (2.4 mM) at 25 °C for 12 min. The reaction was stopped by desalting on Sephadex G-25 at 4 °C. The sample was incubated for a further two hours to allow hydrolysis of any bound DTSSP and then concentrated using a Centricon filtration device (Millipore) with a cutoff of 30 kDa. Similar conditions were used for rabbit muscle actin (Sigma) and recombinant mouse acyl-CoA thioesterase 7 (ACOT7; RefSeq NP_579926) as described by Forwood et al.[16]

Nonreducing Polyacrylamide Gel Electrophoresis (PAGE). The concentrated protein sample was subjected to nonreducing SDS−PAGE using a 12% gel and the NuPAGE Bis-Tris system (Invitrogen). After Coomassie staining the band corresponding to monomeric cortactin was excised and the gel slice prepared for trypsin digestion in the following manner. The gel slice was dehydrated for 5 min in 100% methanol, rehydrated in 30% aqueous methanol for 5 min, washed twice in water for 10 min and finally washed 3 times in 100 mM ammonium bicarbonate containing 30% acetonitrile (10 min per wash). The gel slice was cut into small pieces of approximately 1 cubic millimeter, washed briefly in water and dried under vacuum for 30 min.

Trypsin Digest. Promega sequencing grade modified porcine trypsin was used in a 20:1 mass ratio (cortactin/trypsin) in 50 mM ammonium bicarbonate. The digest was performed in a PCR heating block (MJ Research PTC-200) in the following manner: 2 h at 4 °C, 8 h at 37 °C and 6 h at 50 °C. This digestion regime was designed to maximize digestion of cross-linked proteins. The 4 °C step was intended to allow for diffusion of trypsin into the gel. Digestion at 37 °C is a standard trypsin condition, and the 50 °C step was included to enhance denaturation of the cross-linked protein.

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Peptide Extraction. After the removal of the supernatant, peptides were extracted from the gel pieces by serial extraction using 1.0% formic acid in the first extraction and then 0.1% formic acid in two further extractions. The volume of each extraction was such that the gel pieces were well covered by the solvent. Each extraction started with a brief vortex of the suspended gel pieces, followed by a 20 min incubation at room temperature. The pooled extract was concentrated using vacuum centrifugation and made approximately 2.0% in formic acid prior to nanoHPLC (HPLC = high pressure liquid chromatography).

Peptide Modification with O-Methyl Isourea. A quantity of extracted peptides theoretically equivalent to 25–50 μg of the protein was adjusted to pH 10 with 1.0 M sodium carbonate pH 10. The reaction was performed in a final concentration of 300 mM sodium carbonate. This solution was made approximately 2.0 M in O-methyl isourea by dilution of a freshly prepared aqueous stock. The reaction was performed at 60 °C for 30 min and stopped by acidification with formic acid to approximately pH 2.0.

The methanol generated by the hydrolysis of O-methyl isourea was removed using a vacuum centrifuge, and the concentrated reaction solution was desalted using Millipore C_{18} ZipTips according to the manufacturer's instructions. Acetonitrile in the ZipTip eluant was removed by evaporation in a vacuum centrifuge prior to nanoHPLC.

NanoHPLC/MALDI-TOF/TOF-MS. Samples were analyzed by nanoHPLC on an Agilent 1100 system with fractionation directly onto a MALDI plate, followed by MALDI-TOF/TOF-MS using an Applied Biosystems 4700 Proteomics Analyzer mass spectrometer as described in more detail below. The samples (8 μL) containing peptides originally derived from 5 to 10 μg of cortactin, reconstituted to approximately 2.0% aqueous formic acid, were injected onto a 150 μm to 150 mm Vydac 300A, C_{18}, 5 μm column. A reversed phase linear gradient from 0 to 80% aqueous acetonitrile containing 0.1% formic acid was used to elute the peptides. The elutant was mixed with MALDI matrix (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 25% ethanol and 25% 0.1% aqueous trifluoroacetic acid) and deposited directly onto a 4700 MALDI target plate using an Agilent micro fraction collector (Micro-FC, G1364D) adapted to hold the 4700 Proteomics Analyzer MALDI-TOF/TOF sample plate.

All mass spectra were recorded in positive reflector mode in the automatic control mode of the 4700 Proteomics Analyzer MALDI-TOF/TOF. The 10 most intense precursor ions from each spot were selected for MS/MS spectrum acquisition using a timed-ion selection gate of ±8 Da. The MS/MS spectra were collected using a 1 kV change in voltage (source 1, 8 kV; collision cell, 7 kV) with no additional CID gas.

