Anal. Chem. **2006,** *78,* 8059−8068

Collision-Induced Dissociative Chemical Cross-Linking Reagents and Methodology: Applications to Protein Structural Characterization Using Tandem Mass Spectrometry Analysis

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Chemical cross-linking combined with mass spectrometry is a viable approach to study the low-resolution structure of protein and protein complexes. However, unambiguous identification of the residues involved in a cross-link remains analytically challenging. To enable a more effective analysis across various MS platforms, we have developed a novel set of collision-induced dissociative cross-linking reagents and methodology for chemical cross-linking experiments using tandem mass spectrometry (CID-CXL-MS/MS). These reagents incorporate a single gas-phase cleavable bond within their linker region that can be selectively fragmented within the in-source region of the mass spectrometer, enabling independent MS/MS analysis for each peptide. Initial design concepts were characterized using a synthesized cross-linked peptide complex. Following verification and subsequent optimization of cross-linked peptide complex dissociation, our reagents were applied to homodimeric glutathione *S*-transferase and monomeric bovine serum albumin. Cross-linked residues identified by our CID-CXL-MS/MS method were in agreement with published crystal structures and previous cross-linking studies using conventional approaches. Common LC/MS/MS acquisition approaches such as data-dependent acquisition experiments using ion trap mass spectrometers and product ion spectral analysis using SEQUEST were shown to be compatible with our CID-CXL-MS/MS reagents, obviating the requirement for high resolution and high mass accuracy measurements to identify both intra- and interpeptide cross-links.

Chemical cross-linking combined with mass spectrometry (MS) can provide an alternative to high-resolution techniques such as multidimensional NMR or X-ray crystallography to study the low-resolution structure of protein topology and protein interactions.[1−3] These measurements require relatively small amounts of sample and can be performed under a wide range of conditions to mimic native protein environments not otherwise compatible with high-resolution techniques.[1,4] For cross-linking to be effective for the diverse array of proteins encountered in higher order complexation, a myriad of commercially available cross-linking reagents have been developed. They include a large assortment of bi- and trifunctional reagents that vary in the reactive chemical functional groups which direct the cross-linking reaction to side chains of specific amino acid residues (e.g., Lys and Cys) or vary within the linker region, which determines the maximum distance between the reactive groups.[5]

Although the chemistry involved in covalent attachment of common cross-linking reagents to amino acid residue side chains is well characterized, the MS identification of the chemically modified regions of the protein and the specific amino acid residues that contain the modification remains analytically challenging for several reasons. First, most strategies involve the use of SDS-PAGE to separate cross-linked proteins from non-cross-linked proteins with subsequent in-gel tryptic digestion of the cross-linked protein band. The peptides are extracted from the gel matrix to produce a complex mixture that impairs MS identification of the cross-linked peptides due to the relatively small percentage of desired cross-linked products relative to the other peptides present within the sample. Second, peptide identification using MS/MS analysis is difficult because the resulting fragmentation spectrum of the cross-linked species contains product ions (e.g., b and y ions, corresponding to peptide N- and C-terminal fragment ions, respectively) originating from both peptides involved in the cross-link. This precludes making sequential amino acid assignments for each peptide based on de novo sequencing or database-searching algorithms. As a result of these challenges, most work involving chemical cross-linking and MS analysis is performed using matrix-assisted laser desorption/ionization or electrospray ionization (ESI) with either time-of-flight or Fourier transform ion cyclotron resonance mass spectrometers that are capable of measuring the m/z of the intact cross-linked peptides and their product ions with high resolution and high mass measurement accuracy to enable identification. Even with more advanced MS instrumentation and the assistance

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(1) Sinz, A. *J. Mass Spectrom.* **2003**, *38*, 1225−1237.
(2) Trakselis, M. A.; Alley, S. C.; Ishmael, F. T. *Bioconjugate Chem.* **2005**, *16*, 741−750.
(3) Sinz, A. *Mass Spectrom. Rev.* **2006**, *25*, 663−683.
(4) Back, J. W.; de Jong, L.; Muijsers, A. O.; de Koster, C. G. *J. Mol. Biol.* **2003**, *331*, 303−313.
(5) Wong, S. S. *Chemistry of protein conjugation and cross-linking.*; CRC Press: Boca Raton, FL, 1991.

10.1021/ac0613840 CCC: $33.50
Published on Web 10/28/2006

of liquid chromatographic separations, the identification of the cross-linked species is only moderately increased and is still highly dependent on protein size and complexity. In addition, custom-designed software is often required to characterize or verify the cross-linked species.[6,7]

In an effort to aid in the identification of cross-linked peptides using MS, several different approaches have been suggested. They include cross-linking reagents that incorporate the use of marker ions resulting from low-energy CID,[8] isotope-coding strategies such as proteolytic digestion in ^{18}O-water,[9] isotope coding of the cross-linking reagents[10] or of the proteins,[11] and enrichment of cross-linked products via affinity tags.[12] All of these strategies provide a mass-encoded constraint that promotes identification using mass measurements, yet only offer a remote possibility of assisting in identification of protein cross-links using lower resolution instruments that are present or accessible in many laboratories.

To improve the ability of identifying cross-linked peptides and enable the use of lower accuracy mass analyzers (e.g., ion traps and triple quadrupoles) and commercially available MS data acquisition and analysis software for characterizing cross-linked peptides, we have developed a novel collision-induced dissociative cross-linking reagent and methodology for chemical cross-linking experiments using tandem mass spectrometry (CID-CXL-MS/MS). Our approach enables unequivocal identification of cross-linked complexes, determination of the amino acid sequence of each peptide in the complex using both database-searching and de novo sequencing algorithms, and identification of the site(s) of the covalent chemical modification within each cross-linked peptide. The developed technique should prove to be of great interest to those who study protein−protein interactions using chemical cross-linking and have access to any MS instrument or proteomic facility capable of MS/MS analysis.

EXPERIMENTAL SECTION

Materials. Fmoc-protected amino acids and NovaSyn TGT resin were from EMD Biosciences (San Diego, CA). Essentially fatty acid- and globulin-free bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO) and was used without additional purification. *Schistosoma japonicum* glutathione *S*-transferase (GST) was overexpressed using a pGEX4T2 expression vector from GE Healthcare (formerly Amersham Biosciences) in the BL(21) DE3 *Escherichia coli* cell line. After overexpression and cell lysis, GST was purified by affinity chromatography using immobilized glutathione. GST was eluted with a gradient of glutathione/PBS buffer and purity was monitored using 15% SDS-PAGE. The resulting protein included 20 additional amino acids due to the placement of the stop codon (additional residues: SDLVPRGSPGIPGSTRAAAS). The peptide Ac-MDKVLNRE was purchased from Bachem (King of Prussia, PA) and used without further purification. Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). Acetonitrile (HPLC grade) and formic acid (ACS reagent grade) were from Aldrich (Milwaukee, WI). Water was distilled and purified using a High-Q 103S water purification system (Wilmette, IL). All other chemicals were from Sigma/Aldrich/Fluka unless otherwise stated.

