Collision-Induced Dissociation of Lys–Lys Intramolecular Crosslinked Peptides

Amadeu H. Iglesias,[a,b] Luiz F. A. Santos,[a,b] and Fabio C. Gozzo[a,b]

[a] Center for Structural and Molecular Biology, Brazilian Synchrotron Light Source, Campinas, Brazil
[b] Institute of Chemistry, State University of Campinas, Campinas, Brazil

The use of chemical crosslinking is an attractive tool that presents many advantages in the application of mass spectrometry to structural biology. The correct assignment of crosslinked peptides, however, is still a challenge because of the lack of detailed fragmentation studies on resultant species. In this work, the fragmentation patterns of intramolecular crosslinked peptides with disuccinimidyl suberate (DSS) has been devised by using a set of versatile, model peptides that resemble species found in crosslinking experiments with proteins. These peptides contain an acetylated N-terminus followed by a random sequence of residues containing two lysine residues separated by an arginine. After the crosslinking reaction, controlled trypsin digestion yields both intra- and intermolecular crosslinked peptides. In the present study we analyzed the fragmentation of matrix-assisted laser desorption/ionization–generated peptides crosslinked with DSS in which both lysines are found in the same peptide. Fragmentation starts in the linear moiety of the peptide, yielding regular *b* and *y* ions. Once it reaches the cyclic portion of the molecule, fragmentation was observed to occur either at the following peptide bond or at the peptide crosslinker amide bond. If the peptide crosslinker bond is cleaved, it fragments as a regular modified peptide, in which the DSS backbone remains attached to the first lysine. This fragmentation pattern resembles the fragmentation of modified peptides and may be identified by common automated search engines using DSS as a modification. If, on the other hand, fragmentation happens at the peptide bond itself, rearrangement of the last crosslinked lysine is observed and a product ion containing the crosslinker backbone and lysine (*m/z* 222) is formed. The detailed identification of fragment ions can help the development of softwares devoted to the MS/MS data analysis of crosslinked peptides. (J Am Soc Mass Spectrom 2009, 20, 557–566) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Mass spectrometry for three-dimensional analysis (MS3D) has become a very attractive tool in evaluating protein structure and interactions. In MS3D, proteins are subjected to crosslinking with one of the many reagents available followed by enzymatic digestion and MS analysis [1]. In recent years, this approach has been widely used in the study of protein folding [2–6], identifying binding partners [7–9], monitoring conformational changes upon ligand binding [10–12], characterizing surfaces in protein complexes [13–19], and as probes for solvent accessibility [20–23].

One of the key steps for a successful MS3D analysis relies on the correct assignment of crosslinked peptides. The identification of those peptides is not trivial because they are present in the sample in a low stoichiometric amount. Several approaches are currently being applied to detect these modified peptides [24–27], with one of the most explored methodologies consisting of tagging crosslinked peptides with heavy isotopes, either by using isotopically coded crosslinkers [18, 28–33] or tryptically digesting the protein solution in a mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$ [8, 31, 34, 35]. Affinity-tagged crosslinkers have been synthesized, so that modified peptides can be enriched after reaction [28, 30, 36–38]. Although this seems to be a promising method, relatively little success has been reported so far. Finally, cleavable crosslinkers have proved to be an interesting alternative because, after cleaving the crosslinker, both chains behave like regular modified peptides [33, 39, 40]. Although these methods have proved useful, a more direct and simple method would be the analysis by tandem mass spectrometry (MS/MS), a successful approach used in the study of regular peptides.

Although there are several papers in the literature reporting MS/MS spectra of crosslinked peptides [2, 5, 9, 11, 12, 18, 33, 41–43], few studies discuss the fundamental issue of how these peptides fragment in the gas phase, specifically in the case of intramolecular crosslinkers [44, 45]. Schilling et al. [44] proposed a nomenclature for fragments generated from dissociation of crosslinked peptides, based on the previous nomenclature for regular (linear) peptides proposed by Roepstorff and Fohlman [46] and modified by Biemann [47]. This nomenclature was used to annotate ions

Address reprint requests to Prof. Fabio Cesar Gozzo, State University of Campinas–UNICAMP, CP 6154, Institute of Chemistry, 13083-970 Campinas, SP, Brazil. E-mail: fabio@iqm.unicamp.br

1044-0305/09/$32.00
doi:10.1016/j.jasms.2008.11.009

Published online November 21, 2008
Received September 12, 2008
Revised November 14, 2008
Accepted November 14, 2008

generated from dissociation of model crosslinked peptides. Despite several proposed fragmentation patterns, most ions in the spectra were annotated as regular *b* and *y* ions derived from the dissociation of the linear part of the peptide. In a more recent paper [45], the fragmentation of model peptides was studied as a function of position of the crosslinker within the peptide backbone, charge state, and nature of crosslinker. MS/MS of crosslinked peptides usually presents two distinct series of *b* and *y* ions, making it difficult to differentiate from linear peptides. Therefore, fragmentation features unique to crosslinked peptides would be very desirable. It is worth stating that in this work all peptides used were not tryptic, but they were acetylated at the N-terminus, making them not real tryptic peptides. This modification changes charge distribution over the peptide, which changes the fragmentation pattern compared with that of tryptic peptides [48]. The main reason for choosing this strategy is that once peptides present three groups that are able to react with the crosslinker (N-terminus and two lysine residues), the reaction product would be a mixture of three isobaric species that would not allow separation on the gas phase.

Herein, we report the fragmentation pattern of intramolecular crosslinked peptides (Type 1), in which both connected residues lie in the same chain [44]. These resultant peptides are quite common because once proteins are crosslinked they become very compact, making enzymatic digestion difficult. As a result, it is commonly observed that large peptides with some missed cleavage sites contain an intramolecular crosslink. Moreover, one type of crosslinked peptides not studied in the literature is one in which the binding site is the free N-terminus. In general, this part of the protein is very flexible and knowing other residues that could be connected to this group could give a dynamic sense to the experiment. In other words, knowing residues to which the N-terminus is connected could give an idea of how flexible that portion of the molecule is. To generate tryptic peptides that resemble species found in experiments with proteins, we used a novel approach based on specially designed peptides. Peptides in which only Lys–Lys crosslinking was desired contained in their sequence the N-terminus acetylated followed by an Arg and the two Lys residues. After the crosslinking reaction peptides were both partially and completely tryptically digested, generating intra-crosslinked peptides in which the N-terminus is not blocked.

Experimental

Materials

Disuccinimidyl suberate (DSS) and sequencing-grade modified porcine pancreas trypsin were obtained from Pierce Biotechnology (Rockford, IL, USA) and Promega (Madison, WI, USA), respectively. Peptides AGAKGAERLVKAGVR (PX), Ac-ARKGCREVTKNDLR (P1), Ac-ARGKWPREVKIHR (P2), and Ac-ARYTKDLSQRAFKGMR (P3) were obtained from Proteimax (São Paulo, Brazil). All other reagents were obtained from Sigma–Aldrich (St, Louis, MO, USA) and Tedia Chemicals (Cincinnati, OH, USA) and used without further purification.

Crosslinking Reactions

Peptides were solubilized in buffer containing sodium phosphate 50 mM (pH 7.0) to a final concentration of 40 μM. DSS was dissolved in dimethylformamide (1 mg/mL) and added in 50 molar excess to the previous solution. Reactions were allowed to stand at room temperature for 2 h (to ensure maximum yield of crosslinking), after which Tris buffer (1 M, pH 7.6) was added to a final concentration of 50 mM. Enzymatic digestion was performed 15 min after the quenching reaction, when trypsin was added to a final concentration of 1:50 (weight) and the reaction was incubated at 37 °C for 1 or 3 h to obtain intra- and intermolecular crosslinked species, respectively (Figure 1).