RESULTS AND DISCUSSION

DTSSP Fragmentation. Chemical cross-linking of proteins can provide structural constraints for the modeling of protein domain relationships, both within and between proteins. This technique needs only small amounts of material, and the procedure is rapid and simple, but several experimental obstacles limit its usefulness at present. One major challenge is the low abundance of cross-linked peptides in the complex mixture produced by digestion of the modified protein, which makes picking out cross-linked peptides a difficult task. Another is the complexity of the fragmentation patterns of cross-linked peptides,

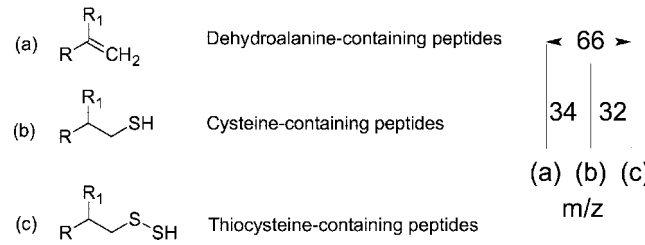

Figure 1. The triplet observed in MALDI-PSD mass spectra arising from the fragmentation of cystine-containing peptides. The symmetric fragmentation product (b) and the asymmetric fragmentation products (a) and (c) are illustrated.

which makes problematic the tandem-MS-based confirmation of their identity. A cross-linked precursor will display a mixed fragmentation series owing to the presence of two peptide sequences, and further complexity is created by the presence of cross-linked fragments, and amino acids modified by the fragmented cross-link. This complexity can be greatly reduced by cleaving the cross-link prior to MS/MS identification, yielding easily identifiable linear peptides. We chose the cleavable disulfide cross-linker DTSSP for this purpose with the intention of using reducing agents to cleave the disulfide. Unexpectedly, we observed facile MS fragmentation at the disulfide in the absence of reducing agent, which provided a distinctive MS/MS signature. As described below, this signature enables the rapid identification of cross-linked peptides in complex peptide mixtures.

We have assigned the dominant 66 Da doublets that we have observed in the MALDI-TOF/TOF-MS data to the products of the asymmetric fragmentation of the DTSSP disulfide. The analogous fragmentation of cystine disulfides has been reported to occur during MALDI-MS of peptides.[17,18] Two fragmentations are described for the cystine disulfide: a symmetric fragmentation that occurs between the sulfur atoms and an asymmetric fragmentation that occurs on either side of the disulfide. The symmetric fragmentation has been reported as a MALDI-MS in-source phenomenon; the products observed in the spectrum are those expected for the chemical reduction of the disulfide and are thought to occur by electron capture followed by protonation. Both the symmetric and the asymmetric fragmentation of the disulfide have also been reported to occur as a postsource phenomenon.[17]

Mechanism of Fragmentation. A characteristic triplet, illustrated in Figure 1, arising from the occurrence of both types of fragmentation in MALDI-post source decay (PSD) analyses has been used to identify cystine-containing peptides.[18] The triplet can be understood as a result of the appearance of the combination of the products of the symmetric fragmentation (cysteine-containing peptides) and the products of the asymmetric fragmentation. The products of the asymmetric fragmentation of the disulfide of cystine cross-linked peptide are dehydroalanine- and thiocysteine-containing peptides. In alkaline solution, these products are known to form as a result of the base-catalyzed β-elimination reaction on the cystine of proteins. This β-elimination is illustrated for cystine cross-linked peptides in Figure 2; the β-elimination occurs from either end of the cystine producing a dehydroalanine-containing

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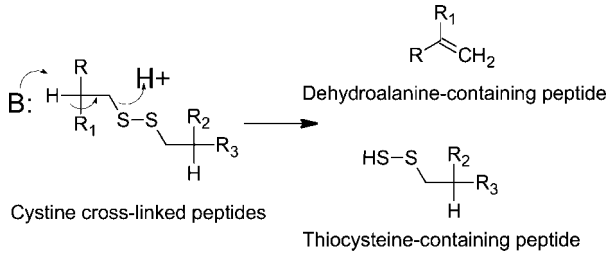


Figure 2. The base-catalyzed β-elimination reaction occurring on cystine cross-linked peptides.