Synthesis of Bisuccinimidyl-succinamyl-aspartyl-prolyl-glycine (SuDPG). Fmoc-Gly-NovaSyn TGT extremely acid-sensitive preloaded resin was used to synthesize the linker region of the SuDPG cross-linking reagent. Fmoc-Pro-OH followed by Fmoc-Asp(OtBu)-OH was coupled to the resin-bound amino acid batchwise using the active ester method. Briefly, following deprotection of the resin-bound amino acid, active esters were formed using Fmoc-amino acid, *N*-hydroxybenzotriazole, and diisopropylcarbodiimide in a 5:5:5.5 molar ratio to the resin−bound amino acid. Following Fmoc deprotection of Fmoc-Asp(OtBu)-OH, succinic anhydride was added to introduce a carboxylate group to the N-terminus of the reagent linker region. The completed linker region was cleaved from the resin with 1% trifluoroacetic acid (TFA) in dichloromethane. Following high-performance liquid chromatography (HPLC) purification, esterification of the dicarboxylic acid was performed using di(N-succinimidyl) oxalate. The esterified product was HPLC purified, dried under vacuum, and stored at −20 °C. Using various concentrations of disuccinimidyl suberate and saponification of the *N*-hydroxysuccinimidyl esters using NaOH, a standard curve was generated for the signal obtained at 260 nm and used to quantify our HPLC-purified product. Before use, the aspartyl side chain *tert*-butyl protecting group was deprotected by addition of 95% TFA/5% water. The mixture was stirred at ambient temperature for 1 h, then dried under vacuum, and subsequently stored at −20 °C.

Synthesis of Bisuccinimidyl-succinamyl-aspartyl-proline (SuDP). Fmoc-Pro-NovaSyn TGT extremely acid-sensitive preloaded resin was used to synthesize the linker region of the SuDP cross-linking reagent. Fmoc-Asp(OtBu)-OH followed by succinic anhydride was coupled to the resin as described for SuDPG. Esterification, purification, quantification of the product, and removal of the aspartyl side chain *tert*-butyl protecting group were performed as described for SuDPG.

In-Source Collision-Induced Dissociation (ISCID) Analysis. To characterize ISCID of a cross-linked peptide complex, a suitable standard had to be synthesized. This was achieved by incubating the cross-linking reagent SuDPG, previously solubilized in dimethyl sulfoxide (DMSO), in the presence of Ac-MDKVLNRE in 100 mM sodium phosphate, 25 mM NaCl, pH 7.5, in a 1:1 molar ratio to produce a final concentration of 5 mM. The reaction was incubated at ambient temperature for 45 min and then quenched with a final concentration of 50 mM Tris, pH 7.5, for 15 min. The resulting mixture was separated on an Atlantis 4.6 × 150 mm C_{18} reversed-phase column (Waters Corp., Milford, MA) using an acetonitrile/0.1% formic acid gradient. Based on the chromatograms generated at 215 nm, fractions corresponding to cross-linked peptides as verified by ESI-MS were pooled and dried under vacuum. The cross-linked peptide was dissolved in 0.2% formic

(6) Schilling, B.; Row: R. H.; Gibson, B. W.; Guo, X.; Young, M. M. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 834−850.
(7) Tang, Y.; Chen, Y.; Lichti, C. F.; Hall, R. A.; Raney, K. D.; Jennings, S.F. *BMC Bioinformatics* **2005**, *6*, S9.
(8) Back, J. W.; Hartog, A. F.; Dekker: H. L.; Muijsers, A. O. Koning, L. J.; Jong, L. d. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 222−227.
(9) Back, J. W.; Notenboom, V.; de Koning, L. J.; Muijsers, A. O.; Sixma, T. K.; de Koster, C. G.; de Jong, L. *Anal. Chem.* **2002**, *74*, 4417−4422.
(10) Petrotchenko, E. V.; Olkhovik, V. K.; Borchers, C. H. *Mol. Cell. Proteomics* **2005**, *4*, 1167−1179.
(11) Taverner, T.; Hall, N. E.; OHair, R. A. J.; Simpson, R. J. *J. Biol. Chem.* **2002**, *277*, 46487−46492.
(12) Hurst, G. B.; Lankford, T. K.; Kennel, S. J. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 832−839.

acid in water/methanol (1/1, v/v) to ~100 ng/μL and infused at a flow rate of 1.5 μL/min through a 150-μm-i.d. fused-silica capillary containing a flamed, pulled capillary tip. An LCQ Deca ion trap mass spectrometer (Thermo Electron, San Jose, CA) was operated in positive ESI mode with an applied voltage of 2.2 kV. During MS scanning, data were acquired for 10 scans (~15 s) for various voltage settings. Additional potential was applied to the multipole region of the instrument in 5-V increments up to 50 V by entering the value in the instrument acquisition method file. The effect of the additional potential of 15 V is diagramed in Figure 2. For postacquisition analysis, the MS data acquired at each potential were integrated over 10 scans.

Cross-Linking, SDS-PAGE, and In-Gel Digestion of *S. japonicum* Glutathione *S*-Transferase. The appropriate cross-linker-to-protein ratio was determined by resuspending SuDPG or SuDP in DMSO to a final concentration of 90 mM. Various cross-linker amounts were added to 15 μL of 30 μM GST in 100 mM sodium phosphate, 25 mM NaCl, 1 mM EDTA, pH 7.5, to produce final protein-to-cross-linker molar ratios of 1:10, 1:42, 1:100, and 1:500. Each reaction was incubated at ambient temperature for 30 min. Each reaction was quenched by the addition of 1 M Tris, pH 7.5, to produce a final concentration of 20 mM, and then the solution was incubated for 15 min at ambient temperature to ensure complete deactivation of the cross-linking reagent. The volume of the resulting mixture was reduced to half via vacuum centrifugation, diluted with loading buffer, and subsequently transferred onto a 15% SDS-PAGE gel. Protein bands were visualized by Coomassie staining.

Protein bands of interest were excised and destained using 1 mL of 40% acetonitrile/60% 50 mM ammonium bicarbonate, pH 8.0. After destaining, each protein gel slice was reduced for 1 h at 37 °C with 5 mM DTT in 50 mM ammonium bicarbonate, pH 8.0, and Cys residues were alkylated with 20 mM iodoacetamide in 50 mM ammonium bicarbonate, pH 8.0, at 37 °C for 1 h. For in-gel proteolysis, trypsin in 50 mM ammonium bicarbonate, pH 8.0, was used at a final 1:5 (w/w) trypsin-to-protein ratio and incubated overnight at 37 °C. Peptides were extracted from the gel three times using 200 μL of 60% acetonitrile/35% 50 mM ammonium bicarbonate, pH 8.0/5% formic acid (v/v/v) with sonication in a water bath for 20 min. Extractions were pooled, dried under vacuum centrifugation, and stored at −80 °C until LC/MS/MS analysis could be performed. Dried peptides were resuspended using 12 μL of 5% acetonitrile/0.1% formic acid, and 8 μL was used for each LC/MS/MS analysis. Identified cross-linked residues using our CID-CXL-MS/MS approach were compared to the X-ray crystallographic structure of *S. japonicum* glutathione S-transferase (PDB: 1U88) using Pymol rendering software (version 0.99, Delano Scientific).

Cross-Linking, In-Solution Digestion, and In-Gel Digestion of Bovine Serum Albumin. SuDP in DMSO was added to BSA in 100 mM sodium phosphate, 25 mM NaCl, 1 mM EDTA, pH 7.5, to produce a final concentration of 10 μM protein to 500 μM cross-linker. The reactions were incubated at ambient temperature for 30 min then quenched by the addition of 1 M Tris, pH 7.5, to produce a final concentration of 20 mM. The solution was then incubated for 15 min at ambient temperature to ensure complete deactivation of the cross-linking reagent. Samples were reduced with 40 mM DTT for 1 h at 37 °C and alkylated with 200 mM iodoacetamide for 1 h at ambient temperature. The protein samples were then either dialyzed overnight against 50 mM ammonium bicarbonate, pH 8.0, using a 3000 MWCO Slide-A-Lyzer dialysis cassette (Pierce Biotechnology, Rockford, IL) or separated using SDS-PAGE. Dialyzed samples were digested overnight at 37 °C with trypsin using an enzyme-to-protein ratio of 1:10. Proteins were separated by SDS-PAGE, visualized with Coomassie staining, and the cross-linked protein band processed as previously described for GST. All digested samples were dried using vacuum centrifugation and stored at −80 °C until LC/MS/MS analysis was performed. Identified cross-linked residues using our CID-CXL-MS/MS approach were compared to the X-ray crystallographic structure of human serum albumin (PDB: 1AO6) using Pymol rendering software (version 0.99, Delano Scientific).