Mass Spectrometry Analysis

Before MS analysis of crosslinked peptides, all reaction products were desalted using an Oasis HLB Cartridge (Waters, Milford, MA, USA) made of a polymeric resin, endcapped with N-vinylpyrrolidone and divinylbenzene, according to the manufacturer's protocol. Samples were dried in a SpeedVac and resuspended in 50% ACN/0.1% trifluoroacetic acid. Matrix-assisted laser desorption/ionization (MALDI)-MS and MS/MS spectra were acquired in MALDI Q-ToF Premier (Waters). Samples analyzed by MALDI positive-reflectron mode were prepared by the dried droplet method, using α-cyano-4-hydroxy-*trans*-cinnamic acid as the matrix. The equipment operates with a 200-Hz solid-state laser. Typical operating conditions used were 250 a.u. (laser energy) and 10 V (sample plate). MALDI-MS/MS spec-

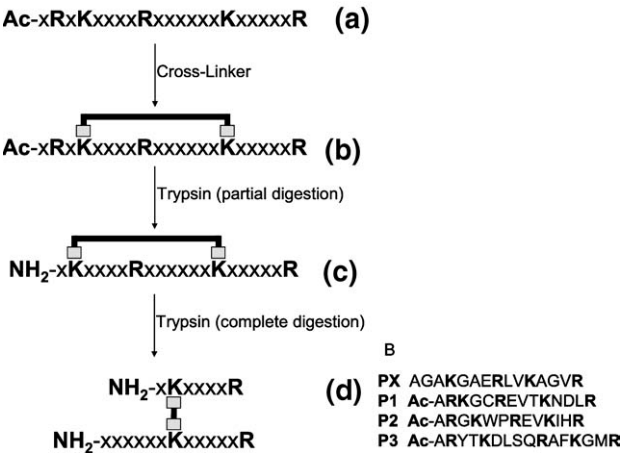


Figure 1. (**a**) Schematic representation of the designed peptides, followed by reaction with DSS and trypsin digestion. "x" represents a variable number and type of amino acids except lysine and arginine. (**b**) Sequence of the peptides used in the present work.

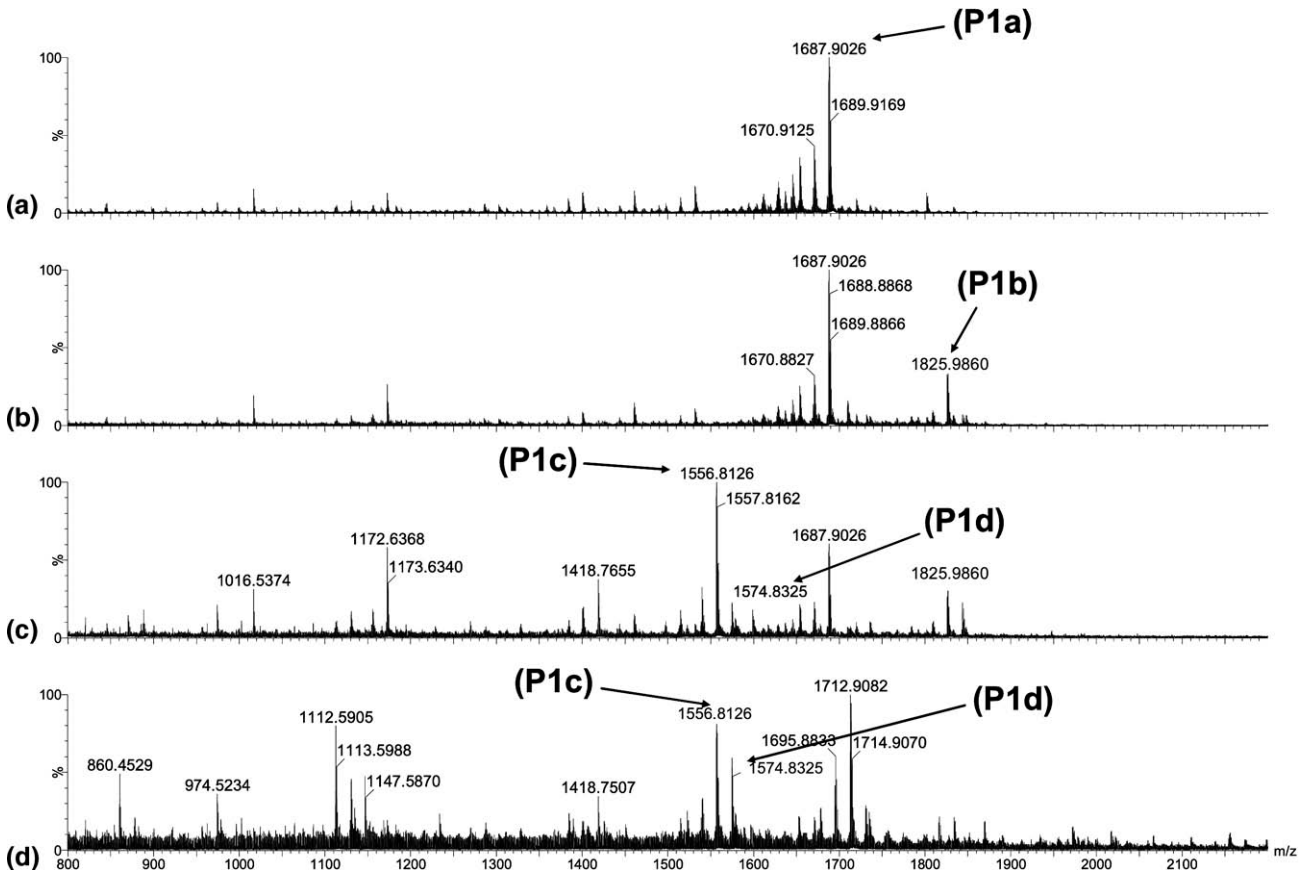


Figure 2. Spectra of P1 before (**a**) and after (**b**) reaction with DSS and consecutive digestion for 1 (**c**) and 3 h (**d**). Ions indicated by arrows are discussed in the text.

tra were manually acquired by increasing the collision energy until the precursor ion was completely fragmented. Argon was used as the collision gas. Whenever possible, spectra were internally calibrated using the mass of the unmodified peptide as lock mass.

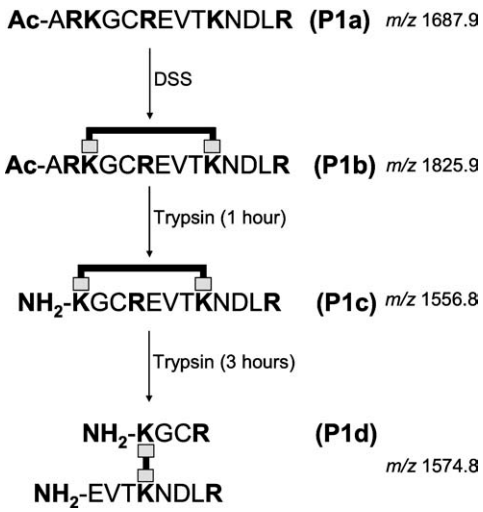
Database Search

MS/MS spectra were converted to .dta files and were then searched against the Swiss-Prot database (~260.000 sequences) containing the sequences of P1, P2, P3, and PX using MASCOT protein identification software (Matrix Science, Boston, MA, USA). The following parameters were used: enzyme trypsin, 4 missed cleavages, 0.1 of mass tolerance for MS and MS/MS, and variable modification of the corresponding mass of DSS. The significance threshold was set at $P < 0.05$, which in this case corresponds to a score of 31.