and a thiocysteine-containing peptide for each of the cross-linked peptides. Kim and Kim[19] thermally promoted the aqueous β-elimination reaction in cystine-containing peptides and then used MALDI-TOF-MS to identify the presence of a triplet corresponding to that observed in MALDI-PSD spectra of cystine-containing peptides. More recently, Schnaible et al.[17] reported a method of screening for native disulfides using LIFT-TOF/TOF-MS that makes use of these cystine disulfide fragmentations but did not attempt to explain the mechanism of formation of these species. The cross-link introduced by reaction with DTSSP is clearly analogous, giving rise to both symmetric and asymmetric fragmentation of the disulfide. For DTSSP reacting with proteins at pH values above 7.0 the major site of reaction is the ϵ-amine of lysine residues.[20]

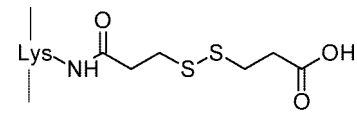
The fragmentation of the disulfide of the chemically introduced DTSSP cross-link is illustrated in Figure 3. For the asymmetric fragmentation (reaction 2) each cross-linked peptide produces an acryloyl-modified peptide and a 3-disulfanyl-propionyl-modified peptide because the fragmentation occurs from either side of the cross-link. These peptides differ in mass by 66 Da and constitute the dominant 66 Da doublets we have observed in the MALDI-TOF/TOF-MS data of DTSSP cross-linked peptides. Although the MS/MS spectra of DTSSP-modified peptides contain evidence for both the symmetric and asymmetric fragmentation of the DTSSP disulfide (and display the characteristic disulfide triplet), we find that typically the asymmetric fragmentation dominates (Figures 5 and 6), resulting in a smaller central peak than those reported for cystine disulfides.[17]

Cleavage across disulfide bonds has also been observed in ESI-QTOF-MS/MS studies by Gaucher et al.,[9] who utilized the cross-linker dithiobis(succinimidylpropionate) (ESI = electrospray ionization). This reagent introduces into proteins the same disulfide-containing cross-link as does DTSSP. In addition to a complex peptide backbone fragmentation, Gaucher et al.[9] report fragmentation of the cross-link between the sulfur atoms and across the cross-link amides during ESI-QTOF-MS/MS but they do not see the asymmetric fragmentation described here. Using MALDI-TOF/TOF-MS we have observed the same two cross-link fragmentations described by Gaucher et al.[9] as minor species, but the MALDI TOF/TOF-MS spectra of DTSSP cross-linked peptides was dominated by 66 Da doublets. Finally, Wells et al.,[21] in an ESI-FTICR-MS study of the fragmentation of intact insulin, demonstrated that cleavage at the disulfide was favored by the $[MH]^+$ ion but not in multiply charged ions, suggesting that one reason this fragmentation event is seen with MALDI-MS/MS but not ESI-MS/MS is that singly charged ions of cross-linked peptides predominate in MALDI-MS spectra but will rarely be selected for analysis in ESI-MS.

Fragmentation of Different Types of DTSSP Adducts. Based upon an understanding of the underlying chemistry, we next compared fragmentation patterns that arise from the various types of adducts formed by DTSSP. The digests of proteins that have been modified with cross-linking reagents will contain three types of modified-peptide products. Schilling et al.[8] proposed the following classification: "type-0" or "dead ends" arise when just one end of the cross-linking reagent has reacted with the protein; "type-1" cross-links produce cyclic peptides and arise when both ends of the cross-linking reagent have reacted with the protein but no trypsin digestion site exists between the sites of reaction; and "type-2" cross-links occur where both ends of the cross-linking reagent have reacted with the protein and a trypsin digestion site exists between the sites of reaction, yielding two linear peptides bridged by a cross-linker.

In order to understand the fragmentation signatures of DTSSP-modified peptides we considered the experimental outcomes of fragmentation of each type of cross-link based upon modification of recombinant proteins being studied within the structural genomics group. In Table 1 we have summarized some results to illustrate that this method yields useful data for several proteins and protein complexes. Each type-2 peptide reported in Table 1 was observed in an MS/MS spectrum that contained two 66 Da doublets. The precise nature of the adducts produced in this experimental series is discussed below.