Microcapillary Reversed-Phase Liquid Chromatography/Tandem Mass Spectrometry. Peptide samples were separated using an Agilent 1100 Series capillary LC system (Agilent Technologies, Inc., Palo Alto, CA) coupled on-line with a LCQ Deca ion trap mass spectrometer (Thermo Electron). The instrument was equipped with an in-house-manufactured ESI interface and operated in positive ion mode with an ESI voltage of 2.2 kV. The reversed-phase capillary HPLC column containing 5-μm Jupiter C_{18} stationary phase (Phenomenex, Torrance, CA) was slurry-packed in-house into a 150 μm i.d. × 50 cm length capillary (Polymicro Technologies Inc., Phoenix, AZ). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. After loading the sample onto the reversed-phase column, the mobile phase was held at 5% B for 20 min and the peptides were eluted at a flow rate of 1.5 μL/min using one of two linear gradients: 1% B/min to 95% B or 0.5% B/min to 95% B.

The data acquisition sequence used for all LC/MS/MS analyses employed a series of six scan events. In this sequence, an MS scan of m/z 500−2000 was performed first. Next, an ISCID MS scan of m/z 500−2000 implementing an additional potential offset of 10 or 15 V was performed. These voltages were experimentally determined based on the ISCID experiment using a synthesized cross-linked complex (refer to Figure 3B for details). Then in a data-dependent manner, the four most intense ions detected in the second MS scan (i.e., the ISCID MS scan) were sequentially captured and subjected to MS/MS using a normalized collision energy setting of 45%. To increase the selective capture of lower abundant precursor ions, a 2-min dynamic exclusion list was employed.

Identification of Cross-Linked and Non-Cross-Linked Peptides. All peptides were identified by searching the product ion spectra against a database containing the *S. japonicum* GST, BSA, and trypsin protein sequences using TurboSEQUEST (BioWorks 3.1, Thermo Electron). All spectra were searched with a dynamic mass modification of +57.0 u on Cys residues, due to the additional mass of carboxamidomethylation, and a dynamic modification of +16.0 u on Met residues, due to oxidation. Samples cross-linked with SuDPG were searched with dynamic mass modifications on the N-terminus, Lys, Ser, and Tyr residues of +154.1 (prolyl-glycine, PG),+197.1 (succinamyl-aspartyl-anhydride, SuD), and +215.1 u (succinamyl-aspartate, SuD + H_2O). Samples cross-linked with SuDP were searched with dynamic mass modifications on the N-terminus, Lys, Ser, and Tyr residues

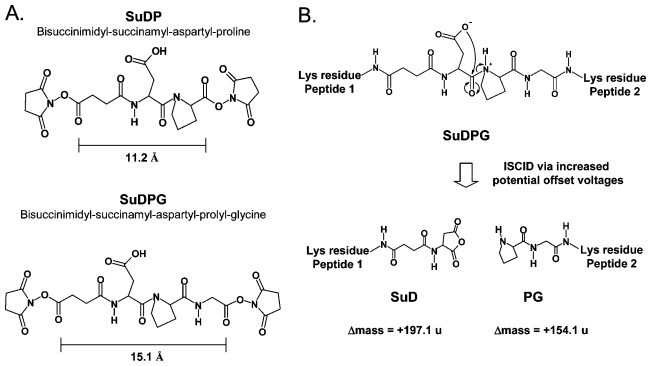

Figure 1. ISCID cross-linking reagents for use in our CID-CXL-MS/MS approach. (A) Synthesized SuDP and SuDPG cross-linking reagents are shown with the calculated distances between succinimidyl ester reactive sites using a trans conformation about the aspartyl−prolyl amide bond. (B) ISCID of the reagent SuDPG. The fragmentation mechanism at the aspartyl−prolyl bond within SuDPG due to the application of additional potential offset voltages between the skimmer lens and multipole region of the mass spectrometer is presented. The resulting mass additions (unified atomic mass unit, u) following SuDPG fragmentation are indicated below the individual peptide products.

of +97.1 (proline, P),+197.1 (SuD), and +215.1 u (SuD + H_2O). To initially identify a peptide, charge-dependent cross-correlation scores (Xcorr) of at least 1.6, 1.5, and 2.9 for +1, +2, and +3 charged species, respectively, and delta-correlation (ΔC_n) scores of 0.1 and higher were used.[13] Manual inspection of the ions was performed to validate all SEQUEST identifications and to ensure acceptable ion coverage.

To identify a pair of cross-link peptides, SEQUEST results were arranged in the order they were acquired by the mass spectrometer (i.e., scan number). Because the residual mass of the fragmented cross-linker remains attached to the lysyl residues, the sorted results were searched for a product ion spectrum that confirmed the presence of a peptide with +97.1 (P) or +154.1 u (PG) mass modification on a lysyl residue that was adjacent to a product ion spectrum for a peptide with +197.1 (SuD) or +215.1 u (SuD + H_2O) modification on a lysyl residue. For these MS/MS pairs, the MS scan prior to their acquisition was observed for the calculated m/z of the intact cross-linked peptide complex. The following ISCID MS scan event was then examined for the emergence of the two individual peptide product ions generated from dissociation at the aspartyl−prolyl bond within the cross-linking reagent. The same analysis was performed for the data obtained using the SuDP cross-linking reagent and its modification masses.

RESULTS AND DISCUSSION

Cross-Linker Design and Rationale. Cross-linking experiments often employ the use of multiple reagents varying in the linker region length to promote a more comprehensive mapping of protein surface topology and interactions. In accordance with this, our initial cross-linker design included two versions, both containing aspartyl−prolyl bonds but varying in linker region length (Figure 1A). The novel feature of incorporating an aspartyl−prolyl bond near the center of the linker region provides a controllable and specific gas-phase cleavage site. Previous studies have shown that these particular amide bonds preferentially fragment during CID and require less energy to effectively achieve fragmentation compared to other peptide bonds.[14−17] This decreased peptide bond stability is thought to be mediated by the transfer of a labile proton from the aspartyl side chain to the basic backbone amine of the adjacent prolyl residue. The carboxy anion on the aspartyl side chain then attacks the carbonyl carbon, resulting in a cyclized anhydride structure for the aspartyl residue and a free prolyl residue.[14] This cleavage reaction was found to be independent of the length of the peptide on either side of the aspartyl-prolyl sequence but was dependent on the amount of additional CID energy, as increasing the voltage between the heated capillary and the source house (cone voltage) resulted in more intensive fragmentation.[17] Because of these properties, we have incorporated this peptide bond into the linker region of our cross-linking reagent to allow for single-site fragmentation within the reagent to produce two unique peptides, each containing an additional mass modification corresponding to the remaining portion of the fragmented cross-linking reagent (Figure 1B). Tandem mass spectrometry of the individual peptides at higher energy CID then allows for peptide identification and determination of the site(s) of modifications via interpretation of the generated y and b ions.