Results and Discussion

Peptide Design

The peptides proposed herein to the study of crosslinking have the common moiety Ac-xxRxxxKxxxRxxxKxxxR, where x represents a variable number of any amino acid except K and R. The use of this set of peptides in which the position and composition of residues varies allowed the unambiguous assignment of fragment ions, which could be isobaric if the sequences consisted of only a single residue repeat [45]. The use of different sequences and compositions also allowed the study of sequence-dependent fragmentation [49], at the same time the rigid postions of K and R residues made it



Scheme 1

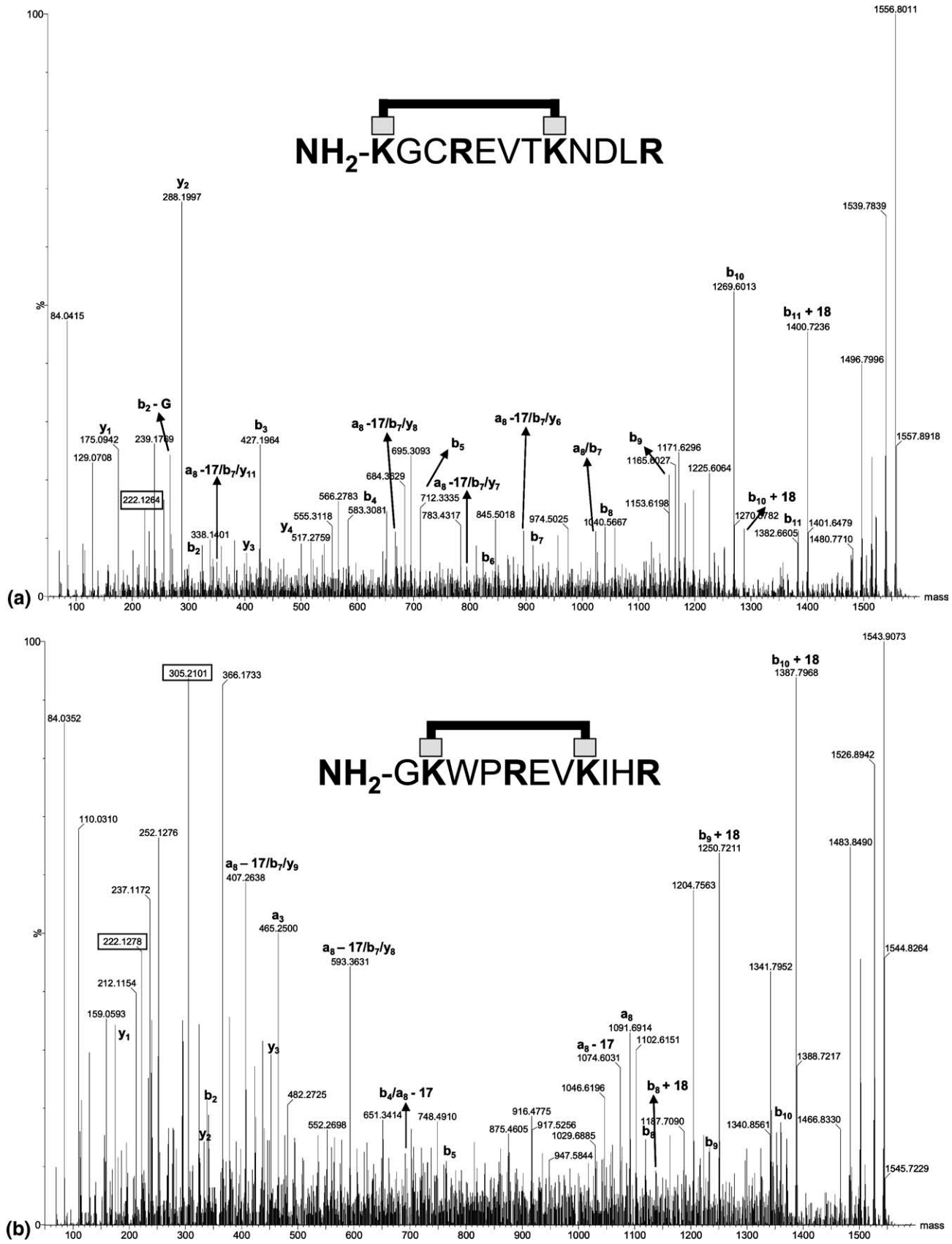


Figure 3. MS/MS spectra of intramolecular crosslinked P1 (**a**), P2 (**b**), and P3 (**c**). For a detailed discussion of *m/z* 222 and 305 refer to text. Ions marked with * are internal fragments.

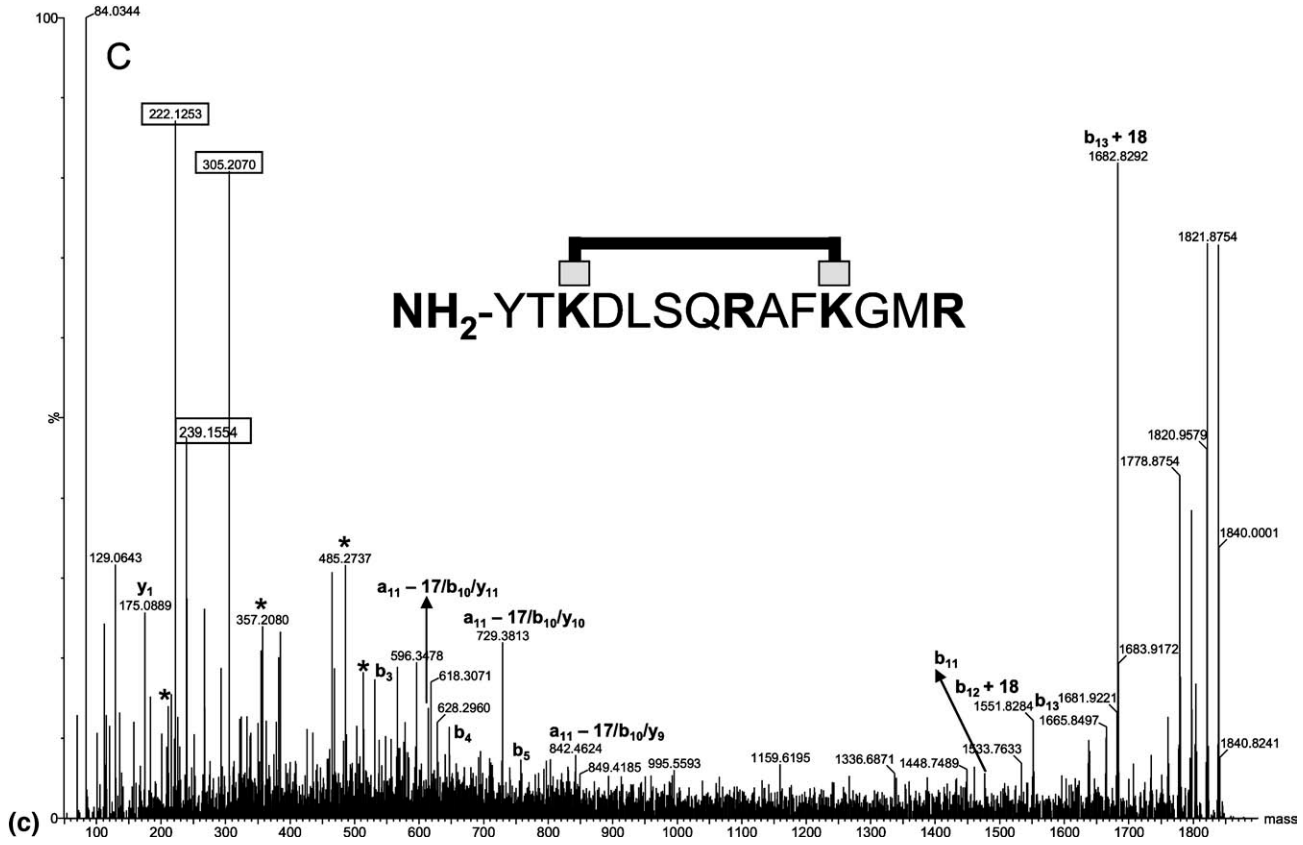
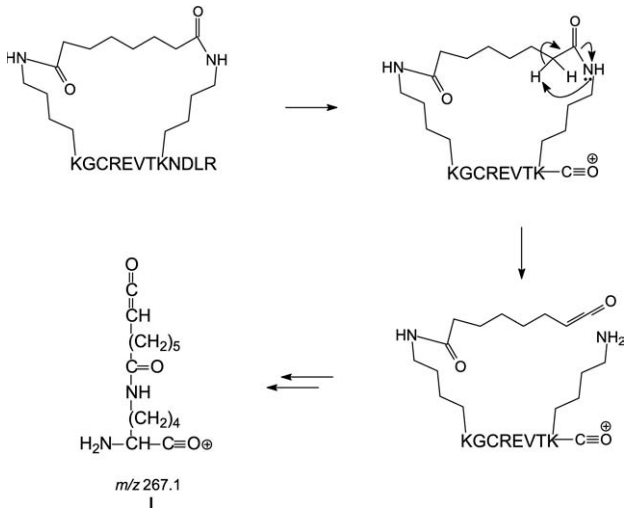