Fragmentation of Type-0 Modifications. A type-0 DTSSP-modified peptide (**II**) arises from the reaction of one end of the bifunctional cross-linker with the ϵ-amine of a lysine residue while the other end of the cross-linker is hydrolyzed either because there is no additional suitable lysine or because the reagent was already hydrolyzed at one end prior to reaction with the protein. A type-0 modification has two consequences: the loss of a cleavage site at the modified lysine (which is no longer recognized by trypsin), and the consequent generation of a longer peptide (upon trypsin digestion of the modified protein), which has an anomalous mass owing to the modified lysine. Type-0 modifications are unavoidable. They yield little information specifically concerning cross-links, although the presence of a type-0 modification means that the site is available as a cross-linking partner. The absence of cross-links *other than* type-0 adducts can suggest that a site is not oriented close to other reactive lysines. In other contexts type-0 adducts can be useful, for example, to assess regions of solvent accessibility and the order of lysine reactivity.



(II)

When the disulfide of a type-0 DTSSP modification fragments symmetrically, the 3-mercapto-propionyl lysine-containing product which is 104 Da lighter than the precursor ion is expected. By contrast, in the case of asymmetric fragmentation the mass losses of 72 and 138 Da are expected for the 3-disulfanyl-propionyl lysine-containing product and the mass loss of 138 Da for the acryloyl lysine-containing product.

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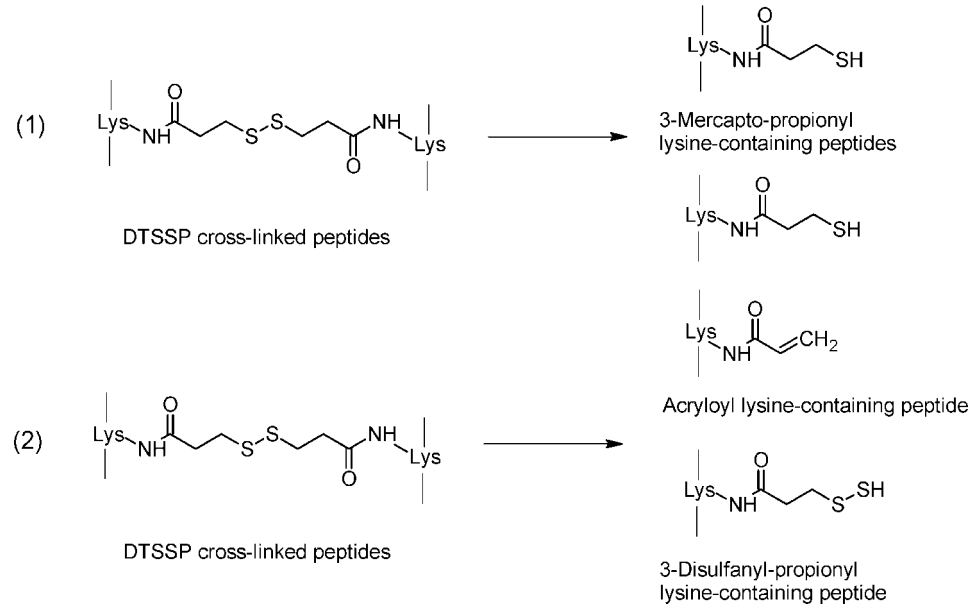


Figure 3. The symmetric and asymmetric fragmentation of peptides cross-linked with DTSSP. The symmetric fragmentation is illustrated in reaction 1 and the asymmetric fragmentation in reaction 2.

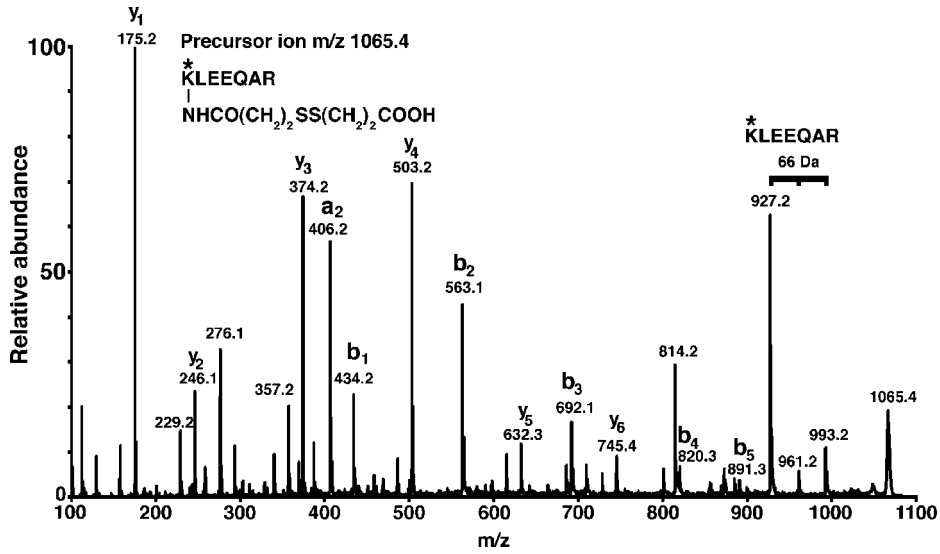
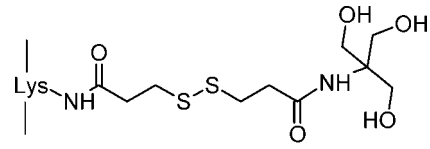