Characterizing ISCID of Aspartyl−Prolyl Bonds in CID-CXL-MS/MS Analysis. Initially, we performed fragmentation analysis of the aspartyl−prolyl bond within the quadrupole ion trap. However, limitations in commercial software control of the LCQ Deca mass spectrometer to implement a data-dependent scanning sequence for the four most intense signals of a full MS scan required that our cross-linking reagents (Figure 1A) be fragmented at the aspartyl−prolyl bond before physically reaching the ion trap portion of the instrument to increase the probability of selecting the peptide product ions for in-trap CID. This was accomplished by increasing the multipole dc offset by applying increased negative voltage to both multipoles (Figure 2). This additional potential offset increases the velocity of the ions traveling between the skimmer lens and the multipole region, which in turn increases the ions translational kinetic energy and frequency of collisions with gases (i.e., H_2O, N_2, CO_2) present within this region of the instrument, thus promoting ISCID. Since the amounts and ratios of these gases cannot be specifically controlled by the user on an unmodified instrument, unlike the helium collision gas within the ion trap itself, the fragmentation efficiency of the aspartyl−prolyl bond using ISCID by applying various addition potential offset voltages needed to be experimentally determined.

To characterize the dissociation of two cross-linked peptides by ISCID at the aspartyl−prolyl bond within our cross-linking reagent, SuDPG was incubated with the peptide, Ac-MDKVLNRE, to generate a cross-linked complex (M-SuDPG-M) as indicated in Figure 3A. The peptide used for derivatization was chosen

(13) Peng, J.; Elias, J. E. Thoreen, C. C.; Licklider, L. J.; Gygi, S. P. *J. Proteome Res.* **2003**, *2*, 43−50.
(14) Yu, W.; Vath, J. E.; Huberty, M. C.; Martin, S. A. *Anal. Chem.* **1993**, *65*, 3015−3023.
(15) Mak, M.; Mezo, G.; Skribanek, Z.; Hudecz, F. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 837−842.
(16) Huang, Y.; Triscari, J. M.; Tseng, G. C.; Pasa-Tolic, L.; Lipton, M. S.; Smith, R. D.; Wysocki, V. H. *Anal. Chem.* **2005**, *77*, 5800−5813.
(17) Skribanek, Z.; Mezo, G.; Mak, M.; Hudecz, F. *J. Pept. Sci.* **2002**, *8*, 398−406.

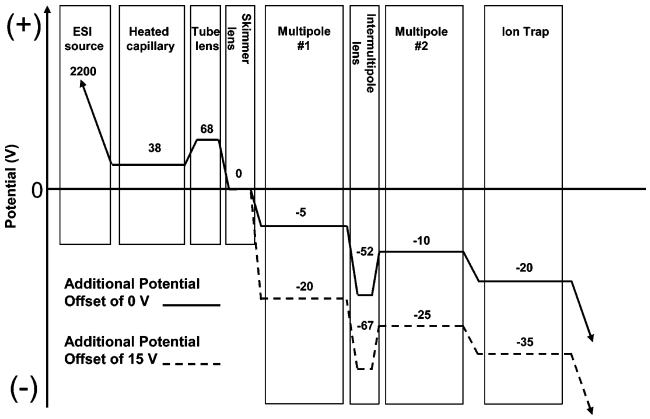

Figure 2. ISCID in the LCQ Deca mass spectrometer. The solid line indicates the potential applied to each portion of the mass spectrometer with no additional potential offset (i.e., additional potential offset of 0 V). Application of ISCID increases the negative voltage applied to the multipole region of the instrument, which includes multipole 1, the intermultipole lens, multipole 2, and the ion trap mass analyzer by a user-defined amount. The dotted line indicates an increase in the multipole dc offset voltage of 15 V (i.e., additional potential offset of 15 V). This increased potential offset between the skimmer lens and the multipole region increases both the translational kinetic energy of the ions and their frequency of collision with gas molecules present in this region of the instrument which leads to CID within the source region.

because (1) it contained only one primary amine that was reactive toward the SuDPG cross-linking reagent due to acylation of the N-terminus and (2) it contained at least one basic residue (Arg) near the C-terminus and thus closely mimics a tryptic peptide product. As shown in Figure 3B, the relative ion intensity of the cross-linked peptide complex and resulting modified peptides due to aspartyl−prolyl cleavage were measured at various additional ISCID voltages. With no additional potential offset, the [M-SuDPG-M + 2H]$^{2+}$ and [M-SuDPG-M + Na + 2H]$^{3+}$ intact cross-linked pair of peptides are the predominant ions in the MS spectrum (Figure 3C); however, the two individual peptides with the addition of either SuD ([M-SuD + H]$^{+}$) or PG ([M-PG + H]$^{+}$) are readily detectable. The optimum potential offset at 15 V to promote ISCID fragmentation results in a decrease in the relative ion abundance of the intact cross-linked peptide complex [M-SuDPG-M + 2H]$^{2+}$ while substantially increasing the relative ion abundance of the peptide product ions [M-SuD + H]$^{+}$ and [M-PG + H]$^{+}$ (Figure 3D). This controlled change in ion abundance generated from specific fragmentation within the cross-linking reagent is important for automation of our CID-CXL-MS/MS approach, since the precursor ions selected for MS/MS will depend on product ion abundance generated during the ISCID MS scan.

Although ISCID is less controllable than in-trap CID, more pronounced fragmentation of the aspartyl−prolyl amide bond within our reagent was observed for the cross-linked peptide complex as compared to the aspartyl−prolyl bond contained within a peptide (data not shown). The result of this increased fragmentation propensity can be observed in Figure 3C where no additional potential offset was required to promote fragmentation. This phenomenon for our CID-CXL-MS/MS derivatives may be due to the ease of product ion formation, the stability of the product ions, or the result of a gas-phase conformation that promotes alternative dissociation channels for fragmentation.

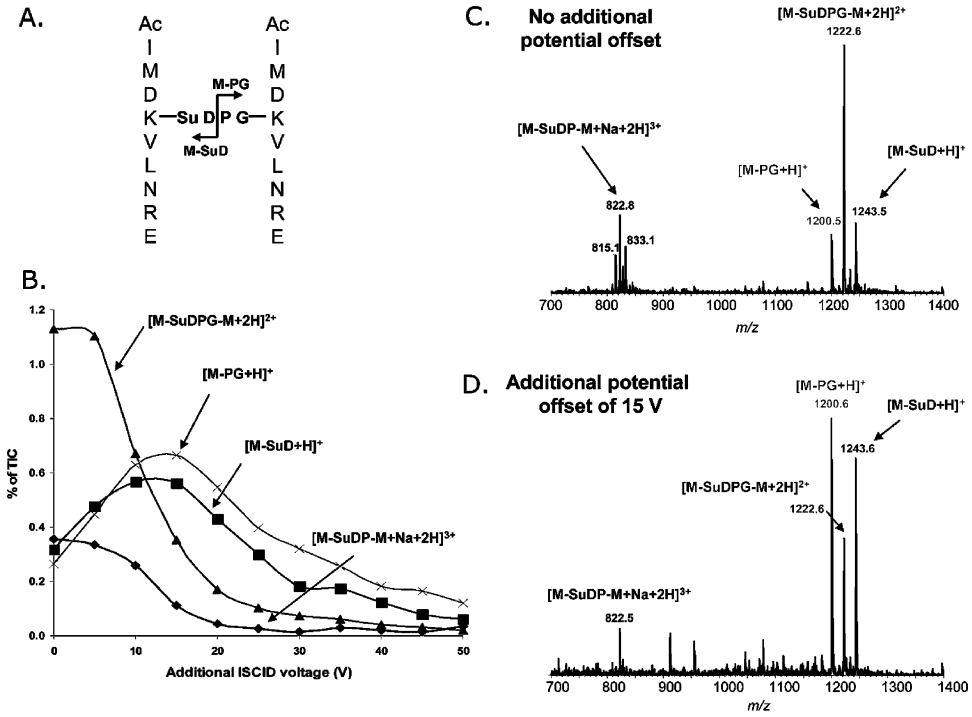

Figure 3. ISCID analysis of the aspartyl−prolyl bond of a SuDPG interpeptide cross-link. (A) Two N-terminal acylated peptides cross-linked at their lysyl side chains with SuDPG (M-SuDPG-M, calculated average mass 2443.6 Da), along with the predicted cross-linker fragmentation to generate product ions M-SuD (calculated monoisotopic mass of 1242.6 Da) and M-PG (calculated monoisotopic mass of 1199.6 Da). (B) The percentage of the total ion current (TIC) for [M-SuDPG-M + 2H]$^{2+}$ (▲), [M-SuDPG-M + Na + 2H]$^{3+}$ (◆), [M-PG + H]$^{+}$ (x), and [M-SuD + H]$^{+}$ (■) ions at additional potential offset voltages between 0 and 50 V. (C) Mass spectrum (integration of 10 individual MS scans) produced without any additional potential offset. (D) Mass spectrum (integration of 10 individual MS scans) produced with an additional potential offset of 15 V. The application of the additional potential offset voltages was applied to the instrument as indicated in Figure 2.