Figure 3. Continued.

possible to obtain several types of crosslinked products from a single peptide precursor. After the reaction of peptide and DSS, generating a crosslink between the two lysine residues, and enzymatic digestion with trypsin, two species can be generated: (1) tryptic, intramolecular crosslinked peptide, by cleavage of the first arginine; and (2) tryptic, intermolecular crosslinked peptide, by cleavage of both arginines. The intra crosslinker can be easily obtained by controlling the digestion time because the crosslink turns the second arginine less prone to digestion because of steric hindrance. Thus the versatility of these peptides allows the formation of both intra- and intermolecular crosslinkers that mimic real tryptic crosslinked peptides found in MS3D (data on intermolecular crosslinked peptides will be shown on a future paper) (Figure 1).

MALDI-MS spectra of P1a after reaction with DSS and digestion with trypsin are shown in Figure 2 (spectra of P2 and P3 are available as Supplementary Material Figures S1 and S2, which can be found in the electronic version of this article). Figure 2a and b show the spectra of P1 before (P1a) and after (P1b) reaction with DSS, respectively. The main reaction product of P1 (*m/z* 1687.8, P1a in Scheme **1**) with DSS is the desired intramolecular crosslinked peptide (*m/z* 1825.9, P1b). It is worth mentioning that the yield of crosslinking reaction as well as the formation of other products showed to be sequence dependent, whereas for P1, which showed a relatively poor crosslinking yield, PX reacted almost completely with DSS. Formation of other species such as *dead-end* (data not shown) is also affected by the peptide sequence.

Figure 2c and d show the spectra of the reaction product after enzymatic digestion for 1 and 3 h, respectively. It is possible to note that controlling the time for enzymatic digestion, the formation of either intra- or intermolecular crosslinks can be favored. In the case of P1,



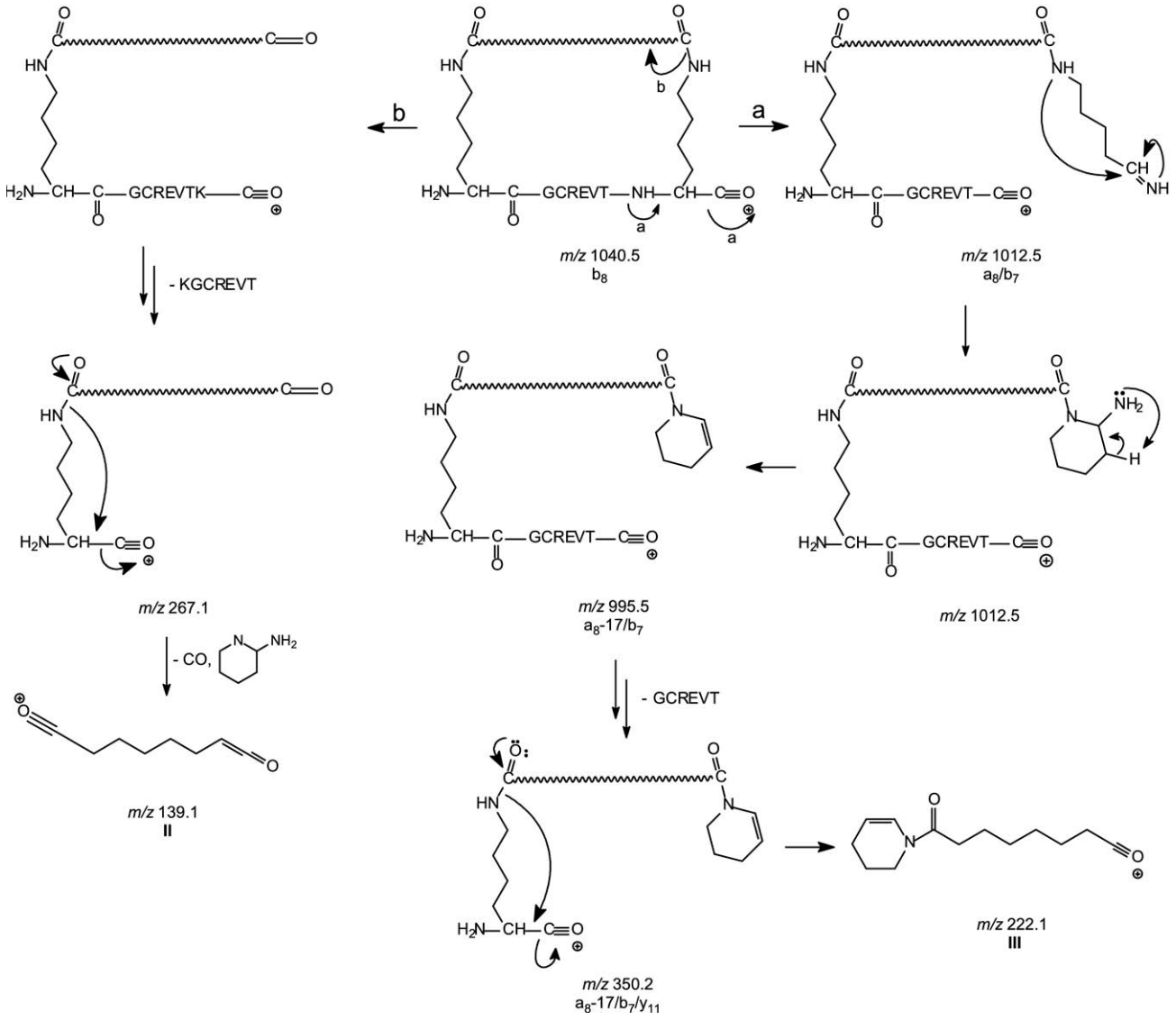
Scheme 2

after 1 h of trypsin digestion the base peak in the MS spectra is the intramolecular crosslinked peptide in which only the first arginine was cleaved (*m/z* 1556.8, P1c in Scheme **1**). After digestion for an additional 2 h equimolar amounts of intra- and intermolecular crosslinking were formed (*m/z* 1574.8, P1d in Scheme **1**). Once again those yields were found to be sequence dependent. As expected, the kinetics of enzymatic digestion is faster on the exposed arginine residue, preferentially generating the unblocked, intramolecular crosslinked peptide on shorter digestion times, although the optimum time is sequence-dependent (data not shown).