Figure 4. MALDI-TOF/TOF mass spectrum of a type-0 DTSSP modified peptide. The precursor ion (*m*/*z* 1065.4) has been assigned to the peptide K*LEEQAR produced by the trypsin digestion of DTSSP-modified cortactin. (K* indicates the site of modification.)

Surveys of our MALDI-TOF/TOF-MS data sets generated by DTSSP modification of cortactin reveal that type-0 modifications did not give rise to the distinct doublet that arises from the asymmetric fragmentation of the disulfide. Instead, the acryloyl-lysine product dominated. All 19 type-0 modifications found in this study fragmented to give this product, which is observed at precursor mass minus 138 u. Six of these spectra also contained evidence for the 3-disulfanyl-propionyl-lysine product although at a diminished intensity relative to the acryloyl-lysine product. Presumably, the presence of a carboxylic acid moiety on type-0 modifications makes the β-elimination less likely to occur from the free end of the modification and there is little or no 3-disulfanyl-propionyl-modified product.

The mass spectrum presented in Figure 4 is that of the type-0 DTSSP modification, K*LEEQAR (*m*/*z* 1065.4; K* indicates the site of modification). The 66 Da doublet, *m*/*z* 927.2 and 993.2, illustrates the dominance of the acryloyl-lysine product in the fragmentation of type-0 modifications.

Incidentally, we have observed that type-0 modifications containing tris(hydroxymethyl) aminomethane ("Tris") adduct (**III**) fragment readily to produce both the acryloyl-lysine and the 3-disulfanyl-propionyl-lysine in approximately equal amounts (data not shown).



(III)

When Tris is used to terminate a cross-linking reaction, an amide is formed at the free end of the cross-linker (**III**). We suggest that the presence of this amide makes the asymmetric

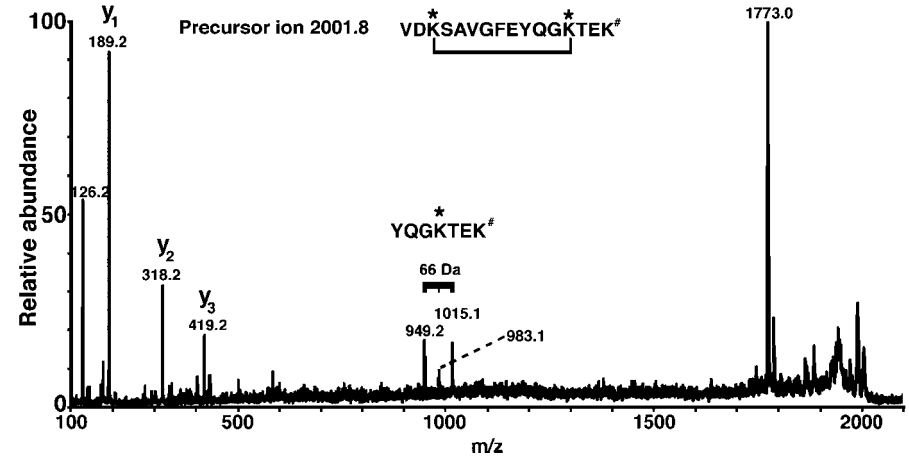


Figure 5. MALDI-TOF/TOF mass spectrum of a guanidinylated type-1 DTSSP cross-linked peptide. The precursor ion (*m*/*z* 2001.82) has been assigned to the internally cross-linked peptide VDK*SAVGFEYQGK*TEK# produced by reaction of the tryptic digest of DTSSP-modified cortactin with O-methyl isourea. K* indicates the sites of DTSSP cross-linking, and K# indicates the site of guanidinylation.