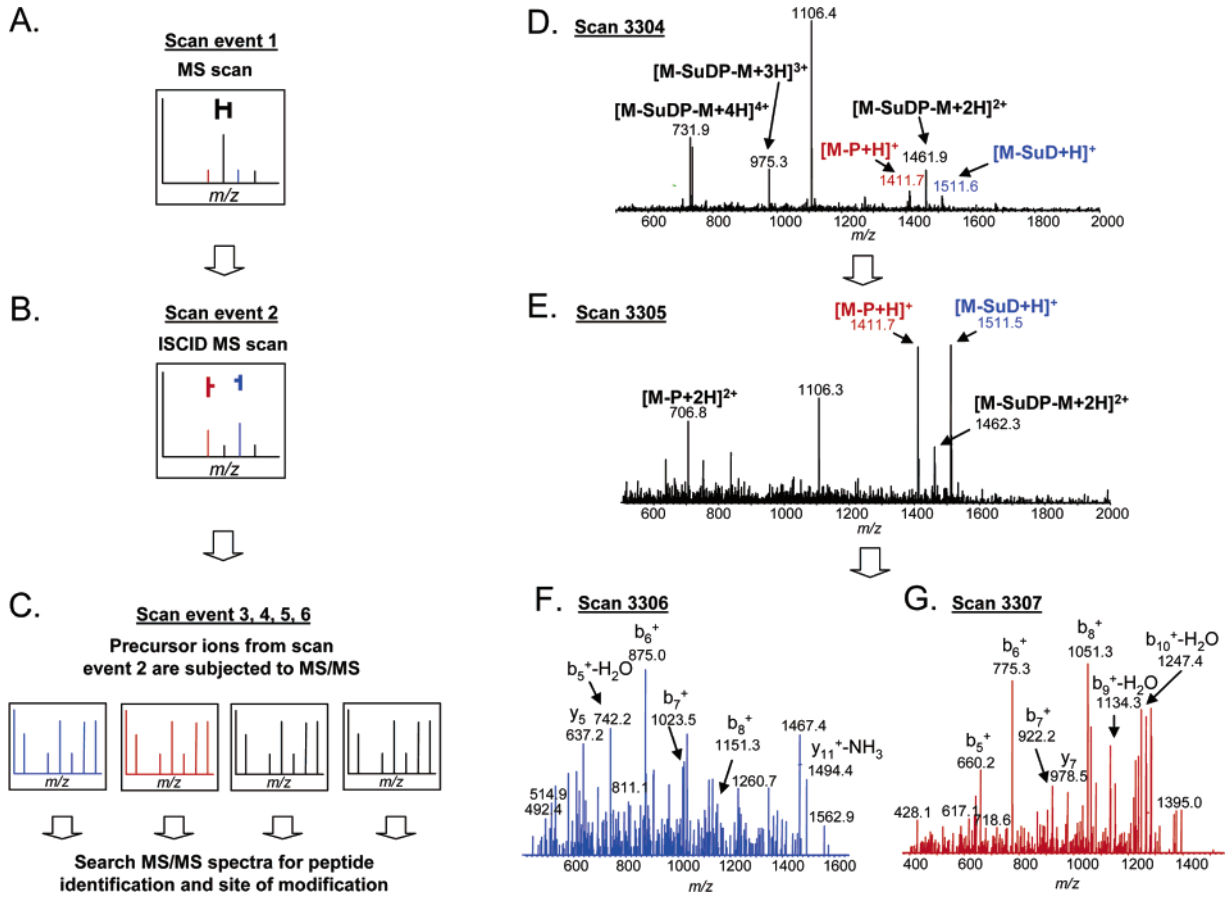

Figure 4. CID-CXL-MS/MS acquisition parameters and data analysis for protein cross-link identification. (A) Scan event 1: MS scan to detect precursor ions without any additional potential offset. (B) Scan event 2: ISCID MS scan with the application of an additional potential offset between the skimmer lens and multipole region of the mass spectrometer to promote fragmentation within the SuDPG or SuDP cross-linking reagents. This scan generates two separate peptide product ions (red and blue ions), each containing an additional mass corresponding to the remaining portion of the reagent. (C) Scan event 3−6: four individual MS/MS scan events of the most abundant ions generated in scan event 2 (i.e., red and blue ions from the ISCID MS scan). The resulting product ion spectra are then searched using the SEQUEST algorithm to identify peptides and the location of the modified lysyl residues introduced by the cross-linking reagent. (D) Scan 3304: MS spectrum from *m/z* 500 to 2000 contains the intact doubly, triply, and quadruply charged cross-linked GST peptides at *m/z* 1461.9, 975.3, and 731.9, respectively. (E) Scan 3305: ISCID MS spectrum with 15 V of additional potential offset demonstrating specific fragmentation at the aspartyl−prolyl bond that produces the increased relative abundance of $[M\text{-}P + H]^+$ (red ion, *m/z* 1411.7) and $[M\text{-}SuD + H]^+$ (blue ion, *m/z* 1511.5). (F) Scan 3306: data-dependent product ion spectrum of the singly charged precursor ion at *m/z* 1511.5 (blue ion) from the previous ISCID MS scan eliciting an Xcorr of 1.840. (G) Scan 3307: data-dependent product ion spectrum of the singly charge precursor ion at *m/z* 1411.7 (red ion) from the previous ISCID MS scan eliciting an Xcorr of 2.743. It should be noted that in addition to the ions resulting from the cross-linked peptides, a non-cross-linked peptide (*m/z* 1106.4) eluted off the column at the same retention time and was therefore detected in the first MS spectrum in (D). This is expected since the sample is composed of both peptide species. However, the negative impact of selecting non-cross-linked peptides instead of those involved in a cross-link is diminished due to the ISCID MS scan event (refer to E), which reduces the relative intensity of the non-cross-linked peptide (*m/z* 1106.3) while increasing the relative intensity of the peptide products (*m/z* 1411.7 and 1511.5) generated from dissociation of the cross-linked complex (compare D and E) and promoting their selection during data-dependent MS/MS analysis.

Further study is required, particularly in regard to normalizing the degrees of freedom using various peptides, to fully understand the nature of the fragmentation mechanism of the cross-linked peptides relative to a peptide containing an aspartyl−prolyl amide bond and its impact in our CID-CXL-MS/MS approach.