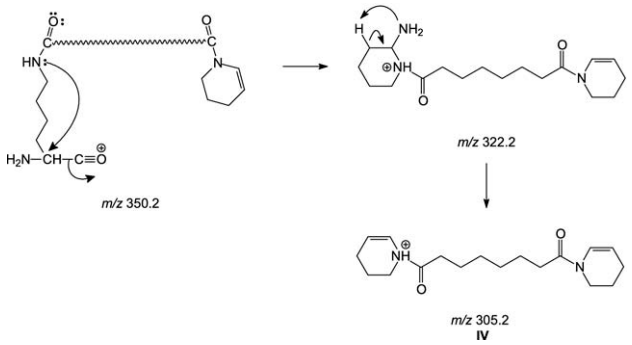
One important issue that should be addressed at this point is that by using our approach, there is the possibility of generating two isobaric species: the *dead-end* crosslinked peptide and the intermolecular crosslinked peptide in which trypsin digested only at the arginine between the two lysine residues. In the case of P1 for example, the *dead-end* version of peptide KGCREVTKNDLR would be isobaric (*m/z* 1556.8) to the intermolecular crosslink between KGCR and EVTKNDLR. This occurrence, however, is very unlikely because the *dead-end* peptide has to be resistant to 3-h trypsin digestion and the intermolecular crosslinked peptide should be digested at, and only at, the central arginine. To exclude this remote possibility, we carried trypsin digestion overnight and compared the MS/MS spectrum of the intermolecular crosslinked peptide with the one obtained after the 3-h digestion (data not shown). Because both fragmentation spectra were identical, it can be concluded that this species is the intermolecular crosslinked peptide.

Collision-Induced Dissociation of Singly Charged Intramolecular Crosslinked Peptide

Figure 3 presents MALDI-MS/MS annotated spectra of intramolecular crosslinked P1c, P2c, and P3c (for no-



Scheme 3



Scheme 4

menclature see Liu et al. [38]). These peptides can be seen as branched cyclic peptides because the DSS–peptide bond is also an amide bond. Fragmentation occurs in two different steps, first on the linear moiety and then on the cycle. Ions generated from the linear part of the peptide (NDLR in the case of P1) are regular *b* and *y* type ions, where the *b* ions are more intense, as expected for singly charged precursor ions.

When fragmentation reaches the cyclic part of the molecule, it can be forked into two different paths. Considering that the peptide–crosslinker bond is also an amide bond, fragmentation can take place at either bond in competing pathways: (1) the crosslinker/peptide bond or (2) the backbone bond. If the crosslinker/lysine side-chain bond fragments, the crosslinker moiety acts like a regular modification at the lysine and fragmentation will continue as in a regular, linear peptide (pathway b in Scheme **3**). This pathway can be clearly seen in all three spectra (Figure 3), but its extent is sequence dependent. In the case of P1c, where the first crosslinked lysine is also the first residue of the peptide, the *m/z* 267.1 ion (**I** in Scheme **2**) can be easily seen (annotated as b_2-G in the spectrum) as well as all other ions of this sequence (b_2: 324.1, b_3: 427.2, b_4: 583.3, b_5: 712.3, b_6: 811.4; and b_7: 912.5).

Ions generated in this pathway can also be seen in the fragmentation spectra of P2c and P3c (Figure 3) but they are not as intense as for P1c. The observation of this sequence is extremely interesting once it shows that this kind of peptide resembles regular modified linear peptides and therefore they can be identified by common automated search engines, using DSS as a modification on lysine residues. In the case of P1, in which this fragmentation pathway is competitive, this modification could be identified using MASCOT with a score of 56. In the case of P2 and P3, however, identification was not possible because this fragmentation pathway is not as intense as in the case of P1.

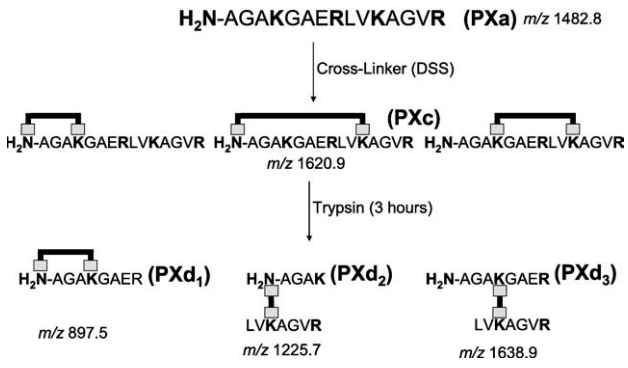
As shown in Scheme **3**, the ion of *m/z* 1040.5 has both lysine residues connected by the crosslinker chain. Fragmentation can take place at either of the lysine residues, depending on where the charge is located. However, in all cases the loss of CO was observed, generating an "a" type ion, followed by the loss of NH_3 and sequential loss of residues until the formation of an ion of *m/z* 222 (**III** in Scheme **3**). This ion has been previously reported [31] to be a signature for DSS crosslinked peptides, although its mechanism of formation has not been elucidated so far.

We propose that once fragmentation reaches the cyclic portion of the molecule there is the cleavage of the peptide bond between the crosslinked lysine and the previous residue followed by CO loss. Then, the ε nitrogen from the former lysine attacks the α carbon of the same residue. This is extremely favorable because of the formation of a six-member ring. This ring contains an amino group (derived from the α nitrogen) whose bond was observed to fragment in all cases, generating a tetrahydropyridine ring. Then, sequential loss of residues occurs until the formation of the acylium ion of *m/z* 222 (pathway a in Scheme **3**). Besides being an intense ion, it is also extremely interesting because there is no overlapping combination of *b*, *a*, or *y* ions for the regular 20 amino acids, making *m/z* 222 a very promising diagnostic ion for any Lys–Lys DSS crosslinked peptides. The preferred fragmentation pathway seems to be sequence dependent because it can be observed in MS/MS spectra, whereas for P1c the amide bond between crosslinker and peptide seems to be the most prone to fragmentation; backbone cleavage is the most favored in the case of P2c and P3c. However, in all cases it is possible to see ions generated from both fragmentation pathways.

Another remarkable feature in MS/MS spectra of P2c and P3c is the rather intense ion of *m/z* 305 (it is also present in the spectrum of P1c with lower intensity), which is 83 Da higher in mass than *m/z* 222. This 83 Da difference is exactly the mass of the tetrahydropyridine ring; we propose that this ion is the formation of a precursor to *m/z* 222 in which both lysine residues rearrange to generate the tetrahydropyridine ring (**IV** in Scheme **4**).

Intramolecular N-terminus–Lysine Crosslinking

Another interesting type of intramolecular crosslinking is that in which the protein N-terminus is connected to a lysine residue. In general, the N-terminus region of a protein is very flexible in solution and analysis of distance constraints involving this part of the protein



Scheme 5

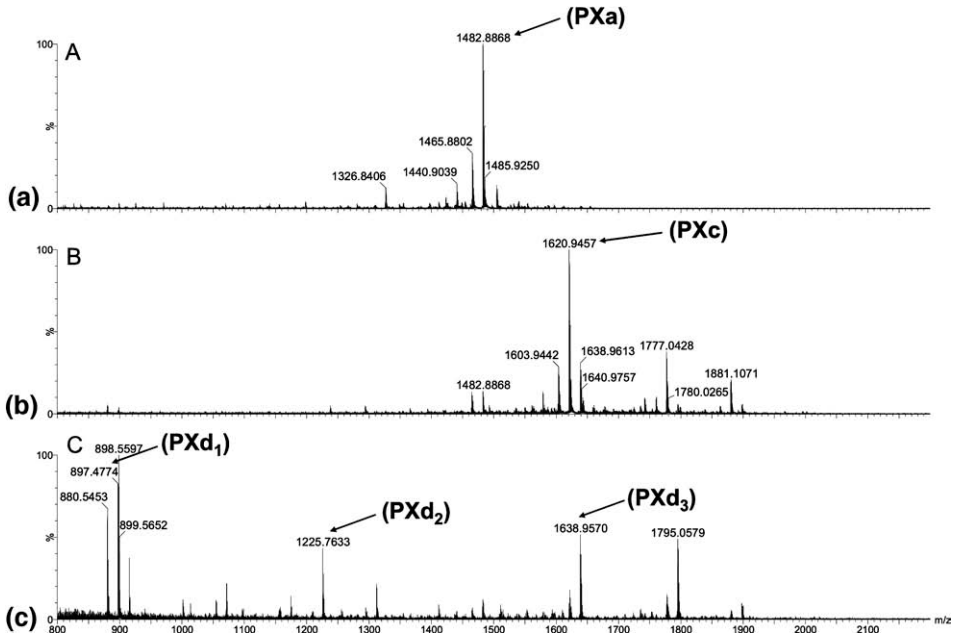


Figure 4. Spectra of PX before (**a**) and after (**b**) reaction with DSS and consecutive digestion for 3 h (**c**).