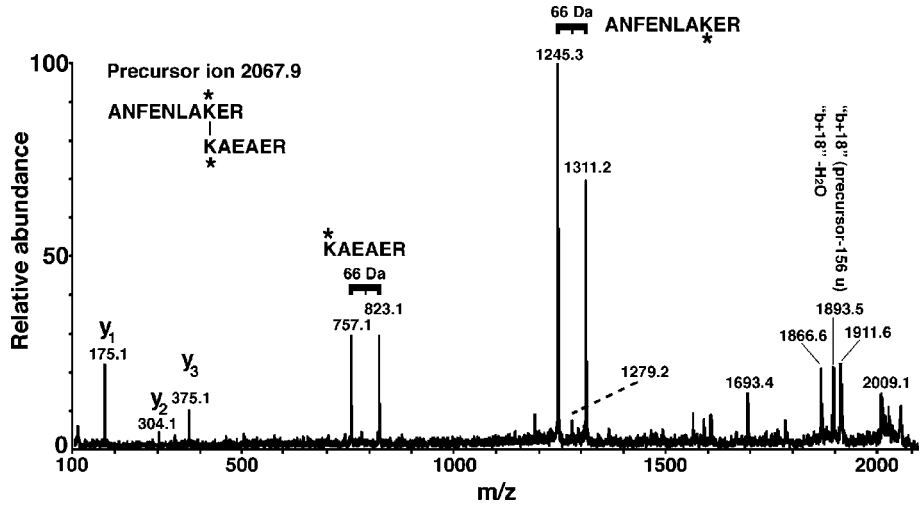


Figure 6. MALDI-TOF/TOF mass spectrum of type-2 DTSSP cross-linked peptides. The precursor ion (*m*/*z* 2067.89) has been assigned to the cross-linked peptides ANFENLAK*ER and K*AEAER. K* indicates the site of DTSSP cross-linking.

Table 1. Peptides Displaying a 66 Da Doublet in MALDI-TOF/TOF-MS Studies of DTSSP Cross-Linked Proteins and Protein Complexes

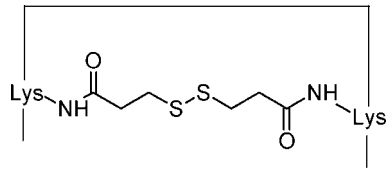
modification[a]	66 Da doublet	protein	66 Da doublet	protein
type-0	MAK*ER	cortactin	K*LEEQAR	cortactin
	K*AEAER	cortactin	GFGGK*YGVQK#	cortactin
	YGVQK*DR	cortactin	ANFENLAK*ER	cortactin
type-1[b]	YQGK*TEK#	cortactin		
type-2[c]	IK*IIAPPER	actin[d]	GFGGK*FGVQMDR	cortactin
	K*LEEQAR	cortactin	M^{ox}AK*ER	cortactin
	AK*K#	cortactin	DK*VDK#	cortactin
	K*AEAR	cortactin	YEAQK*LER	ACOT7
	MAK*ER	cortactin	YLQM^{ox}K*AKR	ACOT7
	ANFENLAK*ER	cortactin	FEEGK*GR	ACOT7
	TVPIEAVK*TSNIR	cortactin	K*R	ACOT7
	YGVQK*DR	cortactin		

[a] Modification symbols: *, site of DTSSP modification; #, site of guanidinylation; M^{ox}, methionine sulfoxide. [b] This peptide is derived from the type-1 (cyclic) cross-linked peptide VDK*SAVGFEYQGK*TEK# which has a propensity to break at the glutamic acid residue, in which case the C-terminal (homoarginine-containing) ion becomes detectable. [c] For type-2 cross-links the cross-linked peptides have been separated into their component linear peptides and each peptide has been reported just once. Each peptide found in a type-2 cross-link was observed in a MS/MS spectrum that contained two doublets which corresponded to both the cross-linked peptides. [d] Although only one cortactin−actin peptide is reported here, an exhaustive analysis of this complex has not yet been carried out.

fragmentation occur readily from either end of the modification, just as it does when a second lysine side chain has reacted with the cross-linker to form a type-2 cross-link, and a 66 Da doublet is then observed.