CID-CXL-MS/MS Methodology and Analysis Using SuDP and SuDPG. The SuDPG and SuDP cross-linking reagents were designed to be used in conjunction with common LC/MS/MS acquisition approaches, such as data-dependent acquisition experiments for peptide identification using ion trap mass spectrometers. In a data-dependent acquisition, the mass spectrometer is programmed to continuously cycles through five scan events. The first scan event is an MS scan to detect precursor ions from which the four most intense ions are selected in a data-dependent manner and separately subjected to MS/MS during the next four scan events. When used in conjunction with our SuDPG or SuDP cross-linking reagents, this method also includes an ISCID MS scan (Figure 4B) that incorporates an additional potential offset voltage applied after the initial MS scan (Figure 4A). During the ISCID MS scan, cross-linked peptides are selectively dissociated into two individual peptides, each containing half of the cross-linking reagent. In a data-dependent manner, the four most intense peaks are selected individually from the ISCID MS scan and are subjected to MS/MS in scan events three through six (Figure 4C).

To illustrate our cross-linking identification strategy from a complex peptide mixture, the four scans used in the identification of the intermolecular [I109−K119]−[I109−K119] cross-link of

Table 1. Cross-Linked Peptides of GST Identified[a] Using Our CID-CXL-MS/MS Cross-Linking Reagents SuDPG and SuDP

reagent	additional potential offset (V)	peptide sequence[b]	cross-linked residue	charge state	scan no.	Xcorr[c]
		Interpeptide Cross-links				
SuDPG	15	[109]IAYSK^DFETLK[119]	K113 (monomer 1)	1	3313	1.641
		[109]IAYSK@DFETLK[119]	K113 (monomer 2)	1	3315	2.194
SuDP	15	[109]IAYSK^DFETLK[119]	K113 (monomer 1)	1	3306	1.840
		[109]IAYSK#DFETLK[119]	K113 (monomer 2)	1	3307	2.743
		Intrapeptide Cross-Links				
SuDP	10	[182]RIEAIPQIDK∧YLK#SSK[197]	K191−K194	2	3386	3.869

[a] This set of cross-linked peptides was isolated from a protein band corresponding to cross-linked GST following SDS-PAGE. Lysyl modifications: @, 154.1 u (PG); #, 97.1 u (P); ∧, 197.1 u (SuD). [c] SEQUEST charge-dependent cross-correlation score.

GST with SuDP (Table 1) are shown in Figure 4D−G. The first MS scan of our LC/MS/MS analysis (Figure 4D, scan 3304) shows the intact doubly, triply, and quadruply cross-linked complexes. With an addition potential offset of 15 V, two peaks emerge in the ISCID MS scan (Figure 4E, scan 3305): one corresponding to the singly charged peptide with an additional mass of 197.1 u for the SuD modification ($[M\text{-}SuDP + H]^+$) and the other corresponding to a singly charged peptide with an additional mass of 97.1 u for the P modification ($[M\text{-}P + H]^+$). In a data-dependent manner, these two peaks were selected and subjected to MS/MS in the subsequent scan events (3306 and 3307) as shown in Figure 4F and G. The peptide sequence and the site(s) of modification were then identified from the product ion spectra. Details regarding the data analysis using these acquisition parameters and SEQUEST appear in the Experimental Section.

CID-CXL-MS/MS Analysis of Glutathione *S*-Transferase Using SuDP and SuDPG. The experimental capability of our CID-CXL-MS/MS reagents and methodology was experimentally demonstrated when both inter- and intrapeptide cross-links were unequivocally identified from protein samples using a low-resolution, commercially available ion trap mass spectrometer and software (LCQ Deca and SEQUEST). The first study examined the homodimeric interaction of *S. japonicum* GST, a member of the Mu family of GSTs that is commonly used as a convenient tag for purification of protein constructs. It is known to form homodimers at protein concentrations and buffer conditions similar to those used for cross-linking experiments.[18] In our phosphate buffer at pH 7.5, GST was completely dimeric as determined by size exclusion chromatography (data not shown). Using these conditions, GST was cross-linked with either SuDPG or SuDP, and the resulting protein mixture was separated using SDS-PAGE (Figure 5). Bands corresponding to GST dimers were excised and in-gel digested. The complex peptide mixture was analyzed by our CID-CXL-MS/MS approach, and the results are presented in Table 1. The same pair of intermolecularly cross-linked residues, K113 in one subunit to K113 of the other, was identified using both SuDPG and SuDP with an additional potential offset of 15 V. Since the peptide sequence containing K113 is unique in the protein amino acid sequence, it is clear that the cross-link was between two monomers. An intrapeptide cross-link

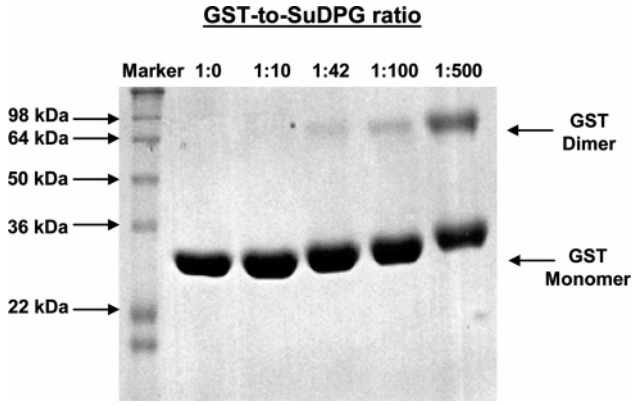

Figure 5. Dose-dependent cross-linking of *S. japonicum* GST cross-linked using SuDPG. To find initial molar ratios of protein-to-cross-linker resulting in an acceptable amount of cross-linked product, 30 μM GST was incubated with 300 μM−15 mM SuDPG (1:10−1:500 protein-to-cross-linker ratio) and qualitatively analyzed by SDS-PAGE. Commassie staining revealed that intermolecular cross-linking between subunits of GST was detectable at 1:42 protein-to-cross-linker ratio and increased in a concentration-dependent manner. At higher concentrations of cross-linking reagent, a slower migration of the monomer band was observed. The additional mass of the monomer was due to the reagent reacting with solvent-accessible lysyl residues that can increase the mass of monomeric GST. For example, the modification of 10 lysyl residues would result in an additional mass of 3.7 kDa. The 1:500 band was excised, in-gel digested, and subjected to LC/MS/MS analysis using our CID-CXL-MS/MS acquisition parameters outlined in Figure 4.

was also identified between K191 and K194 of the same subunit with SuDP using an offset of 10 V.

When mapped to the crystallographic structure of GST, the measured distance between α-carbons of the two K113 lysine residues is 12.8 Å (Figure 6A). To calculate maximal distance constraints of the reagents, both the length of the linker region and the flexibility of the lysyl side chains were used. Therefore, the calculated maximal distance constraints of SuDPG (27.8 Å) and SuDP (23.9 Å) are in agreement with the observed distances in the crystal structure. Experimentally, the presence of the intact cross-linked peptide complex in the MS scan (Figure 4D) and the emergence of the individual peptides in ISCID MS scan (Figure 4E) confirm that the modified peptides identified by the MS/MS scans (Figure 4F and 4G) were covalently cross-linked to one another.

(18) Hornby, J. A.; Codreanu, S. G.; Armstrong, R. N.; Dirr, H. W. *Biochemistry* **2002**, *41*, 14238−14247.