can give some clues on the dynamics of the N-terminal segment of protein, giving information on the interacting span of this part of the molecule. To the best of our knowledge, there is no study on the fragmentation of this type of crosslinking. To obtain such crosslinked species, we used a slight variant of the peptides used so far (PX), that has a free N-terminus. After DSS reaction with PX, three isobaric species could be formed (PXc in Scheme **5**). Trypsin digestion was used to discriminate the isomers and allow MS analysis, as shown in Figure 4 and represented by PXd_1, PXd_2, and PXd_3 in Scheme **5**.

The MALDI-MS spectrum of PXa (*m/z* 1482.8) with DSS shows that the unmodified peptide was totally consumed to generate three major ions of *m/z* 1620.9 (PXc, intramolecular crosslinking), 1777.0, and 1881.1 (*dead-end* species). After trypsin digestion, all three intramolecular crosslinked peptides were observed: Lys–Lys (PXd_3 with *m/z* 1638.9) and N-terminus with both Lys residues (PXd_2 with *m/z* 1225.7 and PXd_1 with *m/z* 897.4). It is worth noting that these three ions were formed in the same proportion, indicating that there is no bias toward the formation of crosslinking on different sites.

The fragmentation spectrum of the N-terminus crosslinked peptide of *m/z* 897.4 (PXd_1) is presented in Figure 5. In this spectrum it is possible to see the formation of the *m/z* 464.2, which corresponds to the b_4 ion containing only the cyclic portion of the peptide. Subsequent fragmentation leads to the loss of CO and NH_3 followed by the consecutive loss of the residues until the formation of the ion with *m/z* 222, following the same pathway as in the case of the fragmentation of intramolecular Lys–Lys crosslinked peptides. In this case, the alternative mechanism in which there is the cleavage of the crosslinker/peptide bond is almost absent. Another interesting feature in this spectrum is the presence of the quite intense ion of *m/z* 240. This was previously described [44] as the immonium ion of DSS-modified lysine losing an amine but was observed only in the PSD spectrum of *dead-end* modified peptides, not in the N-terminal crosslinked peptide. The higher energetic regime of CID compared to PSD can explain the absence of this ion in previous reports, since formation of *m/z* 240.1 requires fragmentation of three bonds (A–K, K–G, and K–DSS bonds).

Conclusions

The fragmentation pattern of intramolecular, tryptic crosslinked peptides was evaluated using MALDI-MS/MS and a more detailed fragmentation pathway was described. By using versatile model peptides, a number of structurally different crosslinked peptides were generated, including intramolecular, intermolecular, and N-terminal crosslinked peptides. The yield of the reaction of DSS with peptides was sequence dependent, once all experiments were performed under the same conditions (pH, buffer, DSS:peptide ratio, time). Also the formation of different products such as *dead-end* crosslinkers depended on the primary sequence of each peptide, varying from almost none (in the case of P1) to about 50% (PX).

Once intramolecular crosslinked peptides resemble branched cyclic peptides, MALDI-MS/MS fragmentation of those species produced mostly *b* and *y* ions of the linear portion of the molecule. On the cyclic part of the peptide, two different pathways are observed: fragmentation between the crosslinked lysine and DSS or the lysine–backbone bond. If the crosslinker/residue bond fragments, the peptide behaves like a linear peptide containing a modified lysine residue. If, on the other hand, the fragmentation occurs between the two

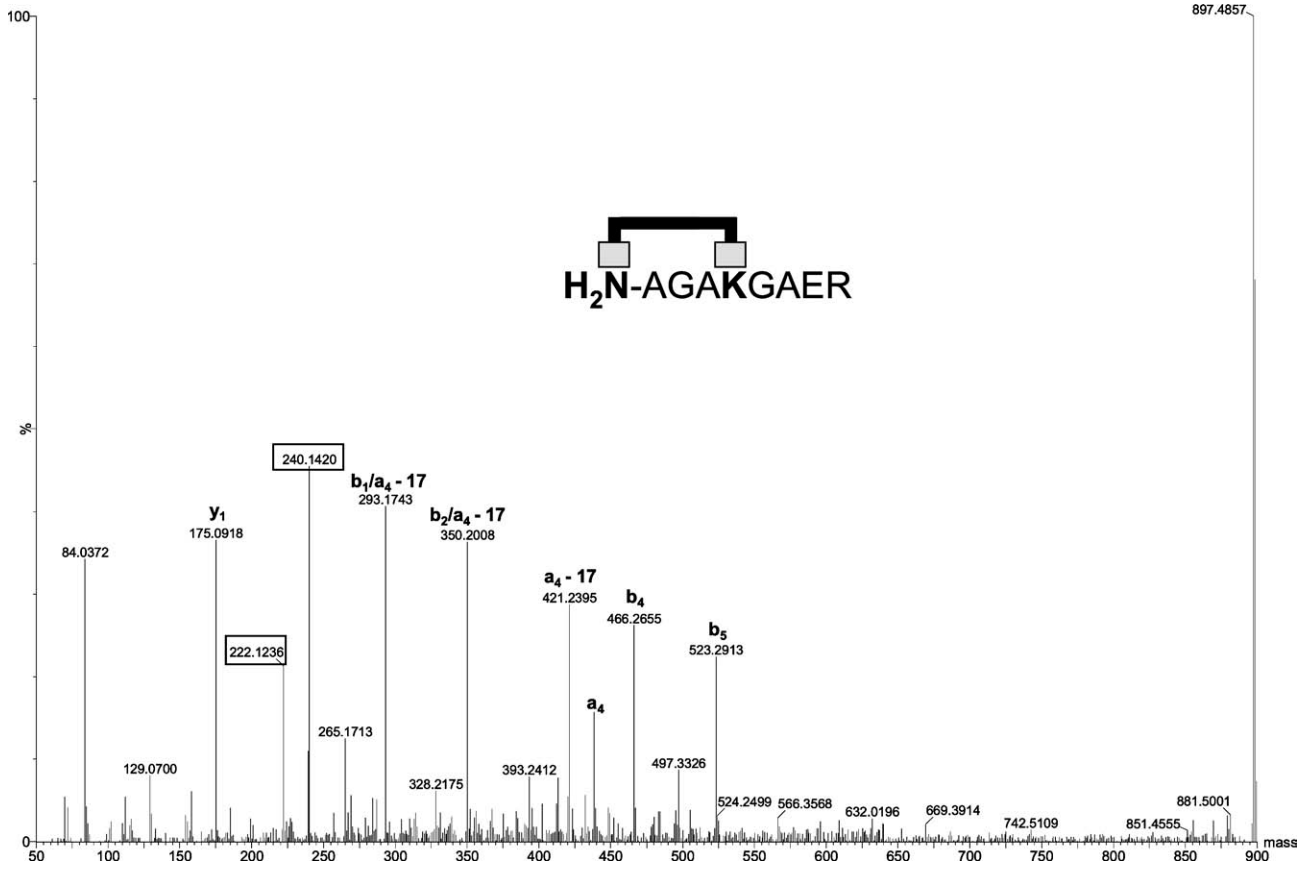


Figure 5. MS/MS spectrum of the intramolecular crosslinked PX (Lys-N-terminus).

residues, one crosslinked lysine rearranges into tetrahydropyridine. Consecutive loss of residues leads to the formation of an intense acylium ion of *m/z* 222, which is formed by the DSS backbone and the rearranged lysine residue.