Nineteen type-0 modifications were identified in this study, 12 arginine-terminated peptides and a further 7 lysine-terminated peptides that were only observed in the analysis of the O-methyl isourea-treated peptides. The reaction with O-methyl isourea increases the peptide coverage obtained when using MALDI-MS techniques.[22] The predominant products of the reaction of O-methyl isourea with peptides are homoarginine-containing peptides which arise from guanidination of the ϵ-amine of lysine residues. Homoarginine-containing peptides appear to be strongly selected in MALDI-MS, just as arginine-containing peptides are strongly selected.[23]

Fragmentation of Type-1 Modifications. A type-1 DTSSP cross-linked peptide (**IV**) arises from the reaction of both ends of the bifunctional cross-linker (DTSSP) with the ϵ-amine of lysine residues that are not separated by a trypsin hydrolysis site. A cyclic cross-linked peptide is generated upon trypsin digestion of the modified protein.

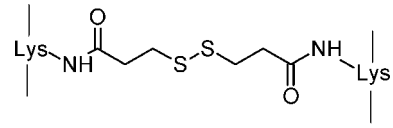


(IV)

When peptides containing a type-1 DTSSP cross-link fragment, a mass gain of 2 Da might be expected for the symmetric fragmentation based on what is seen for linear cross-links. For the asymmetric fragmentation no mass change is expected. If the symmetric fragmentation (2 Da mass increase) occurs, the product is not seen because it will be close to the precursor mass which is routinely suppressed in these MS/MS spectra. However, any fragmentation of the peptide backbone will simulate the introduction of a proteolytic site, and fragmentation of the disulfide will produce discernible products. The mass spectrum presented in Figure 5 is that of a DTSSP type-1 cross-linked peptide that appears to have fragmented in this way. The 66 Da doublet (m/z 949.2 and m/z 1015.1) has arisen from the fragmentation of the precursor ion at the peptide backbone and at the disulfide. We assigned this doublet to the acryloyl-modified and 3-disulfanyl-propionyl-modified guanidinylated precursor C-terminal fragment YQGK*TEK$^{\#}$ (where $^{\#}$ indicates the site of guanidinylation).

As discussed below, type-2 modifications typically produce a pair of 66 Da doublets in each spectrum, one doublet from each of the linear peptides which result from breakage of the cross-link. Similarly, when a type-1 cross-linked peptide fragments at the peptide backbone *between* the sites of cross-linking, two 66 Da doublets might be expected because the products will usually be similar to those produced by the asymmetric fragmentation of a type-2 cross-linked peptide. In the case of the example presented in Figure 5, just one 66 Da doublet was observed, perhaps because the VDK*SAVGFE fragment is unlikely to be protonated. This suggests that type-1 cross-links will only occasionally result in a pair of 66 Da doublets, when the C-terminal fragment is visible in MALDI-TOF/TOF-MS due to a favorable internal residue.

Fragmentation of Type-2 Modifications. Type-2 peptides are most likely to be informative for structure determination, because the sites involved may be remote from each other in the tertiary structure, or on different molecules in a protein complex. A type-2 DTSSP cross-linked peptide (**V**) arises from the reaction of both ends of the bifunctional cross-linker (DTSSP) with the ϵ-amine of lysine residues that are separated within the protein by a trypsin hydrolysis site (or when it is derived from cross-linking of different protein molecules). Trypsin digestion then results in two linear peptides cross-linked through internal lysines.



(V)

When the asymmetric fragmentation occurs on a type-2 DTSSP cross-link, the masses of both peptides may be revealed simultaneously. Two 66 Da doublets are then expected because each linear peptide may comprise a doublet. These doublets reveal the masses of the acryloyl-modified and the 3-disulfanyl-propionyl modified peptides. This information together with the precursor mass is sufficient to assign the identity of the cross-link with a high degree of confidence. Further evidence in support of the assignment may come from the limited but useful y-ion and b-ion series typically found in these mass spectra. Alternatively, following identification using the 66-pair technique, the cross-linked peptides can be reduced with Tris[2-carboxyethyl] phosphine (TCEP) and sequenced conventionally with tandem MS.

The mass spectrum presented in Figure 6 is that of a DTSSP type-2 cross-linked peptide. This spectrum is typical of the type-2 cross-links we have observed in this study, and the presence of the prominent 66 Da doublets has made the identification of these cross-links straightforward. The cross-linked precursor mass (m/z 2067.89) is consistent with ANFENLAK*ER cross-linked to K*AEAER. The 66 Da doublet (m/z 757.1 and m/z 823.1) is consistent with the masses expected for the acryloyl-modified and 3-disulfanyl-propionyl-modified peptide K*AEAER respectively. The 66 Da doublet (1245.3 and 1311.2 m/z) is consistent with the masses expected for the acryloyl-modified and 3-disulfanyl-propionyl modified peptide ANFENLAK*ER respectively.