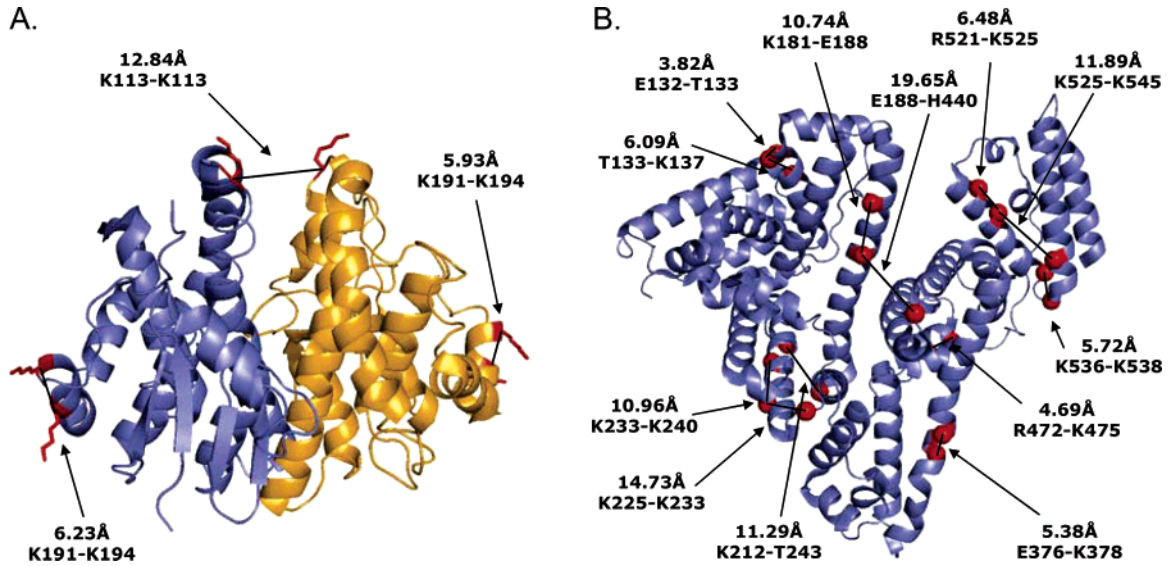

Figure 6. Assessment of CID-CXL-MS/MS identified protein cross-links in GST and BSA. (A) The 3.50-Å resolution X-ray crystallographic structure of *S. japonicum* GST (PDB: 1U88) highlighting the identified cross-linked lysyl residues using SuDPG and SuDP and our CID-CXL-MS/MS approach. The distances between the α-carbons of the indicated lysyl residues are 12.84, 6.23, and 5.93 Å, which are within the calculated maximal distance constraint of SuDPG (27.8 Å) and SuDP (23.9 Å). (B) The 2.50-Å resolution X-ray crystallographic structure of HSA (PDB: 1AO6) highlighting the distances between the α-carbons of the identified cross-linked lysyl resides corresponding to BSA using SuDP and our CID-CXL-MS/MS approach. Although BSA and HSA share 75% sequence identity and 87% sequence homology, several of the identified cross-linked residues in BSA did not have a corresponding lysyl residue at the same position in HSA. Therefore, distances were measured between α-carbons at the corresponding position, regardless of the type of residue present in HSA. The calculated distances between the α-carbons of HSA residue positions corresponding to the identified BSA cross-linked lysyl residues range from 3.82 to 19.65 Å and are within the calculated maximal distance constraint of SuDP (23.9 Å).

Table 2. Cross-Linked Peptides of BSA Identified Using Our CID-CXL-MS/MS Cross-Linking Reagent SuDP

digestion	additional potential offset (V)	peptide sequence[a]	cross-linked BSA residue	corresponding HSA residue[b]	charge state	scan no.	Xcorr[c]	Huang et. al.[d,e]
		Interpeptide Cross-Links						
solution	15	[264]VHK#EC*C*HGDLLEC*ADDR[280]	K266	T243	3	4345	4.145	DSS, DSG
		[233]ALK^AWSVAR[241]	K235	K212	2	4347	2.576	
solution	15	[210]EK^VLTSSAR[218]	K211	E188	1	4201	2.095	ni
		[198]GAC*LLPK#IETMR[209]	K204	K181	1	4202	2.127	
solution	15	[548]K^QTALVELLK[557]	K548	K525	2	5623	3.475	ni
		[562]ATEEQLK#TVMENFVAFVDK[580]	K568	K545	2	5624	3.348	
gel	15	[210]EK#VLTSSAR[218]	K211	E188	1	854	1.641	DSS, BS^3, DSG
		[460]C*C*TK^PESER[468]	K463	H440	2	867	1.732	
solution	15	[548]K#QTALVELLK[557]	K548	K525	2	4819	2.989	ni
		[532]LFTFHADIC*TLPDTEK^QIK[547]	K544	R521	2	4821	2.130	
		Intrapeptide Cross-Links						
solution	10	[387]DDPHAC*YSTVFDK#LK^HLVD-EPQNLIK[412]	K399−K401	E376−K378	3	3595	2.819	BS^3
gel and soln	15	[152]ADEK^K#FWGK[160]	K155−K156	E132−T133	1	4292	2.228	ni
gel	10	[249]AEFVEVTK^LVTDLTK#VHK[266]	K256−K263	K233−K240	2	3769	2.762	ni
solution	15	[246]FPK^AEFVEVTK#LVTDLTK[263]	K248−K256	K225−K233	2	7458	2.030	ni
gel	15	[558]HK^PK#ATEEQLK[568]	K559−K561	K536−K538	2	866	2.402	ni
gel and soln	15	[156]K^FWGK#YLYEIAR[167]	K156−K160	T133−K137	2	5252	3.645	BS^3
gel	15	[490]TPVSEK#VTK^C*C*TESLVNR[507]	K495−K498	R472−K475	2	4621	3.310	DSS, DSG

[a] Residue modifications: #, 97.1 u (P); ∧, 197.1 u (SuD); *, 57.0 u (carboxamidomethyl). [b] The indicated HSA residues correspond to the identified BSA Lys residues as based on sequence alignment. [c] SEQUEST charge dependent cross-correlation score. [d] Cross-linked Lys residues also identified by Huang et al. with the following reagents (linker length): BS^3, (11.4 Å), DSS, (11.4 Å); DSG, (7.7 Å). [e] ni, cross-linked residues not identified by Huang et al.

CID-CXL-MS/MS Analysis of Bovine Serum Albumin Using SuDP. To further characterize one of our cross-linking reagents for structural topology applications, monomeric BSA was cross-linked with SuDP and subjected to our CID-CXL-MS/MS approach using either in-solution or in-gel digestion. The identified cross-links and corresponding lysyl residues are listed in Table 2 with the MS and product ion spectra of the [K548−K557]−[A562-K580] cross-link appearing in Figure 7. To assess our results we compared our BSA cross-links to the study by Huang et al.[19] where bis(sulfosuccinimidyl) suberate (BS^3), disuccinimidyl suberate

(19) Huang, B. X.; Kim, H. Y.; Dass, C. *J. Am. Soc. Mass Spectrom.* **2004**, *121*, 2264−2271.