The intramolecular N-terminus crosslinked peptide evaluated in this report fragmented in the same way as Lys–Lys linked peptides, but with one pathway much more intense; other data must be acquired in other peptides to distinguish it as being preferred for this type of ion or as being biased because of the sequence of this particular peptide. Finally, we believe that the continuous characterization of the fragmentation patterns of every type of crosslinker can improve one of the main bottlenecks of MS3D—the detectability and correct assignment of crosslinked peptides.

Acknowledgments

This work was supported by the São Paulo Proteome Network (FAPESP 2004/14846-0 and FINEP 01.07.0290.00). The authors also acknowledge the support from FAPESP, CAPES, and CNPq.

References

1. Sinz, A. Chemical Cross-Linking and Mass Spectrometry to Map Three-Dimensional Protein Structures and Protein–Protein Interactions. *Mass Spectrom. Rev.* **2005,** *25,* 663–682.
2. Huang, B. X.; Kim, H. Y.; Dass, C. Probing Three-Dimensional Structure of Bovine Serum Albumin by Chemical Cross-Linking and Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2004,** *15,* 1237–1247.
3. Young, M. M.; Tang, N.; Hempel, J. C.; Oshiro, C. M.; Taylor, E. W.; Kuntz, I. D.; Gibson, B. W.; Dollinger, G. High Throughput Protein Fold Identification by Using Experimental Constraints Derived from Intramolecular Cross-Links and Mass Spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **2000,** *97,* 5802–5806.
4. Dihazi, G. H.; Sinz, A. Mapping Low-Resolution Three-Dimensional Protein Structures Using Chemical Cross-Linking and Fourier Transform Ion-Cyclotron Resonance Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2003,** *17,* 2005–2014.
5. Lee, Y. J.; Lackner, L. L.; Nunnari, J. M.; Phinney, B. S. Shotgun Cross-Linking Analysis for Studying Quaternary and Tertiary Protein Structures. *J. Proteome Res.* **2007,** *6,* 3908–3917.
6. Nielsen, T.; Thaysen-Andersen, M.; Larsen, N.; Jorgensen, F. S.; Houen, G.; Hojrup, P. Determination of Protein Conformation by Isotopically Labeled Cross-Linking and Dedicated Software: Application to the Chaperone, Calreticulin. *Int. J. Mass Spectrom.* **2007,** *268,* 217–226.
7. Rappsilber, J.; Siniossoglou, S.; Hurt, E. C.; Mann, M. A Generic Strategy to Analyze the Spatial Organization of Multi-Protein Complexes by Cross-Linking and Mass Spectrometry. *Anal. Chem.* **2000,** *72,* 267–275.
8. El-Shafey, A.; Tolic, N.; Young, M. M.; Sale, K.; Smith, R. D.; Kery, V. "Zero-Length" Cross-Linking in Solid State as an Approach for Analysis of Protein–Protein Interactions. *Protein Sci.* **2006,** *15,* 429–440.
9. Kitatsuji, C.; Kurogochi, M.; Nishimura, S. I.; Ishimori, K.; Wakasugi, K. Molecular Basis of Guanine Nucleotide Dissociation Inhibitor Activity of Human Neuroglobin by Chemical Cross-Linking and Mass Spectrometry. *J. Mol. Biol.* **2007,** *368,* 150–160.
10. Carven, G. J.; Stern, L. J. Probing the Ligand-Induced Conformational Change in HLA-DR1 by Selective Chemical Modification and Mass Spectrometry Mapping. *Biochemistry* **2005,** *44,* 13625–13637.
11. Bhat, S.; Sorci-Thomas, M. G.; Alexander, E. T.; Samuel, M. P.; Thomas, M. J. Intermolecular Contact Between Globular N-terminal Fold and C-terminal Domain of ApoA-I Stabilizes Its Lipid-Bound Conformation. *J. Biol. Chem.* **2005,** *280,* 33015–33025.
12. Huang, B. X.; Kim, H. Y. Interdomain Conformational Changes in Akt Activation Revealed by Chemical Cross-Linking and Tandem Mass Spectrometry. *Mol. Cell. Proteomics* **2006,** *5,* 1045–1053.
13. Gertz, M.; Seelert, H.; Dencher, N. A.; Poetsch, A. Interactions of Rotor Subunits in the Chloroplast ATP Synthase Modulated by Nucleotides and by Mg^{2+}. *Biochim. Biophys. Acta* **2007,** *1774,* 566–574.