One further feature of the mass spectra that we have found useful in the identification of type-2 cross-links is the frequent presence of a major species 156 Da lighter than the precursor ion. In Figure 6, this species appears at m/z 1911. We assign this signal to a b + 18 ion that arises via a rearrangement at the C-terminus which has been previously reported in native peptides.[24] Our experience is that type-2 cross-links are particularly prone to this loss and searching for it leads rapidly to the identification of type-2 cross-links. Because of the ease of fragmentation at this site we rarely see extensive peptide backbone fragmentation obscuring the 66 Da doublet.

Seven type-2 modified peptides were identified in this study: six of these peptides produced MS/MS spectra that contained two 66 Da doublets (that is 66 Da doublets corresponding to both parent peptides of the cross-link). The spectrum that contained

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(23) Baumgart, S.; Lindner, Y.; Kuhne, R.; Oberemm, A.; Wenschuh, H.; Krause, E. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 863–868.

(24) Ballard, K. D.; Gaskell, S. J. *J. Am. Chem. Soc.* **1992**, *114*, 64–71.

an unaccompanied 66 Da doublet was assigned to a cross-linked species that included the peptide AKK as one of the parent peptides. The 66 Da doublet arising from the AK*K peptide of the cross-link was only observed in the MS/MS data after guanidinylation of the peptide.

Role of This MS Technique. The question arises as to the generality of this method and the frequency with which 66 Da doublets can be observed in a variety of different proteins. Our experience suggests that, as expected, cross-linked peptides derived from different proteins generally behave similarly during fragmentation, and we anticipate that most proteins capable of being cross-linked and digested will yield peptides with the 66 Da signature upon MALDI-TOF/TOF-MS. To date we have employed this method for several unrelated proteins, which will be the subject of future publications, and we anticipate that further data will enable more robust conclusions to be drawn about the generality of this method.

CONCLUSIONS AND PERSPECTIVES

The approach presented in this report makes possible a rapid and sensitive identification of DTSSP cross-links in proteins, and it extends previous observations of disulfide fragmentation in MALDI-MS. The prominent 66 Da doublets observed for type-2 cross-links and occasionally for type-1 cross-links and type-0 modifications greatly facilitate the analysis of the cross-link products of DTSSP modified proteins.

Automation of the detection of the 66 Da doublets using a pair finding tool would allow faster processing of the cross-link MS/MS data sets. We currently make effective use of the Applied Biosystem's Peak Explorer software in the neutral loss mode to identify type-0 and type-2 modifications. Type-0 modifications are effectively filtered into mass lists by searching for a 138 Da neutral loss; as discussed earlier we found that type-0 modified peptides preferentially fragmented to produce the dehydroalanine analogue (138 Da lighter than the precursor ion).

As a first pass selection, type-2 modifications are effectively filtered into mass lists by searching for a 156 Da neutral loss from precursor ions. Even though this species also arises from the normal C-terminal rearrangement investigated by Ballard and Gaskell,[24] and may not be specific to DTSSP cross-linked species, in practice six of the seven type-2 modifications found in this study were detected in this manner. Frequently, the loss of the terminal arginine in a neutral loss product will result in an undetectable ion. We suggest that the ongoing presence of a terminal arginine (or homoarginine) in the neutral loss product of a type-2 ion facilitates its observation and accounts for the general usefulness of this filtering approach.

We interpret the mechanism of the asymmetric fragmentation as a β-elimination occurring in the gas phase which implies structural constraints on the types of cross-linkers that may fragment in this way. DTSSP cross-linked peptides fragment in a way analogous to cystine only because DTSSP contains a disulfide on the carbon which is beta to the carbonyl moiety. It does not follow that the presence of a disulfide in a cross-link will inevitably lead to this distinctive fragmentation, because it requires the ability to undergo β-elimination mechanism which is dependent on the presence of a nearby acidic carbon. We note that the β-elimination mechanism proposed here is not dependent on the presence of a disulfide nor on the presence of a carbonyl; all that may be required is an acidic carbon and an appropriately placed leaving group. We are currently designing cross-linkers that exploit this β-elimination chemistry to achieve a similar fragmentation but without a disulfide. The ease of identification of type-2 cross-links offered by this method will be useful in the characterization of protein–protein interactions and in the generation of protein structural constraints.

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