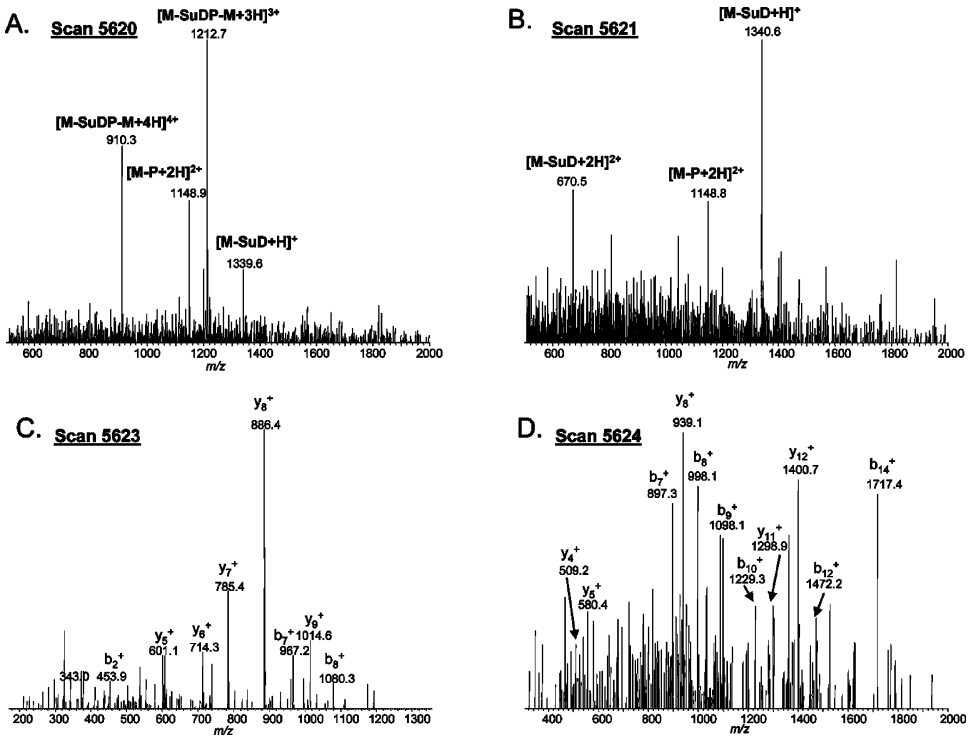

Figure 7. CID-CXL-MS/MS series of scan events identifying the [K548−K557]−[A562−K580] cross-link in BSA using SuDP. (A) Scan 5620: MS spectrum from *m/z* 500 to 2000 detecting the intact triply and quadruply charged cross-linked peptides at *m/z* 1212.7 and 910.3, respectively. (B) Scan 5621: ISCID MS spectrum with 15 V of additional potential offset to specifically fragment the aspartyl−prolyl bond and increase the relative abundance of $[M\text{-}P + 2H]^{2+}$ (*m/z* 1148.8) and $[M\text{-}SuD + 2H]^{2+}$ (*m/z* 670.5). (C) Scan 5623: data-dependent product ion spectrum of the doubly charged precursor ion at *m/z* 670.5 from the previous ISCID MS scan eliciting an Xcorr of 3.475. (D) Scan 5624: data-dependent product ion spectrum of the precursor ion at *m/z* 1148.8 from the previous ISCID MS scan eliciting an Xcorr of 3.348.

(DSS), and disuccinimidyl gluterate (DSG) were used to cross-link BSA. In their analysis, proteolytic isotope coding of peptides using $^{16}O/^{18}O$-water provided a distinctive mass difference between cross-linked and non-cross-linked peptides, permitting the identification of cross-links using high mass accuracy measurements. In terms of linker region length, BS[3] and DSS are 11.4 Å each but vary in reactive group hydrophobicity, whereas DSG has a linker region length of 7.7 Å. Our reagent, SuDP, has a linker region that is only 0.2 Å shorter than that of BS[3] and DSS; however, only 5 of our 12 cross-linked peptides were identified by Huang et al. These data suggest that the remaining seven cross-links are the result of (1) altered flexibility, hydrophobicity, or reactivity of SuDP relative to BS[3] and DSS that affects protein labeling and cross-linking efficiency or (2) our unique CID-CXL-MS/MS approach that enables the cross-linked complex to be more confidently identified than conventional techniques. To further validate our results, the lysyl residues identified as cross-linked were positioned on the X-ray crystallographic structure of human serum albumin (HSA) and their measured distances are indicated in Figure 6B. HSA was used because there is no high-resolution structure available for BSA and these two proteins are thought to be structurally similar due to a 75% sequence identity (87% sequence homology). The maximal distances between the α-carbons at the corresponding HSA positions varied from 3.82 to 19.65 Å and are within the maximal distance constraint of 23.9 Å for SuDP. As shown in Figure 6B, all of our identified cross-links correspond to structurally feasible modifications, thus supporting the cross-links identified using our CID-CXL-MS/MS approach.

Assessing the CID-CXL-MS/MS Approach. The use of CID to aid the identification of cross-linked peptides has previously been attempted by several groups using various custom-designed cross-linking reagents. Although these methods are promising, they have some inherent analytical limitations and require certain MS/MS platforms. The cross-linking reagent developed by Tang et al.[20] is based on the design of incorporating a bisRINK-based linker region into the cross-linking reagent. During CID, this molecule fragments at two positions within the linker region and produces two individual peptides, each with an additional mass on their lysyl residues, and a "reporter ion" generated between the two fragmentation sites. However, this cross-linking reagent is nearly 43 Å in length and precludes its use for informative protein−protein interactions.[3] Because proteins vary greatly in their surface topology and mode of interaction, chemical cross-linking experiments often employ multiple reagents that vary in the length of the linker region to facilitate the yield and variety of cross-linked products for enhanced structural characterization. In this regard, our initial customized cross-linker design included two versions, SuDP and SuDPG, both containing aspartyl−prolyl bonds that have a linker region length of 11.2 and 15.1 Å, respectively. In addition, the reactive ends of each reagent contain succinimidyl esters that selectively target primary amines (i.e., Lys residues) at pH values above 7.0. These attributes are in common with other commercially available cross-linking reagents for probing structural features of monomeric proteins as well as larger protein complexes.[1,3,5]

(20) Tang, X.; Munske, G. R.; Siems, W. F.; Bruce, J. E. *Anal. Chem.* **2005**, *77*, 311−318.

An earlier report by Back et al.[8] also described the use of a reporter ion generated during CID to aid in the identification of cross-linked species, but their reagent does not promote efficient MS/MS analysis. The advantage of our CID-CXL-MS/MS reagents is the ability to selectively dissociate (i.e., bifurcate) a chemically cross-linked complex in the gas phase of the mass spectrometer into two components that can be subsequently fragmented to produce product ions for elucidating structural features (i.e., peptide sequencing), without the need of a reporter ion for confident identification. By generating the product ions via ISCID, the subsequent CID within the ion trap can be performed at the MS/MS stage, as opposed to only the MS/MS/MS stage.

A more recent study on the reporter ion strategy was presented by Gardner and Brodbelt[21] where a photoreactive chemical cross-linker was used in conjunction with infrared multiphoton dissociation implemented on a modified ion trap mass spectrometer. In contrast, our CID-CXL-MS/MS reagent and methodology is amenable to all tandem mass spectrometers and incorporates the features that are most desirable for protein structural characterization. Common LC/MS/MS acquisition approaches such as data acquisition experiments using ion traps and commercially available software to analyze product ion spectra such as SEQUEST are compatible with our CID-CXL-MS/MS reagents, obviating the requirement for high-resolution and high mass measurement accuracy tandem mass spectrometers and custom-designed software to identify cross-linked peptides.

(21) Gardner, M. W.; Brodbelt, J. S. *Proceedings of the 54th ASMS Conference*. Seattle, WA, 2006.

CONCLUSIONS

Overall, the experiments and data presented demonstrate the utility and application of our CID-CXL-MS/MS reagents and approach to unequivocally identify intra- and interpeptide cross-links using a quadruple ion trap, a low-accuracy mass analyzer. By virtue of our CID cross-linker design, identification of cross-linked species by high mass accuracy mass analyzers will also be enhanced, providing unprecedented identification of cross-linked species, including de novo sequencing, as compared to presently achievable measurements using commercially available cross-linking reagents. To enrich cross-linked peptides for more effective LC/MS/MS analysis, we are currently pursuing the synthesis a CID-CXL-MS/MS reagent containing a chemically reactive/affinity tag within the linker region to enable selective capture of modified peptides after proteolytic digestion for increasing the detection of both intra- and interpeptide cross-links.

ACKNOWLEDGMENT

The authors thank the research agencies of North Carolina State University and the North Carolina Agricultural Research Service for continued support of our biological mass spectrometry research. Portions of this work were supported by grants from the National Science Foundation (MCB-0419819) and the United States Department of Agriculture (NRI 2004-35304-14930 and NRI 2005-35604-15420).

Received for review July 27, 2006. Accepted September 21, 2006.

AC0613840