14. Suchaneck, M.; Radzikowska, A.; Thiele, C. Photo-Leucine and Photo-Methionine Allow Identification of Protein–Protein Interactions in Living Cells. *Nat. Methods* **2005,** *2,* 261–268.
15. Taverner, T.; Hall, N. E.; O'Hair, R. A. J.; Simpson, R. J. Characterization of an Antagonist Interleukin-6 Dimer by Stable Isotope Labelling, Cross-Linking and Mass Spectrometry. *J. Biol. Chem.* **2002,** *277,* 46487–46492.
16. Chang, Z.; Kuchar, J.; Hausinger, R. P. Chemical Cross-Linking and Mass Spectrometric Identification of Sites of Interaction for UreD, EreF, and Urease. *J. Biol. Chem.* **2004,** *279,* 15305–15313.
17. Back, J. W.; Sanz, M. A.; Jong, L.; Koning, L. J.; Nijtmans, L. G. J.; Koster, C. G.; Grivell, L. A.; Spek, H.; Muijsers, A. O. A Structure for the Yeast Prohibitin Complex: Structure Prediction and Evidence from Chemical Crosslinking and Mass Spectrometry. *Protein Sci.* **2002,** *11,* 2471–2478.
18. Maiolica, A.; Cittaro, D.; Borsotti, D.; Sennels, L.; Ciferri, C.; Tarricone, C.; Musacchio, A.; Rappsilber, J. Structural Analysis of Multiprotein Complexes by Cross-Linking, Mass Spectrometry, and Database Searching. *Mol. Cell. Proteomics* **2007,** *6,* 2200–2211.
19. Ahrman, E.; Lambert, W.; Aquilina, J. A.; Robinson, C. V.; Emanuelsson, C. S. Chemical Cross-Linking of the Chloroplast Localized Small Heat-Shock Protein, Hsp21, and the Model Substrate Citrate Synthase. *Protein Sci.* **2007,** *16,* 1464–1478.
20. Azim-Zadeh, O.; Hillebrecht, A.; Linne, U.; Marahiel, M. A.; Klebe, G.; Lingelbach, K.; Nyalwidhe, J. Use of Biotin Derivatives to Probe Conformational Changes in Proteins. *J. Biol. Chem.* **2007,** *282,* 21609–21617.
21. Pimenova, T.; Nazabal, A.; Roschitzki, B.; Seebacher, J.; Rinner, O.; Zenobi, R. Epitope Mapping on Bovine Prion Protein Using Chemical Cross-Linking and Mass Spectrometry. *J. Mass Spectrom.* **2008,** *43,* 185–195.
22. Gabant, G.; Augier, J.; Armengaud, J. Assessment of Solvent Residues Accessibility Using Three Sulfo-NHS-Biotin Reagents in Parallel: Application to Footprint Changes of a Methyltransferase Upon Binding its Substrate. *J. Mass Spectrom.* **2008,** *43,* 360–370.
23. Baker, D. L.; Seyfried, N. T.; Li, H.; Orlando, R.; Terns, R. M.; Terns, M. P. Determination of Protein–RNA Interaction Sites in the Cbf5-H/ACA Guide RNA Complex by Mass Spectrometry Protein Footprinting. *Biochemistry* **2008,** *47,* 1500–1510.
24. Back, J. W.; Hartog, A. F.; Dekker, H. L.; Muijsers, A. O.; Koning, L. J.; Jong, L. A New Crosslinker for Mass Spectrometric Analysis of the Quaternary Structure of Protein Complexes. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 222–227.
25. Back, J. W.; Jong, L.; Muijsers, A. O.; Koster, C. G. Chemical Cross-Linking and Mass Spectrometry for Protein Structural Modelling. *J. Mol. Biol.* **2003,** *331,* 303–313.
26. Back, J. W.; Notenboom, V.; Koning, L. J.; Muijsers, A. O.; Sixma, T. K.; Koster, C. G.; Jong, L. Identification of Cross-Linked Peptides for Protein Interaction Studies Using Mass Spectrometry and ^{18}O Labeling. *Anal. Chem.* **2002,** *74,* 4417–4422.
27. Tang, X.; Munske, G. R.; Siems, W. F.; Bruce, J. E. Mass Spectrometry Identifiable Cross-Linking Strategy for Studying Protein—Protein Interactions. *Anal. Chem.* **2005,** *77,* 311–318.
28. Chu, F.; Mahrus, S.; Craik, C. S.; Burlingame, A. L. Isotope-Coded and Affinity-Tagged Cross-Linking (ICATXL): An Efficient Strategy to Probe Protein Interaction Surfaces. *J. Am. Chem. Soc.* **2006,** *128,* 10362–10363.
29. Lamos, S. M.; Krusemark, C. J.; McGee, C. J.; Scalf, M.; Smith, L. M.; Belshaw, P. J. Mixed Isotope Photoaffinity Reagents for Identification of Small-Molecule Targets by Mass Spectrometry. *Angew. Chem. Int. Ed.* **2006,** *45,* 4329–4333.
30. Sinz, A. Isotope-Labeled Photoaffinity Reagents and Mass Spectrometry to Identify Protein–Ligand Interactions. *Angew. Chem. Int. Ed.* **2007,** *46,* 660–662.
31. Seebacher, J.; Mallick, P.; Zhang, N.; Eddes, J. S.; Aebersold, R.; Gelb, M. H. Protein Cross-Linking Analysis Using Mass Spectrometry, Isotope-Coded Cross-Linkers, and Integrated Computational Data Processing. *J. Proteome Res.* **2006,** *5,* 2270–2282.
32. Müller, D. R.; Schindler, P.; Towbin, H.; Wirth, U.; Voshol, H.; Hoving, S.; Steinmertz, M. O. Isotope-Tagged Cross-Linking Reagents. A New Tool in Mass Spectrometric Protein Interaction Analysis. *Anal. Chem.* **2001,** *73,* 1927–1934.
33. Petrotchenko, E. V.; Olkhovik, V. K.; Borchers, C. H. Isotopically Coded Cleavable Cross-Linker for Studying Protein–Protein Interactions and Protein Complexes. *Mol. Cell. Proteomics* **2005,** *4,* 1167–1179.
34. Gardsvoll, H.; Gilquin, B.; Du, M. H. L.; Ménèz, A.; Jorgensen, T. J. D.; Ploug, M. Characterization of the Functional Epitope on the Urokinase Receptor. *J. Biol. Chem.* **2006,** *281,* 19260–19272.
35. Gao, Q.; Doneanu, C. E.; Shaffer, S. A.; Adman, E. T.; Goodlett, D. R.; Nelson, S. D. Identification of the Interactions Between Cytochrome P450 2E1 and Cytochrome b_5 by Mass Spectrometry and Site-Directed Mutagenesis. *J. Biol. Chem.* **2006,** *281,* 20404–20417.
36. Hurst, G. B.; Lankford, T. K.; Kennel, S. J. Mass Spectrometric Detection of Affinity Purified Crosslinked Peptides. *J. Am. Soc. Mass Spectrom.* **2004,** *15,* 832–839.
37. Sinz, A.; Kalkhof, S.; Ihling, C. Mapping Protein Interfaces by a Trifunctional Cross-Linker Combined with MALDI-TOF and ESI-FTICR Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2005,** *16,* 1921–1931.
38. Liu, B.; Archer, C. T.; Burdine, L.; Gillette, T. G.; Kodadek, T. Label Transfer Chemistry for the Characterization of Protein–Protein Interactions. *J. Am. Chem. Soc.* **2007,** *129,* 12348–12349.
39. Davidson, W. S.; Hilliard, G. M. The Spatial Organization of Apolipoprotein A-I on the Edge of Discoidal High Density Lipoprotein Particles. *J. Biol. Chem.* **2003,** *278,* 27199–27207.
40. Kasper, P. T.; Back, J. W.; Vitale, M.; Hartog, A. F.; Roseboom, W.; Koning, L. J.; Maarseveen, J. H.; Muijsers, A. O.; Koster, C. G.; Jong, L. An Aptly Positioned Azido Group in the Spacer of a Protein Cross-Linker for Facile Mapping of Lysines in Close Proximity. *ChemBioChem* **2007,** *8,* 1281–1292.
41. Pearson, K. M.; Pannell, L. K.; Fales, H. M. Intramolecular Cross-Linking Experiments on Cytochrome c and Ribonuclease A Using an Isotope Multiplet Method. *Rapid Comm. Mass Spectrom.* **2002,** *16,* 149–159.
42. Jacobsen, R. B.; Sale, K. L.; Ayson, M. J.; Novak, P.; Hong, J.; Lane, P.; Wood, N. L.; Kruppa, G. H.; Young, M. M.; Schoeniger, J. S. Identification of Novel Quaternary Domain Interactions in the Hsp90 Chaperone, GRP94. *Protein Sci.* **2006,** *15,* 1303–1317.
43. Chu, F.; Maynard, J. C.; Chiosis, G.; Nicchitta, C. V.; Burlingame, A. L. Structure and Dynamics of Dark-State Bovine Rhodopsin Revealed by Chemical Cross-Linking and High-Resolution Mass Spectrometry. *Protein Sci.* **2006,** *15,* 1260–1269.
44. Schilling, B.; Row, R. H.; Gibson, B. W.; Guo, X.; Young, M. M. MS2Assign, Automated Assignment and Nomenclature of Tandem Mass Spectra of Chemically Crosslinked Peptides. *J. Am. Soc. Mass Spectrom.* **2003,** *14,* 834–850.
45. Gaucher, S. P.; Hadi, M. Z.; Young, M. M. Influence of Crosslinker Identity an Position on Gas-Phase Dissociation of Lys-Lys Crosslinked Peptides. *J. Am. Soc. Mass Spectrom.* **2006,** *17,* 395–405.
46. Roepstorff, P.; Fohlman, J. Proposal for a Common Nomenclature for Sequence Ions in Mass Spectra of Peptides. *Biomed. Mass Spectrom.* **1984,** *11,* 601.
47. Biemann, K. Nomenclature for Peptide Fragment Ions. *Methods Enzymol.* **1990,** *193,* 886–887.
48. Paizs, B.; Suhai, S. Fragmentation Pathways of Protonated Peptides. *Mass Spectrom. Rev.* **2005,** *24,* 508–548.
49. Gardner, M. W.; Brodbelt, J. S. Impact of Proline and Aspartic Acid on the Dissociation of Intermolecularly Crosslinked Peptides. *J. Am. Soc. Mass. Spectrom.* **2008,** *19,* 344–357.